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β-Lactams against the Fortress of the Gram-Positive *Staphylococcus aureus* Bacterium

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Abstract

The biological diversity of the unicellular bacteria—whether assessed by shape, food, metabolism, or ecological niche—surely rivals (if not exceeds) that of the multicellular eukaryotes. The relationship between bacteria whose ecological niche is the eukaryote, and the eukaryote, is often symbiosis or stasis. Some bacteria, however, seek advantage in this relationship. One of the most successful—to the disadvantage of the eukaryote—is the small (less than 1 μm diameter) and nearly spherical *Staphylococcus aureus* bacterium. For decades, successful clinical control of its infection has been accomplished using β-lactam antibiotics such as the penicillins and the cephalosporins. Over these same decades *S. aureus* has perfected resistance mechanisms against these antibiotics, which are then countered by new generations of β-lactam structure. This review addresses the current breadth of biochemical and microbiological efforts to preserve the future of the β-lactam antibiotics through a better understanding of how *S. aureus* protects the enzyme targets of the β-lactams, the penicillin-binding proteins. The penicillin-binding proteins are essential enzyme catalysts for the biosynthesis of the cell wall, and understanding how this cell wall is integrated into the protective cell envelope of the bacterium may identify new antibacterials and new adjuvants that preserve the efficacy of the β-lactams.

Graphical Abstract

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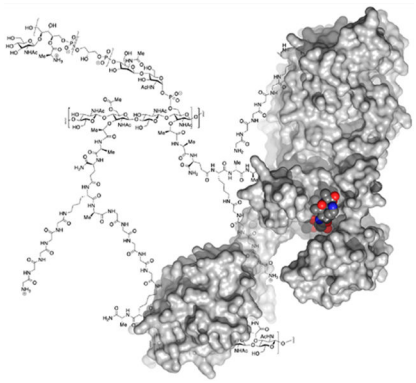

1. INTRODUCTION

The Three Fates of Greek mythology spun, measured, and cut the threads of human lives. Throughout history—and continuing to this day—a reliable blade for cutting the thread of life is infection by the spherically shaped Gram-positive bacterium *Staphylococcus aureus*. While the entry of antibiotics in the mid-20th century into clinical medicine significantly reduced bacterial morbidity and mortality from infections, pathogenic bacteria were not vanquished but became manageable. In the intervening decades successive acquisition of resistance mechanisms have challenged this management. As a result of the continuous refinement of its defensive and offensive capabilities,[1,2] *S. aureus* remains a nefarious pathogen.[3–6] Its adverse impact on human health can be assessed not just in terms of morbidity and mortality but also in terms of the economic cost of hospital length-of-stay and overall cost.[7] As befits its persistent place in both human and animal infection, complementary perspectives on its genetics,[8–11] its biochemistry,[12–14] its resistance mechanisms,[8,15] its virulence,[16,17] and its chemotherapeutic control are compelling topics.[18–23] Of all of the antibiotic classes, the β-lactams offer exquisite affordability, safety, and efficacy.[24] Indeed, *S. aureus* infection was the first indication for penicillin G when the first small quantities of this penicillin became available. Nonetheless, the infectious history of the *S. aureus* is one of successive structural refinement of the β-lactam to counter resistance mechanisms, which emerged in response to β-lactam antibacterial therapy. Whereas once *S. aureus* infection was treatable with the first penicillins (including penicillin G, structure **1** of Chart 1), today only two β-lactams—ceftobiprole (**5**) and ceftaroline (**6**)—are efficacious against the most highly pathogenic *S. aureus* strains. Both entities represent the newest generation of β-lactams of its cephalosporin subclass. This review presents a perspective on the interplay among medicine, microbiology, biochemistry, and medicinal chemistry in order to preserve the advantage of antibiotic chemotherapy (especially that of the β-lactams) in the face of *S. aureus* clinical resistance.

The β-lactams are named for the structural feature that is essential to their mechanism, the four-membered cyclic amide (the β-lactam). This cyclic amide participates in the mechanism-based inactivation of enzymes that are critical catalysts for the biosynthesis of the peptidoglycan cell-wall polymer of the bacterium. This polymer surrounds and encases

the bacterium, and the loss of its integrity is lethal to the bacterium. A late-stage (and possibly final) event in the biosynthesis of this polymer is the cross-linking of peptide stems present on elongating glycan strands. The transpeptidase enzymes that catalyze this cross-linking are inactivated by the β-lactams. β-Lactam recognition by these enzymes exploits the structural similarity of the β-lactams to the D-Ala-D-Ala substrate motif of these enzymes as first discerned by Tipper and Strominger. Their hypothesis persists as a durable theme in β-lactam structural biochemistry.[25–30] Chart 2 shows this structural mimicry and compares the structure of an intact and clinically used cephalosporin β-lactam and the β-lactam-opened structure of an inactivated transpeptidase. Scheme 1 gives a rudimentary kinetic scheme, which contrasts the peptidoglycan substrate and the β-lactam as inactivator. Loss of this transpeptidase enzymatic activity initiates events that culminate, as a bactericidal event, in loss of the structural integrity of this peptidoglycan polymer. Even prior to the point in time that the β-lactams entered into clinical use, the *S. aureus* bacterium had devised, acquired, and near-perfected resistance mechanisms against the β-lactams. Clinical use of the β-lactams has made the resistance mechanisms widespread by the process of selection. The focus of this review is the recent efforts to understand, in terms of its chemistry and biochemistry, these resistance mechanisms. The context for this understanding is the structural chemistry of the peptidoglycan, the identity and mechanism of transpeptidase enzymes (the penicillin-binding proteins or PBPs) that are the targets of the β-lactams, and how the loss of function of the PBPs culminates as a bactericidal event.

In PBP inactivation the cyclic amide is opened by an active-site serine nucleophile of the PBP. In contrast to the reactive acyl-enzyme species provided by its biosynthetic substrate, the acyl-enzyme derived from the β-lactam is stable (Scheme 1, Chart 2, and Figure 1). The PBP is inactivated. *S. aureus* uses multiple resistance mechanisms—both active and (for want of a better word) passive—to prevent this event. The two active mechanisms used by *S. aureus* are well recognized. In the first mechanism, *S. aureus* protects its transpeptidases from inactivation by the efficient hydrolytic destruction of the β-lactam. The enzyme used for this purpose—a β-lactamase enzyme—is evolutionarily related to the PBPs.[30,35–38] Their shared relationship is both structural and mechanistic. The β-lactamase of *S. aureus* is a "serine" β-lactamase. It recognizes the β-lactam, uses a catalytic serine to open the β-lactam to form an acyl-enzyme, and then completes catalytic turnover by transfer of the acyl moiety to water (fundamental similarity to the PBP acyl-transfer mechanism summarized by Scheme 1). The second mechanism is acquisition of a PBP that better distinguishes substrate (as we shall see, that of the peptide segment of the peptidoglycan) so as to disfavor β-lactam inactivation (as structural mimics of that peptide segment). These two mechanisms combine, respectively, audacity and finesse. *S. aureus* strains that have only the first mechanism are termed methicillin-sensitive (MSSA); methicillin is a second-generation penicillin. *S. aureus* strains that have the second or both mechanisms are termed methicillin-resistant *S. aureus* (MRSA). The origin of this terminology is discussed subsequently. Both MSSA and MRSA bacteria are clinically important. Notably, MRSA bacteria are resistant to all but the most recent β-lactam structures and thus present a challenge to antibiotic therapy. A somber note is that the 70 years of clinical use of the β-lactams has eliminated all variants of *S. aureus* that are devoid of a β-lactam resistance mechanism.

The deduction from the preceding statement, that the options for the chemotherapeutic control of the MRSA infection are limited, is correct. *S. aureus* infections account for 10, 000 annual fatalities in the US alone, the most for any single bacterium (2014 data from the Center for Disease Control and Prevention).[39] While clinical management of *S. aureus* infection is often possible,[40,41] the versatility of *S. aureus* to combine both resistance and virulence mechanisms (and it must be noted, *S. aureus* is by no means the only bacterium with this ability) may foretell a future when multiagent chemotherapy will be required for *S. aureus* and other bacterial pathogens.[42] Multiagency could correspond to the concurrent use of an adjuvant structure that itself lacks antibacterial activity but has a mechanism that enhances that of the antibiotic,[43–48] or the combination of antibiotics that have mechanistic synergy.[49–52] For these reasons, a major focus is the study of its active and "passive" resistance mechanisms used by *S. aureus*. Three perspectives guide this focus. The first perspective is the recent progress in the understanding of the complex orchestration of the biosynthetic pathways that create the Gram-positive cell envelope.[53,54] These pathways include (in addition to the peptidoglycan as a component of the cell envelope) the lipoteichoic acids, the wall teichoic acids, and the bacterial membrane. The second perspective is the decision network that has enabled *S. aureus* to survive over eons both as an innocuous human and animal commensal and as an opportunistic pathogen. *S. aureus* can respond to a less-than-lethal antibiotic exposure by coalescence into a protective biofilm[55,56] or by transformation into the torpor states of tolerance or of persistence.[57–61] The third perspective is the longstanding recognition that PBP inactivation by β-lactams is not the ultimate but the initiating event of their bactericidal mechanism.[62–64]

This review is in three parts. The first part is the anatomy of the envelope of the *S. aureus* bacterium, with emphasis on its peptidoglycan component as the structural target of the β-lactams. The second part addresses the four PBPs—the molecular target of the β-lactams—of the β-lactam-sensitive *S. aureus* bacterium and then the two primary resistance mechanisms used by *S. aureus*. As indicated above, the first of these mechanisms is an enzyme that hydrolytically destroys the β-lactam. The second mechanism is acquisition of a fifth PBP that is intrinsically unreactive to inactivation by β-lactams and so able to replace the loss of activity of PBPs susceptible to β-lactams. Mutation of the PBPs to manifest resistance to β-lactams, a resistance mechanism common in other bacteria, was not recognized as an important resistance mechanism in *S. aureus* until recently. In part due to the availability of whole-genome sequencing, and in part due to the progressive refinement of its resistance mechanisms, PBP mutation as a contributory resistance mechanism is now encountered. Although the β-lactams are the central class of antibiotics, the critical importance of the integrity of its cell envelope to the bacterium is also evidenced by other envelope-targeting antibiotics also important to human and animal health.[65] These antibiotics include the glycopeptides (vancomycin, oritavancin, dalbavancin), daptomycin, and fosfomycin as well as a myriad of exploratory structures. The third part of this review discusses how understanding the biochemistry and microbiology of the *S. aureus* bacterium influences the practices of its antibiotic chemotherapy and antibiotic discovery. Although the biochemistry and microbiology of *S. aureus* is that of a typical Gram-positive bacterium —with the emphatic exception of its resistance and virulence pathways—clinical efficacy against bacterial infection requires precision in the selection of chemical structure and in

optimization of its dosing. As bacterial chemotherapy transitions from the use of a single antibiotic structure to concurrent use of synergistic structures, knowledge as to how the interplay among enzyme, pathway, and structure will identify vulnerability is critical to preserving efficacy.

2. GRAM-POSITIVE CELL ENVELOPE

Unicellular bacteria encompass extraordinary diversity of size and shape.[66–69] Pathogenic bacteria largely (but by no means exclusively) have micrometer dimensions that divide among four shapes: cocci (nearly spherical), ovococci (ellipsoidal), rods (cylindrical), and spiral (helical). Each shape embodies the simplest structural characterization of a bacterium, that of a cytoplasm within a cell envelope. The envelope mediates molecular communication with the environment and provides to the bacterium structural stability and protection.[70,71] As the densely packed cytoplasm is not in osmotic equilibrium with the external medium, the envelope contains a significant turgor pressure.[72,73] The cell envelope achieves this containment by interlocking its substructure. Notwithstanding the great diversity (even among species) with respect to the substructures themselves and how they interlock, *S. aureus* is a typical Gram-positive monoderm (single membrane) bacterium with a spherical (coccus) shape of a diameter of approximately 0.6–0.8 μm. The major substructures of its cell envelope are a lipid-bilayer membrane overlaying and surrounding the cytoplasm, that is itself overlaid by a peptidoglycan polymer that is conjoined to the membrane by anionic glycopolymers. The external surface of the Gram-positive bacterium is comprised of these glycopolymers (the peptidoglycan, the “wall” teichoic acids, and the surface polysaccharides). Bacteria with this external surface are colored by certain dyes and consequently are called Gram-positive, after the microbiologist (Gram) who first observed this phenomenon.[74,75] In this coloration, the dye adsorbs to the peptidoglycan substructure.[76] Further interlocking of the membrane and the peptidoglycan is achieved with peptidoglycan-binding membrane proteins and by the second type of glycopolymers (the “lipoteichoic acids”). Lipoteichoic acids (LTA) are membrane-anchored and engage the peptidoglycan noncovalently. Their structure is relatively conserved among the Gram-positive bacteria. In contrast, structural diversity is seen with respect to the wall teichoic acids (WTA). The WTAs attach covalently to the peptidoglycan for the presumptive purpose of organizing and/or stabilizing the peptidoglycan structure and also to contribute to defending the bacterium against antibiotics and phages. Although the covalent structures of both the LTAs and WTAs are known, the molecular basis by which all three—the two glycopolymers and the peptidoglycan—interlock is not known. Nonetheless, the interlocking is important. Its disruption (as accomplished, for example, by inhibitors of teichoic-acid biosynthesis) compromises cell viability and increases susceptibility to antibiotics (as further discussed below). Many Gram-positive bacteria (including *S. aureus* strains) attach covalently proteins and capsular polysaccharides to the exterior surface of their peptidoglycan, in order to promote pathogenic adherence and as a further defense against antibiotics and phages.[54,77–79] As with the wall teichoic acids, the capsular polysaccharides attach covalently to the peptidoglycan.[80] Capsular polysaccharides are absent, however, from two of the most pathogenic MRSA *S. aureus* strains (USA300, USA500).[81,82] The purpose to their absence is not known.

2.1. Cell Envelope of the Gram-Positive Bacterium

The molecular structures of four of the entities that compose the *S. aureus* cell envelope—the membrane, the peptidoglycan as a multilayered surface, the lipoteichoic acids stretching from the membrane to intercalate with the peptidoglycan, and the wall teichoic acids attached to the peptidoglycan—are shown in Scheme 2. This scheme shows structures. It does not address how these structures organize. Additional components, notably the proteins and the capsular polysaccharides that also attached to the peptidoglycan, are not shown. The structures of Scheme 2 are complemented by the pathway summaries within the cartoon of Figure 2. The gap between the membrane and the peptidoglycan is called the inner-wall zone (IWZ; the functional equivalent of the periplasm in Gram-negative bacteria). Whereas Gram-negative envelopes preserve a defined structural space between their two membranes (their cell wall is within this space),[83–85] Gram-positive bacteria appear to maintain a less well-defined space between their cell wall and their single membrane only where active cell-wall synthesis occurs.[86] Where this zone is found, it is populated by the membrane proteins (including the PBPs) and by the lipoproteins of cell-wall biosynthesis. At every point in the cell cycle of *S. aureus* its cell envelope structure is in dynamic flux. Its membrane surface expands as it is populated with lipids and membrane proteins. Proteins are secreted through the membrane to the inner-wall zone,[87] many for attachment to the peptidoglycan.[88] Lipoteichoic acids are assembled in the inner-wall zone upon translocation to the outer-membrane leaflet of a Glc_2-diacylglycerol lipid. The glycophosphate substructure of the LTA is built upon this lipid in order to intercalate with the peptidoglycan. The wall teichoic acids are assembled in the cytoplasm and translocated across the membrane for attachment to the peptidoglycan. The capsular saccharide is assembled both in the cytoplasm and in the inner-wall zone also for attachment to the peptidoglycan. Lastly, the monomer building unit for peptidoglycan biosynthesis—the lipoglycopeptide Lipid II—is assembled in the cytoplasm and translocated across the membrane for PBP-dependent polymerization in the IWZ. All of these events occur concurrently and in harmony. Even after its initial assembly, active remodeling of the cell envelope occurs in response to the cell cycle, to changes in the media (notably nutrients, pH, and osmotic strength), and following encounter with antibiotics. The dominant mass of the cell envelope is the peptidoglycan polymer, with the mass of the teichoic acids only somewhat less. This dominance is reflected by a frequently encountered synonym for the peptidoglycan, the “cell wall”. *S. aureus* has a three-dimensional peptidoglycan. Peptidoglycan is synthesized using the repetitive polymerization of Lipid II. Lipid II is biosynthesized in the cytoplasm, translocated across the membrane, and assembled into a polymer by progressive transglycosylation of its glycosyl segment to form glycan strands, that are subsequently cross-linked by transpeptidation reactions of its peptide segment. The enzyme catalysts of the polymerization are membrane proteins, localized in the outer leaflet of the bacterial membrane, and projecting their active sites facing the membrane surface of the IWZ, or projecting into the IWZ.[85] The relative thickness of the Gram-positive peptidoglycan—for *S. aureus*, a thickness of approximately 25 nm—implicates a layering mechanism for the peptidoglycan. Given the location of the enzyme catalysts, this layering must occur by inside-to-outside growth.[94] Analysis of the overall peptidoglycan surface, using the complementary methods of transmission and atomic force microscopies, shows a highly porous ultrastructure.[95,96] High-resolution atomic force microscopy of live *S. aureus*

bacteria divide the peptidoglycan layer into a less dense outer layer having large pores (up to 60 nm) that taper in some cases to the membrane surface and a denser inner layer of peptidoglycan (pores of less than 7 nm diameter).[97] This distinction—a less cross-linked outer or mature peptidoglycan and an inner layer largely retaining the greater cross-linking of nascent peptidoglycan—is consistent with the long-understood necessity for a mechanism that would relax the outermost peptidoglycan to accommodate the progressive increase in surface area as peptidoglycan layers move outward. The mechanism for this relaxation is the release of hydrolytic enzymes to the exterior peptidoglycan layers for the discrete cleavage of either the glycan strands or their peptide cross-links.[98] We return subsequently to the possible loss of coordination among these biosynthetic processes as a key concept to the understanding of antibiotic mechanisms.

2.1.1. Lipid II Monomer of Peptidoglycan Biosynthesis.—β-Lactams block the biosynthetic completion of the peptidoglycan polymer. The structural touchstone for the understanding of this mechanism is Lipid II.[99–103] Lipid II is assembled in the cytoplasm and then translocated across the membrane to the outer leaflet of the membrane, and exposed to the surface—to the inner wall zone—of this leaflet. Its glycan, stem peptide, and stem bridge substructures may be recognized within its structure (the structure of Lipid II of *S. aureus* is shown in the lower left corner of Scheme 2). The molecular center of Lipid II is a β-(1 → 4)-linked disaccharide of two *N*-acetylglucosamines, one of which is uniquely functionalized. Its proximal *N*-acetylglucosamine is differentiated from its distal *N*-acetylglucosamine (GlcNAc or NAG) in three key respects. These respects are critical to understanding the pathway for the assembly of the cell wall, to β-lactam resistance, and to the possible enhancement of β-lactam efficacy. The first respect is functionalization of the C-3 alcohol of the proximal GlcNAc by an ether-linked D-lactyl moiety. This distinctive substitution suffices for this saccharide to be named not as GlcNAc but as *N*-acetylmuramic acid (abbreviated as either MurNAc or as NAM). To the carboxylate of the lactyl moiety of the MurNAc are added, in amide linkages, a *stem* pentapeptide. The first amino acid is L-alanine; the second is an *iso*-D-glutamine (the amide bond with the L-alanine is to α-amine of the *iso*-D-glutamine); the third is L-lysine (attached by its α-amine to the δ-carboxylate of the *iso*-D-glutamine); and the fourth and fifth are D-alanines. This peptide stem is appended to the MurNAc in the cytoplasm by four ATP-dependent reactions corresponding to sequential addition of L-alanine (catalyzed by the MurC enzyme), D-glutamate (MurD), L-lysine (MurE), and D-alanines as the D-Ala-D-Ala dipeptide (MurF).[104–106] The amidation reaction that transforms the *iso*-D-glutamate of the stem peptide to *iso*-D-glutamine is catalyzed by the GatD/MurT protein complex.[107–111] As loss of GatD/MurT activity correlates directly with decreased peptidoglycan cross-linking and greater β-lactam susceptibility, this activity is a possible target of a β-lactam adjuvant.[111] The molecular basis for the increased susceptibility is unknown.

The second modification of Lipid II in *S. aureus* is the presence of a pentaglycine *bridge* peptide attached to the ε-amine of the L-lysine of the stem peptide.[112] Peptide bridge extensions to the L-lysine of the stem are commonly encountered in Gram-positive bacteria, with different bacteria using different short oligopeptide sequences for their bridge.[113,114] Cytoplasmic modification of Lipid II to install the Gly_5 bridge is accomplished by the

FemXAB enzyme family: FemX adds the first glycine; FemA the second glycine; and FemB the third, fourth, and fifth glycines.[112,115] For all three enzymes the glycyl donor is Gly-tRNA^{gly}.[116,117] The Fem designation to these enzymes (and to their genes) has significance: F*actors* e*nhancing* m*ethicillin* (β-lactam) resistance.[118] FemX, the first enzyme in bridge peptide elongation, is an essential enzyme.[119] Deletion of the FemA and FemB activities gives viable but severely growth-impaired *S. aureus* strains,[120] characterized by substantial reduction in the extent of peptidoglycan cross-linking, altered metabolism, and hypersusceptibility to antibiotics (including the β-lactams).[121,122] The basis for the poor viability is structural. As we discuss shortly, PBP catalysis of transpeptidation involves the transfer of the acyl moiety of the penultimate D-Ala to the amine terminus of the pentaglycine bridge (Scheme 2). The polymeric peptidoglycan that is made in the absence of a complete pentaglycine bridge gives an *S. aureus* bacterium that is osmotically fragile and unable to constrain the turgor pressure of its cytoplasm.[123] Although *S. aureus* has the ability to substitute selectively serine for glycine as a possible resistance mechanism against antimicrobial peptides, this substitution is not yet seen as a resistance mechanism against other antibiotics.[124] The Fem enzymes also are targets for inhibition in order to enhance the efficacy of the β-lactams (and of other antibiotics).[118,125–127]

A third structural aspect of the Lipid II structure is its diphosphate moiety. On one side is the NAM with an anomeric α-glycosyl diphosphate moiety that is recognized—correctly—as poised for glycosyl transfer. On the other side of the diphosphate is a lipophilic C_{55}-polyprenol segment. The historical name for its free alcohol (bactoprenol) is superseded in the current literature by the more systematic name, undecaprenol. Undecaprenol is the universal carrier of bacterial saccharide-containing structures—capsular polysaccharides,[78] wall teichoic acids, and Lipid II—of the Gram-positive cell envelope.[128–131] Each of these three structures translocates across the membrane as conjugates of undecaprenol diphosphate. As the number of undecaprenol diphosphate molecules in the bacterium is limited,[90] constant and frenetic recycling of the undecaprenol is required to support its multiple biosynthetic roles.[89,92,93,132] While key aspects of this recycling are known, other key aspects are not. *S. aureus* is one of several bacteria used for the study of undecaprenol diphosphate recycling. The conclusion from collective experimental study is that the efficient recycling of undecaprenol diphosphate to undecaprenol phosphate and return translocation of undecaprenol phosphate from the outer leaflet to the inner leaflet (by an unknown mechanism) are critical to balanced cell-envelope biosynthesis.[93] One context supporting this conclusion is Lipid II biosynthesis. Undecaprenyl diphosphate is biosynthesized in the cytoplasm from farnesyl diphosphate and eight equivalents of isopentyl diphosphate by a single synthase enzyme (UppS).[133,134] Following its insertion to the inner leaflet of the *S. aureus* membrane, the diphosphate moiety is trimmed by an intramembrane phosphatase (UppP) to give undecaprenol phosphate.[135] In Lipid II biosynthesis, this phosphate accepts a MurNAc phosphate having an assembled peptide stem and peptide bridge, catalyzed by the enzyme MraY.[136] The product of this reaction is the monosaccharide MurNAc-α-diphosphate-undecaprenol (known as Lipid I).[137] Transfer of a GlcNAc to the C-4 of the MurNAc pyranose, catalyzed by the enzyme MurG, completes Lipid II synthesis within the inner leaflet of the membrane.[138] Each of these four enzymes—UppS,[139–148] UppP,[149–151] MraY,[152–158] and MurG[138,159–161]—have been explored

intensively with respect to inhibition by both synthetic and natural product (antibiotic) molecules. The renewed interest in structure–activity development of the nucleoside-mimetic natural product inhibitors of MraY[157,162–166] and the recognition that numerous antibiotics interfere with peptidoglycan biosynthesis by complexation with the undecaprenol diphosphate (in both Gram-positive[135,167–169] and Gram-negative bacteria[170,171]) and/or Lipid II[99,102,103,172–178]) are notable recent developments. Indeed, the recognition of the fastidiousness of the intertwined biosynthetic cycles for Lipid II synthesis,[179,180] for Lipid II assembly into the peptidoglycan, and for peptidoglycan recycling (with preeminent natural product antibacterials targeting within each cycle)[181–185] have led to substantial effort toward answering whether concurrent inhibition of two of these cycles (and their regulatory systems)[186] achieves meaningful antibiotic synergism.

2.1.2. From the Lipid II Monomer to the Peptidoglycan Polymer.—

Peptidoglycan biosynthesis occurs by sequential transglycosylations and transpeptidations of multiple Lipid II molecules. An indisputable point for securing antibiotic activity is the transpeptidation cross-linking reactions, as this reaction is the target of the β-lactam antibiotics. In its simplest form, peptidoglycan cross-linking is the (presumptively isoenergetic) cleavage of an amide bond of the stem peptide, followed by transfer of the acyl group to the amine of the bridge peptide of an adjacent strand, to reform an amide bond as a cross-link. The reality is more complex. The bacterial peptidoglycan is in a constant state of depletion and excision, remodeling, and repair and accretion by both forward and outward growth. These events are precisely coordinated with a legion of concurrent events defining the cell cycle (many, but not all, as noted previously) and across multiple locations within the cell.[187] Bacteria have multiple degradative enzymes and multiple assembling and modifying enzymes for the peptidoglycan.[188] Accordingly, bacteria have multiple penicillin-binding proteins. The key substrate substructure manipulated by the PBPs is the D-Ala-D-Ala terminus (fourth and fifth amino acids of the stem peptide). PBPs divide between two subclasses defined by their molecular mass, either relatively low (LMM) or relatively high (HMM). The HMM subclass further divides between PBPs that are bifunctional and those that are monofunctional. Class A PBPs (HMM aPBPs) are bifunctional with their two active sites spatially separated from each other. One active site has transglycosylase activity while the second active site has transpeptidase activity. Class B PBPs (HMM bPBPs) have a single transpeptidase active site. The low-molecular mass (LMM, or Class C) PBPs usually have a carboxypeptidase function that exerts a control over the extent of possible cross-linking of the peptidoglycan. Leaving in place the terminal D-Ala allows maximal cross-linking, while its removal (by a Class C carboxypeptidase) lowers the possible extent of cross-linking. All bacteria have at least one PBP from each of the three classes. *S. aureus* is unusual in that it has only four PBPs in total: one Class A (PBP2, an essential PBP), two Class B (PBP1, an essential PBP; and PBP3, a nonessential PBP), and one Class C (PBP4, a nonessential PBP, that functions primarily not as a carboxypeptidase but as a transpeptidase). By comparison, the rod-shaped Gram-positive bacterium *Bacillus subtilis* has more than 20 PBPs.[189] The assignment here between essential and nonessential PBPs for *S. aureus* is with respect to laboratory viability. Pathogenic *S. aureus* requires all four of its genomic PBPs, if not a fifth (as discussed below).

PBP enzyme nomenclature is historical (numbered from highest to lowest molecular mass). All PBPs have a membrane-binding domain and a transpeptidase/carboxypeptidase domain. Their transpeptidase (or carboxypeptidase) domains project above the inner wall zone. The transglycosylase domains of the aPBPs are located near the membrane surface of the inner wall zone. β-Lactams mimic the D-Ala-D-Ala stem terminus. The latter substrates are competent for acyl-transfer, by initial transfer of the penultimate D-Ala to an active-site serine to form an acyl-enzyme, that is then transferred in the second half-reaction to the fifth glycine (in *S. aureus*) to effect cross-linking. In contrast, acylation of the serine nucleophile at the transpeptidase/carboxypeptidase active sites of the PBPs by β-lactam antibiotics is irreversible.[190,191] Thus, upon exposure of PBPs to high concentration of radioactive β-lactams (as the relative affinity of a given β-lactam for a given PBP is variable), all of the PBPs of a bacterium are inactivated by this acylation.[192,193] These radiochemically labeled PBPs are sorted by molecular mass using electrophoresis. This analysis is done today using labeling with fluorescent β-lactams.[194–198] The highest molecular mass PBP of the bacterium is designated as its PBP1. Hence, the PBP number does not indicate function. A fundamental barrier (even to this day) to the understanding of the PBPs is the near-complete lack of understanding of their mechanistic roles, including the higher-order basis for PBP recognition of its substrate (beyond the -D-Ala-D-Ala terminus of the stem peptide). Four reasons contribute to this persistent ignorance. PBPs are never found in the bacterium as solitary enzymes, but are either enzyme components of multiprotein and multienzyme complexes with extensive protein–protein contact and regulation (Class A and Class B), or function separately also with extensive protein–protein contact and regulation (Class C). The two primary complexes for the Class A and Class B PBPs in most bacteria are the elongasome (primarily tasked to sidewall peptidoglycan synthesis and remodeling) and the divisome (primarily tasked to septal peptidoglycan synthesis and remodeling).[199–201] *S. aureus* as a coccus bacterium is different in that it uses only a divisome complex (and not an elongasome complex). For these reasons catalytic assay of PBPs is challenging experimentally.[202,203] No three-dimensional structures of the peptidoglycan polymer (of the sidewall; of the septum; of the sidewall to septum transition; of the newly synthesized versus mature peptidoglycan; of the spore; as correlated with shape; as correlated with resistance; and so forth) are known.[204] Lastly, extensive manipulation of the PBP genes of all bacteria shows extensive functional redundancy and cooperativity within their PBP family. Redundancy and cooperativity characterize all four of the PBPs of *S. aureus*.[205,206]

Peptidoglycan polymerization is described concisely. The first step of peptidoglycan biosynthesis is glycan strand elongation, accomplished by repetitive transglycosylations of Lipid II molecules. The PBPs catalyze the second event of peptidoglycan biosynthesis from Lipid II, that of the cross-linking of the peptide stem of one strand to the peptide bridge of another stem.[129,191,203] Peptidoglycan biosynthesis is not, however, a simple combination of these two reactions. Rather, bacteria have several different PBP-dependent processes for peptidoglycan synthesis. In all cases Lipid II, originating from the biosynthetic pathway that culminates at the inner leaflet of the membrane by the completion of its synthesis (as catalyzed by MurG and MraY), is supplied as the substrate. The transmembrane MurJ protein translocates Lipid II from the cytoplasm to the inner wall zone in a proton-motive-force-dependent event.[207–210] In rod-shaped bacteria (such as the Gram-negative *Escherichia*

coli and the Gram-positive *Bacillus subtilis*) a multiprotein, multienzyme complex called the elongasome lengthens the bacterium by sidewall peptidoglycan growth. The elongasome assembly is organized upon dynamic strand repeats of a cytoskeletal protein, MreB, which localizes at the interface of the cytoplasm and the inner membrane. At the elongasome, Lipid II synthesizes glycan strands using the transglycosylase enzyme of the elongasome, RodA. RodA partners with a Class B PBP (that is, a PBP having only transpeptidase activity) enzyme to effect initial cross-linking of the stem peptides.[211] Complete polymerization is the result of an intimate coupling of the Class B PBP with a Class A PBP. Prior to cell division a peptidoglycan septum is created. Many bacteria (including *E. coli*, *B. subtilis*, and *S. aureus*) divide at midcell subsequent to septal synthesis. The divisome is the multiprotein, multienzyme complex used for septal peptidoglycan synthesis. This complex is organized upon dynamic strands of repeats of the tubulin-like cytoskeletal protein FtsZ, wherein the essential protein FtsZ organizes into a ring (the Z-ring) in a GTP-dependent process.[212] Divisome complexes intersperse on the dynamic Z-ring filaments at midcell, against the interface between the inner membrane and the cytoplasm (Figure 3). Proteins are recruited to the divisome sequentially: first FtsZ; then additional cytoskeletal proteins; then the MurJ flippase for Lipid II translocation; and then the remaining proteins of the divisome. Among these latter proteins is the notable pairing of the FtsW enzyme with a bPBP. When this pairing happens, FtsW initiates sequential transglycosylation of Lipid II molecules to begin peptidoglycan biosynthesis. The glycan strands that emerge from FtsW catalysis are cross-linked by the bPBP.[85]

2.1.3. Peptidoglycan Biosynthesis and the *S. aureus* Cell Cycle.—

The particulars of peptidoglycan biosynthesis in *S. aureus* are summarized concisely. *S. aureus* has four PBPs. Three are biosynthetic PBPs: PBP2 is an aPBP (bifunctional transglycosylase and transpeptidase), and PBP1 and PBP3 are bPBPs (monofunctional transglycosylases). PBP4 is a cPBP with a monofunctional transpeptidase active site. PBP4 acts to remodel biosynthetic peptidoglycan. Although (as noted previously) only two of these four PBPs (PBP1 and PBP2) are essential, pathogenic *S. aureus* uses all four PBPs to advantage. Accordingly, this review does not distinguish among the PBPs as essential or nonessential.[205] The function of the four PBPs of *S. aureus* is presented first in the form of a cartoon schematic for the cell cycle of this bacterium (Figure 3).[213,214] In the first panel the bacterium is represented schematically as a cytoplasm surrounded by a membrane (gray), itself surrounded by the peptidoglycan (turquoise). The cell cycle divides in three phases across a timeline of approximately 66 min for the strain that was studied.[214,215] The first phase (in terms of time, the longest of the three, is approximately half of the cell cycle) is dominated by chromosome replication. As replication proceeds to its conclusion, GTP-dependent FtsZ filament formation (the Z-ring, in dashed orange in Panel B) occurs at the midcell circumference against the surface of the inner membrane leaflet.[216,217] Disruption of Z-ring formation using FtsZ as target is an area of extensive ongoing research[218–222] and synergy of exploratory FtsZ inhibitors with the β-lactams is seen.[223–227] A detailed analysis of the coordination of repetitive *S. aureus* cell cycles with respect to chromosome separation and cell-envelope completion is found elsewhere.[228] For simplicity the following discussion addresses a single cell cycle.

The first event in septal peptidoglycan synthesis is recruitment by the Z-ring of a PBP to initiate the spatially orthogonal (with respect to the existing peptidoglycan) inward growth of septal peptidoglycan. This initial thickened belt of peptidoglycan is termed the "piecrust" (red of Panel C). It is identifiable on the surface of the bacteria following division (red "ribs" of the lower left panel).[229–231] Divisome recruitment to the Z-ring, at the piecrust, initiates the second phase. Septal peptidoglycan synthesis completes by the progressive circumferential motion of divisome assemblies, guided initially by the constricting Z-ring (Panel C).[232] This peptidoglycan growth occurs at a leading edge, behind which a thickened and structurally more robust peptidoglycan is made. A concentric (and presumably inward spiraling) pattern of the septal peptidoglycan that is created by the leading-edge PBP is evident from atomic-force microscopy imaging.[95,229–231,233] The thickened peptidoglycan that is synthesized upon the leading-edge peptidoglycan (dark blue of Panel D) has a different texture (that of randomly oriented strands with a 6 nm spacing).[97] Dual layering of the septal peptidoglycan—a heavier layer upon a leading layer that shows concentric ring growth—is supported by microscopy studies on *Staphylocccus warneri*.[234] A critical structural feature within this nascent septal peptidoglycan, with respect to ultimate cell division and separation, is the creation of midzone area (white line within the dark blue of Panel D) of less dense peptidoglycan.[235] This midzone demarcates paired septal plates that are joined at the piecrust. Leading-edge peptidoglycan synthesis converges at the center of the sphere (annulus fusion of Panel E) to complete the second phase. Maturation of the septal peptidoglycan leads to a uniform thickness (Panel F). The piecrust is then progressively weakened by the controlled formation of perforations across the entire ring of the piecrust.[95] At a critical point of this weakening of the piecrust it fractures—termed "popping apart" as it occurs on a millisecond time frame—to separate the daughter cells, as the final event of the cell cycle (Panel H).[214,232] The hemisphere-like shape of the daughter cells relaxes rapidly to the spherical shape of the coccus.[215] The same turgor pressure that effects cell separation enforces transformation to spherical cells. Following septation, the piecrust peptidoglycan appears as an annular exterior rib.[229,231,232,236,237]

2.1.4. PBP Catalysis of Transpeptidation and Inactivation by β-Lactams.—The entire basis for the antibiotic efficacy of the β-lactams is the inhibition of the PBP-catalyzed transpeptidation that cross-links the peptidoglycan. The presence of D-amino acids in the peptide stem (the δ-linked D-isoGln that is adjacent to the two L-amino acids and the D-alanine pair) is widely understood to prevent hydrolytic cleavage of the stem peptide by digestive peptidases (with their requirement of L-amino acids for substrate recognition). PBP transpeptidation occurs as a result of transfer of the acyl moiety of the penultimate D-alanine to the active-site serine nucleophile of the PBP, using lysine as the general-base catalyst for acyl transfer. The ultimate D-alanine departs as the leaving group in the formation of this serine acyl-enzyme. Transpeptidation is completed by the engagement of the primary amine terminus of the bridge peptide (in *S. aureus*, that of the fifth glycine) of an adjacent glycan strand, reforming an amide bond to cross-link the strands. In this two-step reaction of acyl-enzyme formation and acyl-enzyme transfer, the β-lactam ring is a superlative mimic with respect to both structure and reactivity of the stem D-Ala-D-Ala substructure in the acylation step.[25,28,238–240] As the β-lactam presents a ring structure in its acylation half-reaction, its amine leaving group is not released but is retained at the PBP active site.

The steric congestion of the resulting acyl-enzyme prevents the approach of the nucleophilic amine (for PBP catalysis of transpeptidation reactions) or water (for PBP catalysis of carboxypeptidase reactions).[28,30,239,241,242] The PBP is trapped as the acyl-enzyme and is inactivated. The abundance of bacterial pathogens for which β-lactams are preeminent chemotherapy and the diversity of β-lactam structures indicate that this brief mechanistic summary is simplification. As is evident from the preceding discussion, some PBPs (those that are essential) are better targets than other PBPs (those that are nonessential). As each PBP has nuance to its active site, effective chemotherapy of a pathogenic bacterium requires a β-lactam structure that in its totality matches to the active site of an essential PBP. For some time, however, this minimum is not enough: the β-lactam also must evade the resistance mechanism(s) of the bacterium.

2.1.5. PBP Roles in *S. aureus* Peptidoglycan Biosynthesis.—The *S. aureus* cell cycle incorporates precise orchestration of peptidoglycan synthesis that is continuous throughout the cell cycle and peptidoglycan degradation at the end of the cell cycle. PBPs provide the former activity. In the first phase, PBP catalysis of sidewall peptidoglycan growth expands the cell volume, and in the middle phase PBP catalysis as a component of the divisome effects septal peptidoglycan growth. Observations with respect to these peptidoglycan biosyntheses suggest four types of peptidoglycan (notwithstanding that each is made from the same starting material, Lipid II): those of the mature sidewall; the piecrust; the septal leading edge; and the matured septum wherein peptidoglycan is synthesized over the leading-edge peptidoglycan. Moreover, a mechanism must be surmised for the remodeling of septal peptidoglycan into mature peptidoglycan. No experimental data address the differences at the molecular level among these peptidoglycans. Exceedingly few experimental data address the mechanistic character of the four PBPs of *S. aureus*. Nonetheless, exquisite studies over the past years have suggested the localization of these four PBPs that in turn is suggestive with respect to their function.[213,243]

2.1.5.1. PBP3.: PBP3 (*pbp3*) is a nonessential bPBP and is of known structure (as the soluble protein, without its membrane-binding domain).[34] Its absence has minimal effect on cell growth, has undetectable change in the distribution of peptidoglycan fragments generated by lysozyme degradation (muropeptide profiling), and results in a more spherically shaped cell. In the presence of a sub-MIC concentration of a β-lactam, a Δ*pbp3 S. aureus* strain showed significantly impaired growth coinciding with disoriented septa within abnormally sized and shaped cells.[244] The basis for spherical shaping of the cell followed recognition that the transpeptidase activity of PBP3 is paired with the transglycosylase activity of the RodA enzyme.[245] PBP3 is believed to be one of the first enzymes recruited (and is followed by RodA) to midcell following the initial formation of FtsZ filaments. Its location is identified in the cartoon of Figure 4. The structure of a RodA·PBP complex from *Thermus thermophilus* was reported recently.[211,246] RodA·PBP3 recruitment is followed (as assessed by the incorporation of fluorescent D-amino acids into Lipid II and then into the peptidoglycan) by sidewall elongation of the peptidoglycan.[245] This elongation accounts for the coccus-shape of the *S. aureus* bacterium and the near-spherical shape of the Δ*pbp3 S. aureus* bacterium. The function of the peptidoglycan synthesized by RodA·PBP3 might be preparation of the sidewall peptidoglycan to enable

the spatially orthogonal growth of septal peptidoglycan. Regardless of possible function, the ability of Δ*pbp3 S. aureus* to sustain near normal growth in the absence of β-lactam antibiotics indicates that another PBP can assume this function. The dramatic impairment of Δ*pbp3 S. aureus* in the presence of β-lactam antibiotics identifies the inclusion of PBP3 within the inhibition profile of a β-lactam having optimal *S. aureus* activity. PBP3 is a co-PBP target of the clinically approved MRSA cephalosporin ceftobiprole.[247,248] Resistance mutations of PBP3 are described.[249,250]

2.1.5.2. PBP1.: PBP1 (*pbpA*) is an essential bPBP of as-yet-unknown structure. It is a PBP of septal peptidoglycan biosynthesis. The experimental studies that established its essentiality identified structural and catalytic roles for PBP1.[251,252] As a bPBP, PBP1 catalyzes the transpeptidation step of peptidoglycan biosynthesis in partnership with and following glycosyltransferase catalysis by FtsW, itself also an essential protein.[253] At a fundamental mechanistic level the operation of the FtsW·PBP1 complex with respect to peptidoglycan polymer synthesis is thought identical to the operation of the RodA·PBP3 pair.[254] The temporal sequence of protein recruitment at midcell, following sidewall peptidoglycan elongation by RodA·PBP3, is PBP1, then FtsW, and then MurJ (the Lipid II flippase) to initiate septal peptidoglycan biosynthesis.[208] In the absence of complex formation with PBP1, FtsW lacks catalytic activity.[254] In the absence of the FtsW·PBP1 complex, the divisome protein assemblies delocalize away from midcell. This delocalization is seen also with a catalytically impaired PBP1 protein, suggesting the FtsW·PBP1 complex as a structural edifice for the organization of the many additional proteins and enzymes of the divisome.[85] The further observation that FtsW·PBP1 catalysis does not contribute significantly either to the total mass or character of the bacterial peptidoglycan[252] is suggestive of an assignment to the FtsW·PBP1 pair as the catalyst that templates formation of the septal peptidoglycan. A possible function is synthesis of the leading-edge peptidoglycan (Figure 4). Initial septal peptidoglycan biosynthesis is dependent on Z-ring contraction, but final septal peptidoglycan closure is Z-ring independent.[245] A functional PBP1 is also critical for septation, possibly as a result of dysregulation of the autolysis enzymes required for the orderly perforation of the piecrust peptidoglycan.[245,252]

2.1.5.3. PBP2.: PBP2 (*pbp2*) is the essential aPBP of *S. aureus* and is of known structure (as the soluble protein, without its membrane-binding domain).[255] All data are consistent with PBP2 as the workhorse PBP for the synthesis of the septal peptidoglycan.[256] The pairing of a bPBP as an initiator of peptidoglycan biosynthesis followed by an aPBP to complete, or to repair and/or remodel, peptidoglycan biosynthesis has broader implication.[85,201] An aspect with respect to remodeling is the structural integration of other key envelope substructure (notably the teichoic acids) to create the total cell envelope. In *S. aureus* PBP2-catalyzed formation of the structurally robust septal peptidoglycan would follow (and build upon) the leading-edge peptidoglycan created by FtsW·PBP1 catalysis (Figure 4). A key uncertainty is whether the aPBP is an enzyme of the divisome, or is autonomous. In the first phase of the *S. aureus* cell cycle, PBP2 distributes across the entirety of the bacterial membrane. Following MurJ recruitment to the divisome for Lipid II translocation, PBP2 localizes to the nascent septum through Lipid II substrate-dependent recruitment.[216,257] As discussed below, PBP2 is an intrinsically β-lactam-sensitive PBP

and its loss-of-function by clinical levels of β-lactam antibiotics renders *S. aureus* β-lactam-susceptible. Its mechanistic complementation by catalytic coordination with a dedicated transpeptidase, PBP2a (formerly PBP2′) that is intrinsically β-lactam-nonsusceptible, is the key resistance mechanism of MRSA, the β-lactam-resistant *S. aureus*.

2.1.5.4. PBP4.: PBP4 (*pbp4* or *pbpD*) is the nonessential cPBP of *S. aureus* and is of known structure (as the soluble protein, without a membrane-binding domain).[258,259] Notwithstanding its nonessentiality, PBP4 is an important component to antibiotic resistance by *S. aureus*. In most bacteria cPBPs act as carboxypeptidases toward the D-Ala-D-Ala stem terminus of nascent peptidoglycan as substrate. Removal of the terminal D-Ala from the stem precludes (and thus controls the extent of) overall peptidoglycan cross-linking. *S. aureus* is distinctive as a bacterium, however, in the exceptionally high cross-linking (90%) of its peptidoglycan.[260] This value relegates a smaller mechanistic role for the D-Ala-D-Ala carboxypeptidase activity. Indeed, PBP4 acts preferentially as a transpeptidase and contributes prominently to the high cross-linking value.[261,262] A direct correlation between its contribution to high cross-linking of the peptidoglycan, resulting in a stiff peptidoglycan, was seen.[263] Highly cross-linked peptidoglycan has value with respect to antibiotic resistance. For example, the glycopeptide antibiotic vancomycin is still used for the treatment of β-lactam-resistant (MRSA) infection.[264] Vancomycin disrupts peptidoglycan biosynthesis by formation of a stable complex with the D-Ala-D-Ala segment of the stem terminus of Lipid II and of nascent peptidoglycan.[265,266] A mechanism used by *S. aureus* to attain vancomycin resistance is to simultaneously thicken its peptidoglycan cell wall and to reduce PBP4 expression. This pairing achieves a surface abundance of D-Ala-D-Ala-containing stems that trap vancomycin at the cell surface in order to limit access of vancomycin to the inner-wall zone location, where the same complexation would inhibit peptidoglycan synthesis.[267–269] Conversely, mutation of the *pbp4* promoter to effect high level PBP4 expression preserves high-level cross-linking and imparts (PBP2a-independent) high-level β-lactam-resistance.[259,270–273] An additional contributing factor to this PBP4 effect is an ability (albeit limited) to effect the hydrolytic destruction of β-lactams (that is, a β-lactamase activity, similar to what is seen for selected cPBPs of *Escherichia coli* and *Pseudomonas aeruginosa*).[258] PBP4 also augments β-lactam resistance, particularly in community-acquired *S. aureus* infection,[274] of PBP2a-possessing *S. aureus* (MRSA).[250,275,276] A resistance role for PBP4 by MRSA bacteria is consistent fully with the observation that PBP4 is unreactive to inactivation by the newest generation cephalosporin β-lactams that are optimized structurally for the concurrent inactivation of PBP2 and PBP2a.[250,270,271,277] PBP4 may, however, be susceptible to inactivation by the emerging 7-oxo-1,6-diazabicyclo[3.2.1]-octane-2-carboxamide (DBO) class of β-lactamase inhibitors.[278]

An explanation for these observations is PBP4 acting as a “perfecting” transpeptidase in septal peptidoglycan biosynthesis, whose activity trails catalysis by PBP2. Substantial data support this explanation.[206,262,279] For example, the USA300 MRSA strain is fully resistant to the β-lactam oxacillin (MIC 256 mg L^{-1}). Addition of a membrane-disrupting small molecule collapses the proton-motive force (PMF) with concurrent delocalization of PBP2 and PBP4 from the septum, with a 256-fold reduction in the MIC of oxacillin.[225] The

delocalization of PBP2 under this circumstance can be understood in terms of disrupted Lipid II availability (as MurJ function is PMF-dependent). PBP4, however, is not easily understood as an enzyme responsive to Lipid II availability. The concurrence of PBP2 and PBP4 delocalization is more easily interpreted as suggesting an association mechanism (direct or indirect within the divisome) between the two PBPs. Moreover, the possibility that the structural character of the cross-links formed by PBP4 may be distinctly different from the structural character of the cross-links formed by PBP2 is suggested by the unusual cyclic peptidoglycans formed in vitro by PBP4 catalysis.[262,280] A mechanistically compelling observation is the loss of PBP4 localization at the septum upon small-molecule disruption of WTA biosynthesis.[281,282] This loss of PBP4 localization is also seen upon exposure of *S. aureus* to cationic polymers that interfere with cell-envelope formation by complexation to the WTA.[283–285] As was the case with PMF disruption, disruption of WTA biosynthesis results in a significant lowering of β-lactam MIC values for the MRSA strains.[282,286]

The entire sense of the preceding discussion coincides with a septal location for PBP4. The ability of some PBPs to recognize and incorporate fluorophore-functionalized D-amino acids in place of D-Ala of the stem of the peptidoglycan has emerged as a powerful method for probing peptidoglycan synthesis.[287–292] As the only PBP of *S. aureus* with this ability is PBP4, this method of fluorescent imaging revealed its location.[293–295] Gratifyingly, this imaging confirmed a substantial septal location for PBP4 but additionally showed a diffuse, but equally substantial, presence of PBP4 across the entirety of the *S. aureus* lateral wall (Figure 4). As seen also from the previous studies, concurrent WTA synthesis was required to preserve localization of PBP4 to the septum.[295]

2.1.5.5. PBP2a.: All bacteria have multiple PBPs, and the different β-lactam structures (whether penicillin, cephalosporin, carbapenem, or monobactam) have different affinities for these PBPs. A given β-lactam structure may inhibit only a nonessential PBP and thus lack clinical utility. A different β-lactam structure may inhibit modestly an essential PBP. It, too, will lack clinical utility. Given the complementary value brought to *S. aureus* by each of its four PBPs, one might conjecture that the most efficacious β-lactam for MRSA chemotherapy is the β-lactam structure that inhibits potently all four simultaneously. If it were possible to translate conjecture into chemical structure, one would have this result. Such translation is not, however, possible. Nor is a pan-PBP inactivator necessarily desirable. Cell-envelope creation is subjected to exquisite control and monitoring, and all bacteria respond to antibiotic interference by activation of stress and resistance networks.[296] The design of β-lactams that are selective for PBP inactivation is both desirable and structurally attainable. Thus, notwithstanding the fundamental evolutionary identity among all PBPs, the sequence variations presented by each enable selectivity for a given β-lactam structure for inactivation of one (or for a limited copy number of PBPs) to the exclusion of the other PBPs of the bacterium. By empirical manipulation of structure, β-lactams progress to the clinic as a result of (among many other criteria) an optimized pairing between PBP essentiality and PBP vulnerability. The PBP with this pairing for *S. aureus* is PBP2. As noted above, the most recent and the most effective β-lactams for MRSA chemotherapy are cephalosporins with high efficacy for PBP2 inactivation but lack efficacy for PBP4 inactivation. Given the above discussion of the PBPs of *S. aureus*, the loss of function

effected by such a cephalosporin to PBP2 (as an aPBP) will be loss of its transpeptidase activity but not loss of its transglycosylase activity (as this active site is separate from that of its transpeptidase and this active site is not inhibited by β-lactams). One consequence of this loss of transpeptidase catalysis will be a failure to synthesize a structurally robust septum.

Evolution selects answers governed neither by simplicity nor by logic but selects answers that work. The answer selected by MRSA is remarkable: the acquisition, from another *Staphylococcus* species, a gene for a bPBP (having only a transpeptidase active site) that is intrinsically less reactive to β-lactam inactivation and that spatially accommodates with PBP2 as well as the other proteins and enzymes required for peptidoglycan creation.[275,297–300] Circumstantial evidence implicates formation of a PBP2·PBP2a complex,[257,301] wherein the septal peptidoglycan synthesis is completed by cooperative transglycosylase catalysis (provided by PBP2) and transpeptidase catalysis (provided by PBP2a). The number of copies of PBP1–PBP4 per bacterium is not significantly different comparing susceptible (MSSA) and resistant (MRSA): approximately 175 copies of PBP1; 450 copies of PBP2; 175 copies of PBP3; and 290 copies of PBP4. The major PBP of MRSA is PBP2a with 825 copies.[192] Moreover, there is implicit cost to the presence of PBP2a. Possession of the gene alone for this enzyme is insufficient for the MRSA phenotype. The gene is governed by an elaborate regulatory mechanism that ensures its expression only when β-lactams are present. The complexity of this regulatory mechanism, the structure of the PBP2a enzyme, and the allosteric regulation of its enzymatic activity are complementary phenomena. This complementation is discussed below.

2.2. *S. aureus* Cell Envelope beyond the Peptidoglycan

The seamless integration of peptidoglycan biosynthesis with creation of the three other entities of the cell envelope—the wall teichoic acids, the lipoteichoic acids, and the membrane—is not merely important but is critical to the viability of the *S. aureus* bacterium. Failure of any one entity can be lethal (such as β-lactam inactivation of the PBPs). Disabling (as distinct from causing failure) the proper interlocking of these four can increase the sensitivity of the bacterium to an antibiotic, to which the bacterium would otherwise be resistant. Over the past decade a deluge of studies has addressed essential interconnections among each structure of the cell envelope: the LTAs, the WTAs, the peptidoglycan, and the membrane. In this section, we outline the present status of the complex relationship among the WTA, LTA, the peptidoglycan, and the β-lactam antibiotics.

2.2.1. Wall Teichoic Acids.—The “acid” component of LTA and WTA nomenclature reflects their chemical identity as polymeric phosphodiesters, with the phosphodiester having an acidic proton and thus at neutral pH for both (if not further modified structurally) highly anionic character. The structures of the *S. aureus* WTA and LTA are shown in Scheme 2. Whereas LTA structure is relatively conserved among Gram-positive bacteria, WTA structure is not. Variations among the teichoic acid structures are reviewed.[302] Given the emergence of the teichoic acids as essential to an understanding of antibiotic resistance, their role (no longer “secondary”)[303] as polymers of the cell wall has been reviewed from different vantages.[53,54,78,131,304–309] These reviews show a transition in the understanding of the teichoic acids from incidental structural entities of the cell envelope, to recognition

that each (the WTA and the LTA) confers critical and essential character. The LTAs of the cell envelope are long recognized as structurally essential under almost all circumstances,[310] whereas the WTAs are not.[311] Nonetheless, WTAs are essential with respect to virulence and antibiotic resistance.[312] Accordingly, the identification of vulnerable enzymes within the teichoic acid biosynthetic pathways using potent small-molecule inhibitors has confirmed the potential of these inhibitors to subvert antibiotic resistance.[42,44,286,313–315] We address this theme in terms of WTA structure, biosynthesis, and character.

The presumption that WTA biosynthesis is tightly integrated with peptidoglycan biosynthesis is proven.[187,316,317] A schematic summary of the enzymes found at the cell membrane involved in WTA biosynthesis (and also peptidoglycan and lipoteichoic acid biosynthesis) is given in Figure 5. Foundational parallels between peptidoglycan and WTA biosynthesis include the use of undecaprenol as the lipid carrier with biosynthetic assembly in the cytoplasm, translocation across the membrane of penultimate intermediates, and further structural maturation as the WTA is incorporated covalently into the peptidoglycan.[77,318] *S. aureus* WTA biosynthesis is divided between cytoplasmic and inner-wall zone events. The first step of TarO-catalyzed synthesis of Lipid III (undecaprenyl-pyrophosphoryl-GlcNAc, sometimes referred to as Lipid α) is followed by TarA-catalyzed synthesis of Lipid IV (undecaprenyl-pyrophosphoryl-GlcNAc-ManNAc, sometimes referred to as Lipid β); TarB-catalyzed addition of a short repeat of the glycerol phosphate (GroP) linker; TarF-catalyzed priming addition of a ribitol phosphate repeats followed by TarL-catalyzed poly ribitol elongation; TarM or TarS addition of flanking GlcNAc saccharides to the ribitol phosphate segments; and ATP-dependent translocation across the membrane by the TarGH transporter to the inner wall zone.[307,319–323] Two events occur in the inner-wall zone. The WTA is further decorated by D-Ala esterification of the ribitol phosphate (as shown in the structure in Scheme 2). Transfer of the entire glycosyl assembly to the C-6 alcohol of the MurNAc of the peptidoglycan is catalyzed by the so-called LCP enzymes.[324] The LCP abbreviation derives from three proteins (LytR, CpsA, Psr noted originally in *B. subtilis* as encoded within a family of genes) involved in WTA transfer to the peptidoglycan.[325,326] *S. aureus* has three LCP enzymes, LcpA–LcpC. Although each is capable of WTA transfer to the peptidoglycan,[327] there is a hierarchy. The primary LCP catalyst for WTA transfer is LcpA.[327–329] LcpC is the primary catalyst for the transfer of capsular polysaccharides to the same MurNAc alcohol locus.[80,330] The function of LcpB is uncertain but may correspond to recognition of particular structural modifications made to the WTA (or to the peptidoglycan) for WTA addition to the peptidoglycan.[331] Following transfer, the WTA percolates through the polymeric peptidoglycan to represent a significant structural entity of the *S. aureus* cell surface.[91,332] Substitution by D-Ala of the WTA, in the form of an ester linkage, is catalyzed by the enigmatic Dlt pathway interconnecting the LTA and WTA structures.[333] This terse summary of WTA biosynthesis and its incorporation into the peptidoglycan might appear to be topics unrelated to β-lactam resistance by *S. aureus*, but for the fact that both modifications to the ribitol phosphate segments of WTA—those of GlcNAc glycosylation and of the D-Ala esterification, as well as completion of WTA biosynthesis by successful Lcp transfer to the MurNAc saccharide—are profoundly consequential to the antibiotic efficacy of the β-lactams (and other antibiotics) and to *S. aureus* virulence. This interrelationship is summarized.

The GlcNAc saccharides appended to the WTA are not decoration. The two glycosyltransferases of *S. aureus* (TarS and TarM) differ with respect to the resulting stereochemistry of the anomeric linkage.[334–336] TarS catalysis incorporates a β-GlcNAc (as shown in the structure in Scheme 2) while TarM catalysis incorporates an α-GlcNAc.[337,338] GlcNAc presence (regardless of the anomeric nature) is required for nasal colonization.[339] Otherwise, TarM/TarS function is regulated by the environmental circumstance of the bacterium. For example, TarS activity is favored in media with high salt, and in pharmacological assay MRSA Newman strains showing initially preferential TarM activity transform to preferential TarS activity during infection.[340] This change may reflect both structural adaptation (combining a steric effect and alteration of the electrostatic surface of the WTA by the positive charge of the D-Ala) and adaptation for immune evasion (a subtopic with broader ramifications, including with respect to vaccine development).[341–345] To the point of the theme of this review, genetic deletion of TarS transforms MRSA from β-lactam-resistant to β-lactam-sensitive.[346,347]

The pathway for D-Ala substitution of the WTA is enigmatic largely as a result of its complexity. Strong circumstantial evidence suggests that the D-Ala esters of the WTA originate from D-Ala esters of LTA.[348,349] As LTA biosynthesis occurs in the inner wall space, a mechanism is required for translocation of D-alanine (presumably, in the form of an active ester) from the cytoplasm to the inner-wall space, via the transmembrane DltB protein of the DltABCD pathway, for DltD-catalyzed LTA esterification.[333,350] The mechanism for D-Ala transfer from the LTA to the WTA is uncertain. Enhanced D-Ala esterification of the WTA is a key component of the resistance pathway to the multimechanism (pleiotropic) and peptidoglycan-pathway-interacting antibiotic daptomycin, a calcium-dependent lipopeptide. The principle mechanism for daptomycin is suggested to be disruption of undecaprenol phosphate recycling, as the result of its formation of a stable complex among undecaprenol diphosphate-containing entities (of peptidoglycan, WTA, and capsular saccharide biosynthesis) in the presence of the phosphatidylglycerol lipid of the membrane.[351] The complex resistance response of *S. aureus* to daptomycin involves upregulation of the formation of WTA and the DltA activity and increased peptidoglycan thickness.[352–357] A molecular mechanism basis for these alterations is not known. Small molecule inhibition of DltB sensitizes *S. aureus* to aminoglycosides and cationic antimicrobial peptides and is lethal when combined with an inhibitor of WTA biosynthesis.[45,358] The relationship of the D-Ala content of the teichoic acids and β-lactam-resistance has not been well studied. Among the *fem*-resistance responses of *S. aureus* to β-lactams is expression of the PBP-like enzyme FmtA, that acts as D-esterase to reduce the D-Ala content of the teichoic acids.[359,360] In contrast, deletion of the Dlt pathway in *Enterococcus faecalis* sensitized this bacterium to β-lactams.[361]

Nonetheless, the importance of the teichoic acids as intact structural entities to *S. aureus* is proven with respect to a host of properties, including β-lactam resistance. WTAs block antibody recognition,[362,363] prevent lysozyme access to the peptidoglycan,[364] block infection by some phages,[365] reduce dye sensitivity,[366] enable colonization,[366–368] and facilitate horizontal gene transfer.[369] Disruption of WTA biosynthesis in *S. aureus* by genetic deletion of LcpA gave an altered cell morphology, loss of virulence, and increased β-lactam sensitivity accompanied by release of the WTA to the medium.[326,370–372] Likewise, deletion

of the LcpC enzyme gave morphological changes, increased the sensitivity of MRSA and MSSA to both β-lactam and glycopeptide antibiotics, and reduced their ability to colonize epithelial cells.[79] The basis for these effects (whether the result of impairment of WTA or to capsular polysaccharide incorporation) was not determined. The potential value of Lcp enzyme inhibition—enzymes without a eukaryotic equivalent—with respect to *S. aureus* chemotherapy is evident.[79,329] Equally compelling data emerged across a series of papers examining inhibitors of the enzymes of WTA biosynthesis. Tunicamycin is a natural product inhibitor of TarO, the first enzyme of WTA biosynthesis, and blocks WTA incorporation into the *S. aureus* cell envelope and causes septal-growth defect.[43,372–375] It also inhibits MraY.[155,158,163,376] Nonetheless, tunicamycin is not suitable as an antibiotic due to eukaryotic toxicity. Its structural modification favorably altered this balance to give tunicamycin analogs showing β-lactam synergy,[377] confirming previous observations showing a 16–64-fold MIC decrease for β-lactams for MRSA bacteria in the presence of 0.4 mg L^{-1} tunicamycin.[43] Consistent synergy is seen between β-lactams and TarO inhibitors, across TarO inhibitors of different structure.[378–380] Genetic deletion of TarO from MRSA strains restores β-lactam susceptibility.[282] Extensive SAR optimization of a TarO inhibitor toward improved drug-like character gave structures lacking antibacterial activity but efficacious in combination with thienamycin (a carbapenem) in a pharmacological assay of MRSA infection.[347] Restoration of β-lactam efficacy is also observed with inhibitors of the TarGH transporter.[381,382] The most studied TarGH inhibitor, targocil, has intrinsic antibacterial activity (MIC 2 mg L^{-1} for both MSSA and MRSA).[383] Although targocil failed to synergize with β-lactams against MRSA in vitro,[384] the pairing (and especially with a targocil derivative) was beneficial in pharmacological assays of *S. aureus* infection.[385] Moreover, while resistance development to targocil was relatively facile, the presence of subinhibitory β-lactam concentrations (0.2 × MIC) prevented the emergence of targocil resistance.[384] Targocil additionally suppresses MRSA autolysis by a mechanism suggested to coincide with entrapment of Atl, the major autolysin of *S. aureus*, in the membrane as a WTA complex. Failed WTA translocation results in failed Atl delivery.[386] The development of robust screening assays for WTA synthesis[387,388] will identify enzyme targets whose inhibition will synergize with inhibitors of WTA biosynthesis,[146] identify structures that are less protein-bound and less prone to resistance development, and achieve superior β-lactam synergy.[385]

The remaining topic is how WTA biosynthesis integrates with that of the peptidoglycan. Evidence for intimacy between the two biosynthetic pathways was presented previously with respect to the function of PBP4. Two studies offer further insight. TarO, the first enzyme of WTA biosynthesis, is recruited to the *S. aureus* divisome prior to PBP4.[281] In the absence of TarO, the *S. aureus* peptidoglycan is significantly less cross-linked and is more susceptible to lysozyme degradation. The recruitment of the remaining enzymes of WTA biosynthesis is presumed coincident with that of TarO. Second, Lcp-catalyzed transfer of the WTA to the peptidoglycan requires un-cross-linked peptidoglycan (Lipid II is not an Lcp substrate).[331] WTA incorporation into the peptidoglycan may be understood as a mechanism for positioning across the septum of the Atl autolysin, so as to enable its eventual activation for the controlled degradation of the septal peptidoglycan that is required for cell separation.[43,281,389] Final cross-linking by PBP4 (as PBP4 catalysis

follows that of PBP1 and PBP2/PBP2a) contributes to this positioning and secures the nearly complete cross-linking of the peptidoglycan that advantages *S. aureus*. The selective affinity of PBP4 for septal WTA, but not that of peripheral wall WTA, implicates a key point of structural difference between the two WTAs.[281] Scanning-electron microscopy clearly shows a smoother surface texture of new septal cell envelope compared to a much more textured mature cell-envelope surface.[214,215] Whether this difference reflects maturation of the WTA structure is not known. WTA incorporation must follow initial peptidoglycan strand synthesis by FtsW·PBP1 (wherein only selective cross-linking of the peptidoglycan must occur) and presumably precedes PBP2/PBP2a catalysis. Recognition of the WTAs as temporal and spatial regulators of peptidoglycan cross-linking is now well-supported.[43,281,309,331] How this regulation fits into the biosynthetic transition of nascent peptidoglycan to a three-dimensional, multilayered peptidoglycan is not yet known. For the moment, it is sufficient that inhibition of the biosynthesis of the WTAs is a viable means of restoring β-lactam efficacy.

2.2.2. Lipoteichoic Acids.—The lipoteichoic acids (LTAs) are the second of the two glycopolymers of the cell envelope of the Gram-positive bacterium. Their structures are more conserved among Gram-positive bacteria as compared to the WTAs.[348,390] Whereas loss of WTA biosynthesis is disabling, loss of LTA biosynthesis profoundly compromises bacterial viability. LTAs contribute to a host of essential cell properties including growth, stability, virulence, and division.[391] The assertion that the LTAs and WTAs have complementary function has support.[392,393] The relative conservation of LTA structure has additional consequences. The LTA structure is the target of antibiotics (notably antimicrobial peptides),[394,395] is exploited in immune recognition and evasion,[306,396] and offers candidacy for vaccine development.[397,398] The structure of the *S. aureus* lipoteichoic acid is shown in Scheme 2 (right border of the scheme). It is anchored to the outer leaflet of the membrane by a specific diglucosyl lipid (the Glc_2DAG glycolipid), functionalized by repeating glycerol phosphate (GroP) units that are decorated with D-Ala esters. This latter decoration is critical to their structural character.[45,349,399] Alternative decoration of the LTA by glycosylation occurs as a stress response of *S. aureus*.[400] The completed LTA structure intercalates into the peptidoglycan polymer but does not reach to the cell surface.[348,401]

The LTA biosynthetic pathway[308,348,402–405] in *S. aureus* in key respects is distinctive from WTA biosynthesis.[322,392,406] On the cytoplasmic side of the membrane UgtP-catalyzed sequential glucosylation of diacylglycerol gives Glc_2DAG, that is translocated to the inner-wall zone by the membrane transporter LtaA.[407] Addition of the $(GroP)_n$ units is catalyzed by LtaS with catalysis of D-Ala acylation by DltD of the DltAC/DltB/DltD system discussed earlier. In contrast to the covalent addition of WTA to the peptidoglycan that occurs to un-cross-linked peptidoglycan that remains subsequent to PBP1- and PBP2/PBP2a-catalyzed synthesis of septal peptidoglycan, the enzymes of LTA biosynthesis interact with each other and are proximal to, if not members of, the divisome.[401,408] Indeed, perturbation of LTA biosynthesis directly affects *S. aureus* peptidoglycan biosynthesis as evidenced by an increase in cell lysis and an increased sensitivity to β-lactams (MRSA COL, oxacillin MIC of 128 mg L^{-1}; MRSA COL Δ*ltaA*, oxacillin MIC of 16 mg L^{-1}; MRSA COL Δ*ugtP*, oxacillin MIC of 2 mg L^{-1}).[393]

This observation underscores the credibility of the argument (advanced by many of the authors cited) that concurrent inhibition of teichoic-acid biosynthesis could restore β-lactam efficacy against MRSA. However, evidence in support of this argument in the form of small molecule inhibition of LTA biosynthesis is limited. An inhibitor of LtaS (IC_{50} 10 μM) was growth inhibitory and active in pharmacological models of *S. aureus* infection.[409] Substituted *N*-benzoyl-5-phenyl-1,3,4-oxadiazol-2-amine LTA inhibitors have MIC values as low as 0.125 mg L^{-1} against MRSA bacteria.[315,410] These structures synergized with tunicamycin (concurrent inhibition of WTA), but synergy with β-lactams is not yet reported. Naclerio and Sintim argue forcefully that disruption of the interconnection between the peptidoglycan and the LTAs (as well as the other pathways of the cell envelope) is an opportunity for antibacterial discovery.[411]

2.2.3. Membrane.—A theme to the preceding discussion is the importance of order—however poorly understood—to every aspect of bacterial-envelope biosynthesis. The final component of the Gram-positive envelope, the membrane, is involved in this order. Ordering of bacterial-envelope biosynthesis includes protein–protein interactions on and within the membrane, and as the milieu for substrate availability. An example of the former is the dissociation (variously described as mislocalization or delocalization) of PBP2 from the divisome upon disruption of Z-ring formation by FtsZ-binding inhibitors.[223] An example of the latter is the Lipid II biosynthesis as the basis for recruitment of PBP2 to the divisome.[257] The membrane also is central to antibiotic mechanisms. Daptomycin is a clinically used antibiotic monotherapy against resistant Gram-positive bacteria (including MRSA)[412] that acts primarily to complex the undecaprenol diphosphate segment of these intermediates (including Lipid II) in cell-envelope biosynthesis.[351] Daptomycin in combination with a β-lactam (ceftaroline, itself with MRSA efficacy) shows improved clinical efficacy for MRSA bacteremia compared to vancomycin monotherapy,[413,414] as is also seen in vitro (with other β-lactams) in previous studies.[415,416] Resistance mechanisms against daptomycin are complex.[417] With respect to combination with β-lactams, however, increased daptomycin resistance correlates to increased β-lactam susceptibility (a seesaw effect).[418–420] Circumstantial evidence correlates this seesaw effect with daptomycin-induced alterations in the composition (or microdomains) of the bacterial membrane.[421–423] Additional studies are consistent with alterations in the lipid composition of the membrane as a mechanism for daptomycin resistance.[424–426]

Other studies demonstrate enhanced β-lactam efficacy toward *S. aureus* when nonantibiotic, potentiator structures closely associated with membrane binding are copresent. These potentiator structures include farnesol,[427–429] epicatechin gallate (a flavanol ester),[430–433] baicalein (a trihydroxyflavone),[434] other flavones,[435] clerodane (an oxygenated diterpene),[436] a 2-(trifluoromethyl)quinoline-4-ol derivative,[225,437] and cidazine.[438–440] Altered lipid composition (loss of cardiolipin) of the *S. aureus* membrane contributes to thioridazidine resistance.[441] However, the in vitro synergy of thioridazine with the β-lactams was not seen in pharmacological models of infection,[442–444] and β-lactam synergy was lost upon structure–activity study for the optimization of the MIC value.[445] The inability to translate the in vitro phenomenon to in vivo performance is never a surprise. Here, however, uncertainty as to the active structure (thioridazine or a

photochemistry-derived product) may account for this uncertainty.[446] SAR development of the flavone kaempferol (notably by homologation with a pair of arginine residues) gave a dicationic derivative with comparable efficacy (at different concentrations) as vancomycin in a *S. aureus* murine corneal infection assay.[447] This same study verified a membrane mechanism. Additional perspectives on this topic are provided by the venerable lantibiotic, nisin, that is widely used in food preservation, and the recently discovered nonribosomal depsipeptide, teixobactin.[448] The central event in the mechanism of both nisin[103,449–451] and teixobactin[176,177,452] is Lipid II binding. Nisin and β-lactams synergize.[453] Although synergy between teixobactin and β-lactams has not been shown, teixobactin suppresses the biosynthesis of both the peptidoglycan and the teichoic acids.[454]

In many of these studies (including those showing enhancement of β-lactam efficacy) the mechanistic commonality among structure, membrane, and effect is not identified. This identification is extraordinarily difficult experimentation, and ultimately it is clinical performance rather than mechanistic understanding that is paramount. Membrane effects can encompass pore formation, potential dissipation, and bilayer destabilization in addition to alteration of protein–substrate or protein–protein interactions critical to an essential pathway. The membrane is an increasingly recognized antibacterial target to achieve a multitargeting effect so useful to the suppression of antibacterial resistance.[455,456]

3. RESISTANCE MECHANISMS OF *S. AUREUS* AGAINST THE β-LACTAMS

The emphasis of the preceding discussion is the cell envelope of *S. aureus* as a structurally integrated and interdependent network of the peptidoglycan, the teichoic acids, and the membrane. The historical preeminence of the peptidoglycan, as the target of the β-lactams, is now understood to reflect the preeminence of the β-lactams as antibiotics, rather than as a measure of a greater importance of the peptidoglycan as a component of the cell envelope. As discussed in this section (and reiterated in a later section of this review) future chemotherapy of *S. aureus* infection will likely involve multiagents to incapacitate simultaneously multitargets, or single agents that engage multitargets of the cell envelope. This future reality is a consequence of the current, and already powerful, ability of *S. aureus* to dissipate the effectiveness of the β-lactams as antibiotics. In this section we address this ability as context for the β-lactams as chemotherapy.

3.1. Pathogenic *S. aureus*

Clinical *S. aureus* is not monolithic. We introduced previously the clinical division between *S. aureus* that is β-lactam-susceptible (“methicillin-susceptible”, MSSA) and *S. aureus* that is much less β-lactam-susceptible (“methicillin-resistant”, MRSA). In practice additional divisions are meaningful, notably the MRSA strains that are encountered in the community (community-acquired MRSA or CA-MRSA) and in the hospital (hospital-acquired MRSA or HA-MRSA). The division between MSSA and MRSA is distinctive, as MSSA has one primary resistance mechanism to β-lactams, whereas MRSA has two mechanisms. Although CA-MRSA and HA-MRSA are currently clinically distinctive (different virulence mechanisms to abet their common resistance mechanisms) this distinction is likely to blur in the future. *S. aureus*, in all of its guises, is a clinical challenge.[39,457,458] The reality of

S. aureus as a spectrum of strains with perceptible geographic preferences and virulence mechanisms[459] is not the primary focus of this review. Our focus is the factors that define its β-lactam resistance.

Prior to introduction of the first penicillin (benzylpenicillin) to clinical use during the early 1940s, in a historical context, the then-common variants of *S. aureus* were broadly susceptible organisms. The first *S. aureus* resistance mechanism to penicillin was indeed identified prior to broad clinical use of the first-generation penicillins.[460] This mechanism was the production of a class A β-lactamase that hydrolytically destroyed the antibiotic. As described below, this mechanism resulted in obsolescence of the first-generation penicillins, which are susceptible to the action of this resistance enzyme. It was also the impetus for the first campaigns in the pharmaceutical industry to generate by semisynthesis additional analogs of penicillins that were not turned over by the *S. aureus* β-lactamase. These efforts resulted in methicillin, nafcillin, oxacillin, and cloxacillin, among others, in the late 1950s. Availability of these more-effective penicillins led to clinical selection of resistant strains of *S. aureus*, which came to be known as MRSA. This took place first in the United Kingdom in 1962, but shortly after dissemination was global, a scourge that persists to the present day. How the ever-changing clinical challenge has resulted in newer generations of β-lactam antibiotics has been reviewed.[24]

3.2. β-Lactam Resistance of Methicillin-Sensitive *S. aureus*

3.2.1. MSSA and MRSA.—The primary mechanism for β-lactam resistance in MSSA is possession of a *bla* operon containing the *blaZ* gene. BlaZ is a class A serine β-lactamase.[461,462] Expression of BlaZ is not constitutive. When expression of the *blaZ* gene is enabled, BlaZ efficiently hydrolyzes these penicillins (by the acylation–deacylation mechanism of Scheme 2) and thus protects the PBPs of MSSA from inactivation. First-generation cephalosporins (such as cefazolin) are poorer substrates of BlaZ (see, however, the discussion below). Second-generation penicillins (such as methicillin) are very poor substrates of BlaZ. Hence, MSSA is susceptible to these newer β-lactams. While MRSA almost always retains the BlaZ enzyme, it achieves resistance toward all but the newest cephalosporin β-lactams as a result of a second resistance enzyme. This enzyme is a new (additional) PBP termed PBP2a (formerly PBP2′). PBP2a is a bPBP having a single active site, used for the transpeptidase cross-linking of peptidoglycan strands. PBP2a uses a sophisticated allostery-controlled mechanism for its physiological peptidoglycan cross-linking reaction. In the absence of this allosteric trigger, the active site exists in a closed conformation, which precludes its inhibition by the typical β-lactam antibiotic.[463] The fifth-generation cephalosporins ceftaroline and ceftobiprole would appear to be an exception. As documented for ceftaroline, it indeed binds to the allosteric site to subvert allostery.[464] In the detailed analyses of conformational changes documented by mechanistic studies, X-ray analysis, and computation, this interaction leaves the active site accessible to another molecule of the β-lactam antibiotic, and this molecule inactivates the enzyme.[464–466] The typical β-lactams of earlier generations cannot inactivate the transpeptidase activity of PBP2 as they do not bind to the allosteric site effectively to trigger the requisite conformational change. Simultaneously, the closed active site deprives the antibiotic from inhibiting the enzyme. This failure of inhibition of PBP2a by β-lactam antibiotics leads to septal

peptidoglycan biosynthesis proceeding by the complementary mechanistic pairing, achieved by PBP2·PBP2a, of the transglycosylase activity of PBP2 with the transpeptidase activity of PBP2a. The evolving statuses of MSSA and of MRSA are presented in the following sections.

3.2.2. BlaZ and MSSA β-Lactam Resistance.—The staggering clinical success of the first-generation penicillins against *S. aureus* (at first, limited only by the availability of the penicillins) was short-lived. The acquisition by *S. aureus* of a plasmid containing the *blaZ* operon enabled *S. aureus* to detect the presence of β-lactams and to respond by derepression of the *blaZ* gene of this operon (discussed in section 3.2.4). The ability of the BlaZ β-lactamase to counter the efficacy of these penicillins (such as benzylpenicillin, Chart 1) was addressed by medicinal chemists through empirical structure–activity exploration. Replacement of the phenylacetic acid side chain of benzylpenicillin with an *ortho*-substituted benzoic acid side chain gave penicillins that were poor BlaZ substrates. One of the earliest of these new penicillins was methicillin. Accordingly, notwithstanding the presence of the BlaZ β-lactamase, these *S. aureus* strains were methicillin-susceptible. The abbreviation MSSA followed. The vast majority of modern MSSA strains produce BlaZ, and those that do not are uncommon. In short order, however, methicillin was replaced with the structurally similar penicillins oxacillin and flucloxcillin (Chart 1). These penicillins (referred to as antistaphylococcal penicillins) had superior oral and pharmacokinetic properties compared to methicillin. Methicillin is no longer used clinically, but its appellation with respect to *S. aureus* β-lactam resistance persists. Historical and evolutionary perspectives on this progression, and continuing with the acquisition of the *mec* operon for expression of PBP2a to give the MRSA organisms, are reviewed elsewhere.[8,462,467–469]

3.2.3. BlaZ β-Lactamase and Current β-Lactam Chemotherapy for MSSA.—BlaZ is encountered both as a lipoprotein and as a soluble protein, representing two separate processing pathways.[461,462,470] Recognition of an N-terminal “lipobox” in the BlaZ sequence results in the covalent attachment of BlaZ using a cysteine within the lipobox sequence to a lipid of the outer leaflet of the membrane.[470–472] As a result these BlaZ enzymes colocalize with the PBPs in the inner-wall space of the cell envelope. The BlaZ lipoprotein is present in membrane vesicles[473] released by *S. aureus* as a virulence mechanism.[474–477] An equal portion of BlaZ is released to the media.[470] Clinical surveys of MSSA strains often show 90% as BlaZ-positive,[478] but with different proportions of the four common BlaZ isozymes.[479,480] One isozyme was one of the first β-lactamases to have its structure solved crystallographically.[481] The BlaZ isozymes accept first-generation penicillins as substrates, and as poorer substrates also first-generation cephalosporins.[479,482] As a consequence, the in vitro MIC values for these strains for the clinically used cephalosporin used to treat MSSA infection, cefazolin, show an inoculum effect.[478,483,484] The causative role of BlaZ is proven by the disappearance of the effect in the presence of clavulanic acid, the clinically used inactivator of many serine β-lactamases including BlaZ.[485] Since the renal safety of cefazolin is superior to that of the antistaphylococcal penicillins[486–488] the possibility for clinical failure with cefazolin is a topic of current discussion.[482,489–491] This possibility—and the further possibility that use of cefazolin will

select for MSSA strains with BlaZ-conferred resistance—is supported by the appearance of β-lactam "borderline-resistant" MSSA strains[492] that combine PBP mutation and BlaZ hyperexpression toward clinical β-lactam resistance.[493–495] The recent isolation of an oxacillin-resistant MSSA strain (that is, lacking PBP2a, but having the diagnostic phenotype of a MRSA strain as the result of six point mutations within its BlaZ enzyme) is interpreted as a troubling indicator of yet further challenge with respect to future β-lactam chemotherapy of *S. aureus* infections.[496] It is an oddity that mutations in the β-lactamase gene that would confer broader resistance to β-lactam antibiotics, as commonly are seen in Gram-negative bacteria, was not seen previously in *S. aureus*.

3.2.4. BlaI of the *blaZ* Operon.—Our introduction of BlaZ as the protein, with only passing mention of its gene, was purposed. While BlaZ is the primary β-lactam resistance mechanism of MSSA and PBP2a is the defining resistance mechanism of MRSA, at the genetic regulatory level the two resistance mechanisms have profound similarity and commonality. In MSSA the *bla* operon encodes three proteins (BlaZ, BlaR, BlaI). In MRSA the *mec* operon encodes three proteins (PBP2a, MecR, MecI). Some strains might have both operons. Both operons are found on mobile genetic elements. The *bla* operon is typically found on a plasmid or on an integrated transposon (infrequently, is chromosomal) while the *mec* operon is most commonly on an integrated transposon. The primary function of each protein of these operons is known.[498] BlaI is the repressor protein of the *bla* operon, and MecI is the repressor protein of the *mec* operon.[499,500] BlaR and MecR are β-lactam sensor/signal transducer proteins. That is, both BlaR and MecR are transmembrane proteins possessing both a cytoplasmic domain and an inner-wall zone domain. For both, their inner-wall zone domain senses the presence of β-lactams by covalent chemistry and transduces its sensing through the membrane so as to activate the cytoplasmic domain. Structural and functional homology is found between BlaI and MecI and between BlaR and MecR. In point of fact, most clinical MRSA strains control the *mecA* gene (for PBP2a) expression through BlaI as the repressor protein for both operons,[501] suggesting an importance to the coordinated expression of BlaZ and PBP2a.[502–504] There is circumstantial evidence in favor of BlaZ as a coprotective mechanism against β-lactams even in the presence of PBP2a. BlaZ can be exported (whereas PBP2a is a membrane-associated enzyme), and BlaZ is a less demanding biosynthesis as it is a protein of 257 amino acids, compared to the 668 amino acids of PBP2a.[503]

The repression mechanism used by BlaI is association to the DNA of the promoter of the operon. Although crystal structure analysis of the BlaI·DNA association shows that BlaI associates to synthetic palindromic DNA as a homodimer (under the crystallization conditions, and likewise for MecI association),[500,505,506] evaluation of the mono $\rightleftarrows$ dimer equilibrium (and also the BlaI·MecI heterodimer) indicates monomer involvement in transcription repression.[507–509] The mechanism for derepression of the operon is BlaI proteolysis, catalyzed by the cytoplasmic domain of BlaR (or MecR) following activation of BlaR (or MecR) by reaction with a β-lactam on the surface domain.[501,510–512] Regulation of BlaI may be anticipated to have multidimensional control. The affinity of the BlaI protein of *Bacillus licheniformis* (its *bla* operon is highly similar to that of *S. aureus*) with respect to DNA binding is reduced in the presence of peptidoglycan fragments. Full

derepression of the operon is suggested to involve both this allosteric regulation and BlaI proteolysis.[513] BlaI is a component of the resistance mechanism used by MRSA against the LL-37 antimicrobial peptide. Proteolytic degradation of BlaI, as a result of activation of BlaR by reaction of BlaR with a β-lactam, sensitizes MRSA to the LL-37 peptide.[514] Although this observation has not been connected at the molecular level to the cell envelope, it suggests that there are circumstances where transcription of the *blaZ* gene, in the absence of a β-lactam threat, has a fitness cost. As intimated by the preceding discussion, expression of BlaZ is regulated by the third protein of the operon, BlaR.

3.2.5. BlaR of the *blaZ* Operon.—BlaR is distinct from BlaI and BlaZ by its size, its transmembrane character, and its mechanistic complexity. Its complete structure is not known. Abundant circumstantial evidence indicates that the BlaR structure may be 3-fold parsed. One structural component is a C-terminal sensor domain, positioned by the transmembrane helices against the membrane surface and projecting into the inner-wall zone space. A gene construct of the sensor domain expresses a soluble protein (discussed below).[515,516] The second structural domain of BlaR (the N-terminus) is composed of the membrane helices. A computational model (supported by experimental data) for the homologous MecR protein postulates four N-terminal helices that transverse the membrane.[497] The fourth of these helices exits the membrane as the sensor domain. Two additional helices (between the third helix and the fourth helix) enter the membrane from the cytoplasm, bend, and exit the membrane into the cytoplasm. The cytoplasmic residues between the third and the fourth transversing helices organize in the cytoplasm to form the third structural domain, postulated as that of a zinc-binding gluzincin domain with proteolytic activity toward BlaI. The suggested organization of BlaR, as a presumed parallel to MecR, is shown in Figure 6. The current hypothesis for the function of BlaR is detection of the presence of β-lactams by the sensor domain; signal transduction through the helices so as to activate the gluzincin domain for recognition of BlaI as substrate. Proteolytic processing of BlaI results in derepression of the *bla* operon, leading to transcription of the *blaZ* gene.

Experimental interrogation of the soluble C-terminal sensor domain of BlaR clarified the sensing mechanism. Sequence analysis of this domain (*B. licheniformis*) showed homology to the Class D β-lactamases.[517] The mechanistic relevance of this homology was confirmed by comparison of the X-ray structures of the *B. licheniformis* sensor domain[518] and the *S. aureus* sensor domain[516,519,520] to the structures of Class D β-lactamases. Moreover, exposure of the soluble sensor domain to β-lactam antibiotics resulted in acylation (with ring-opening of the β-lactam) of the active-site serine.[521] A key contrast is that Class D β-lactamases are catalytic,[522–524] via a mechanistic sequence of serine acylation followed by acyl-enzyme hydrolysis, whereas serine acylation of the sensor domain is functionally irreversible. One structural feature of the Class D β-lactamases explains this difference. For both proteins serine acylation by the β-lactam is the first event. Nucleophilic character is imparted to both serines by the same general-base activation mechanism: prior reaction of an active-site lysine with CO_2 to form a carbamate functional group ($RNHC(O)O^-$).[516] The carbamate anion hydrogen bonds to the alcohol functional group of the serine acting as the general base for serine activation as a nucleophile.[525] Carbamate formation is, however,

reversible. Its reversal (by CO_2 release) abolishes not only the ability of the serine to undergo β-lactam acylation but also the ability of the lysine carbamate to activate water for hydrolysis of the resulting acyl-enzyme. In Class D β-lactamases carbamate reversal during catalysis is infrequent. In the case of the sensor domain of BlaR, loss of the carbamate by decarboxylation (to give lysine as a catalytically incompetent amino acid) follows immediately after carbamate-catalyzed acylation of the serine by the β-lactam.[516,526–528] This β-lactam-derived acyl-serine is stable, and the β-lactam is now sensed.

While the molecular mechanism for signal propagation is that of a protein conformational change at the surface domain altering the conformation of a cytoplasmic domain, discerning the conformational path is challenging. BlaR is no different. Although the *S. aureus* and *B. licheniformis* sensor domains show differences in in vitro behavior, they show mechanistic consensus. Moreover, the MecR sensor domain is also included in this consensus.[529,530] Comparison of the crystal structures of the unacylated and β-lactam-acylated sensor domains unexpectedly showed only a subtle structural difference with respect to the protein. The key difference is adjustment of the entire BlaR protein to the now stably incorporated acyl moiety of its sensor domain. Altered contact between the sensor domain and the rest of the BlaR protein[518–520] and also for MecR[529] as the basis for receptor signaling is consistent with the observation that the strength of the signal propagation depends on the structure of the β-lactam that acylates the sensor domain. The proposed locus for this altered contact is the interface between the sensor domain and the loop that interconnects, in the inner-wall zone, the second and third transmembrane helices (the “L2” loop).[531,532] Steric conflict contact between the loop—organized as an amphiphilic peptide embedded on the surface of the membrane—and the covalently bound antibiotic is consistent with dynamic NMR evaluation of the complex between a peptide matching the loop sequence and the sensor domain[533–535] and combined computational and experimental study of full-length MecR.[497] As discussed in the following section, BlaR is awoken as a catalyst.

It is noted in passing that protein constructs of the BlaR sensor domain are of interest for the analytical detection of residual β-lactams in food.[536,537]

3.2.6. BlaI Proteolysis Following BlaR Activation.—BlaI represses transcription of the *blaZ* gene, and in many clinical strains of *S. aureus* BlaI also represses the *mecA* gene for PBP2a.[504,509,538,539] In *S. aureus* (the mechanism for *B. licheniformis* may not be a full parallel) derepression results from the intracellular loss of BlaI as the result of BlaR-catalyzed proteolysis.[510,540–542] The mechanism for the acquisition of proteolytic activity by BlaR, as a result of sensor-domain acylation by a β-lactam, is better studied in the homologous MecR system. With reference to the current model for *S. aureus* MecR,[497] the polypeptide sequence (amino acids 147–314) between the third and fourth transmembrane helices has a zinc-binding gluczincin protease motif.[543,544] The polypeptide is postulated to have both cytoplasmic and intramembrane organization, with an intramembrane location for the zinc-containing active site.[497] Detachment of the sensor domain from the L2 loop effects a structural reorganization of the gluczincin motif to proteolytic competency with respect to BlaI as substrate. Turnover of BlaI by this hydrolysis event exposes the genes of its operon for transcription. The mechanism for deactivation of the proteolytic activity of BlaR—that is, reversal of the antibiotic-resistance phenotype—is autoproteolysis.[498,541]

The longevity of BlaR sensor domain acylated by β-lactam antibiotics often exceeds the duration for several generations of *S. aureus* growth.[516] As such, once the *bla* and/or *mec* operons have been activated, reversal cannot be achieved by hydrolysis and dissociation of the signaling entity (the β-lactam antibiotic) from the surface domain. When the antibiotic challenge is absent, BlaR undergoes proteolytic degradation at three known sites to reverse expression of the *blaZ* gene for BlaZ (and *mecA* for PBP2a) resistance enzyme(s).[498,541] In a noteworthy evolutionary selection, the *bla* and *mec* operons, even in the presence of their repressor proteins, are basally "leaky". Thus, there is gradual production of BlaR (MecR) and BlaI (MecI) anew.[508,509] Replenishment of BlaR enables its resumption as the vanguard sentinel for future β-lactam encounter, as BlaI represses the transcription of genes whose products are no longer needed.

3.2.7. PBP2a as the Primary Resistance Mechanism of MRSA.—The phenotypic differences between MSSA (with BlaZ as the primary resistance mechanism against β-lactams) and MRSA (with BlaZ as the secondary resistance mechanism and PBP2a as the primary resistance mechanism against β-lactams) are substantial. Notwithstanding the fact that the *mec* operon in many MRSA strains is coregulated by BlaR of the *bla* operon, the *mec* complex (that includes the *mec* operon encoding PBP2a) has greater genetic variability and greater complexity compared to the *bla* operon.[21,545,546] The greater genetic complexity of the *mec* complex contributes significantly to MRSA virulence, albeit in many cases (for example, the beneficial acquisition of the arginine catabolic mobile element and the genes for the Panton–Valentine leucocidin) the molecular mechanisms that contribute to the virulence are uncertain.[547] A specific example of variability is the difference between the dominant operon (*mecA*) in MRSA and the appearance (in 2011) of a new *mec* operon (*mecC*).[10,11,548,549] The basis for β-lactam resistance for both *mecA* and *mecC* MRSA is complementation of the endogenous PBPs by an additional, and an intrinsically β-lactam-unreactive, PBP. The *mecA* PBP is PBP2a. PBP2a is a monofunctional transpeptidase. In the presence of a β-lactam the transpeptidase activity of the intrinsic PBP2 of *S. aureus* is lost to β-lactam acylation, while its transglycosylase activity is unaffected. In MRSA PBP2 and PBP2a complex to sustain peptidoglycan polymerization by synchronizing transglycosylation (catalyzed by PBP2) with transpeptidation (catalyzed by PBP2a). The mechanism for peptidoglycan polymerization by the PBP2c of *mecC* is different. While *mecC* MRSA human infection remains uncommon (it remains primarily zoonotic),[550] many clinical assays used to detect *mecA* MRSA do not detect reliably *mecC* MRSA and allow *mecC* MRSA to be misidentified as MSSA.[551–553] Although the PBP2c of *mecC* MRSA is homologous (63% sequence) to the *mecA* PBP2a and its gene expression is also β-lactam-inducible (but with different responses to oxacillin and cefoxitin as inducers),[549] the functional integration of *mecC* PBP2c into the PBP family for peptidoglycan biosynthesis does not involve complementation of PBP2.[554,555] *mecC* MRSA is susceptible to the non-β-lactams used clinically against *mecA* MRSA.[556] At this time neither the β-lactam unreactivity of PBP2c nor its protein–protein interactions as a PBP is understood.

In contrast, mechanistic study of PBP2a has progressed. A notable advance is the recognition, from crystallographic studies of PBP2a, that allosteric regulation is a fundamental component of its β-lactam resistance. At clinical concentrations of β-lactams

PBP2a distinguishes between substrate (favoring the peptide stem of its peptidoglycan substrate) and inactivator (excluding the β-lactam). A basis for the discriminating ability of PBP2a emerged from crystal structure studies.[557,558] These studies show a substantive conformational change coincides with catalysis.[559–562] Control of this conformational change occurs as a result of occupancy of an allosteric site on the PBP2a enzyme that is located 60 Å from the active site (Figure 7).[463,465,563] Ligand binding at this allosteric site effects a sweeping conformational motion that propagates from the allosteric site and culminates at the active site with the displacement of a gatekeeping loop (Figure 8). In analogy with other examples of allosteric regulation in proteins, the ligand bound at the allosteric site may stabilize a conformational state coinciding with an open active site, rather than effecting the active-site opening. Displacement of the gatekeeper loop gives access to the active site by substrate. The observation that the allosteric site in the PBP2a enzyme can be occupied by peptidoglycan (Figure 7)[463] suggests how allosteric regulation may govern the catalytic cycle of the PBP2·PBP2a pair. In this catalytic cycle sequential glycopolymer elongation by PBP2 requires positioning by PBP2a (now as an acyl-enzyme with respect to the peptide stem) for cross-linking transfer of the PBP2a acyl moiety to a neighboring peptidoglycan strand. However, release of the cross-linked strand does not complete the cycle. The PBP2·PBP2a pair must then translocate, in response to a constricting Z-ring, to an adjacent site of the peptidoglycan. During translocation, the allosteric site is unoccupied and the PBP2a active site is occluded by the gatekeeper loop. Only after successful translocation of the PBP2·PBP2a pair is the pair “in register” and the allosteric site reoccupied by peptidoglycan. Allosteric conformational change displaces the gatekeeping loop to enable PBP2a catalysis. Within the catalytic cycle when the PBP2·PBP2a pair is out of register and translocating, the active site of PBP2a is closed. PBP2a is protected during this time from inactivation.[464]

3.2.8. Antibacterial Disruption of PBP2a Allostery.—Discovery of the allosteric regulation of PBP2a was a key prelude to new Gram-positive antibacterial structures. Moreover, the experimental path identified by this prelude exemplifies a now fundamental approach toward the identification of structures with intrinsic antibacterial activity or as antibacterial adjuvants. Computational analysis of binding sites to identify small molecule ligands has proven value.[564] Computational analysis of the allosteric site of the PBP2a structure identified two new antibacterial classes. Optimized structures of both classes alone are antibacterial, and selected structures of each class additionally show synergy with β-lactams against *S. aureus* in murine pharmacological assay. The first class has a central 1, 2, 4-oxadiazole ring (Chart 3).[565] Comprehensive SAR exploration[566–568] led to exemplary structure **7** with potent MRSA bactericidal activity (MIC 1–4 mg L^{-1}), and in the mouse low clearance, a high volume of distribution, 41% oral bioavailability, and activity (at 40 mg kg^{-1} in the mouse neutropenic thigh model) against both oxazolidinone-sensitive and oxazolidinone-resistant MRSA strains.[569] A structure closely related to **7** was synergistic with oxacillin as the β-lactam.[570] Activity against other Gram-positive pathogens was also notable (*Staphylococcus epidermis*, MIC 1 mg L^{-1}; *Enterococcus faecalis*, 4 mg L^{-1} including a vancomycin-resistant strain; *Enterococcus faecium* 1 mg L^{-1}).[569,571] An empirically discovered *N*-acyl-1,3,4-oxadiazol-2-amine class, with similarity to **7**, has comparable antibacterial activity. This class is exemplified by structure **8** (MSSA, MIC 2 mg

L^{-1}; *S. epidermis*, MIC 0.062–0.25 mg L^{-1}; *E. faecium*, MIC 0.62–0.25 mg L^{-1}; *E. faecalis*, MIC 0.12–1 mg L^{-1} but with weaker activity against *Mycobacterium abscessus* MIC 32–64 mg L^{-1}).[572] Structures related to **9** are also active against *S. aureus* (MSSA, MIC 0.25 mg L^{-1}; MRSA, MIC0.125–1 mg L^{-1}) with either additivity or synergy with both β-lactams and daptomycin.[573] The target of these structures is not known. Experimental data are consistent with cell-wall targeting (as might occur as a result of disruption of PBP2a allostery).[574]

The second antibacterial class identified from computational search of the PBP2a structure is that of the 2,3-disubstituted quinazolin-4(3*H*)-one. This generic structure has broad Gram-positive antibacterial activity, with optimal activity coinciding with *meta*-substitution by a hydrogen bond donor/acceptor of a 3-phenyl ring, and *para*-substitution to the phenyl of a 3-styrenyl (or 3-phenylethyl) substituent at C-2.[575–579] Prototype structure **10** (Chart 4) has a carboxylate as the substituent of its N-3 phenyl and a 4-cyanostyrenyl substituent at C-2.[580] Quinazolinone **10** (and closely related structures **11–13**) potently inhibits *S. aureus* including MSSA, MRSA, vancomycin-resistant, and oxazolidinone-resistant strains. In almost all cases the MIC values are ≤0.25 mg L^{-1}.[581,582] Excellent activity was seen in murine models of MRSA infection. Extensive experimental evaluation of this structure confirmed the computational basis underlying its identification. Quinazolinone **10** is a cell-wall-acting antibacterial. Application of a high-frequency transposition assay for validation of the mode of action of *S. aureus* antibacterials[583] confirmed the PBPs as its MRSA targets, and PBP2a as the PBP with highest affinity. It bound to PBP2a at the allosteric site as seen by crystallographic analysis.[580] Related quinazolinone structures are active against both MSSA and *Mycobacterium tuberculosis* (such as **14**),[584–586] and scaffold-hopping (from a nitroquinazolinone) gave thieno-[3,2-*d*]pyrimidin-4(3*H*)-one structures (exemplified by **15**) that were active against *Clostridioides difficile*.[587] Phenyl substitution at N-3 in this structure was disadvantageous.

Three questions follow from these studies. The first question is how to extract from PBP structures, such as PBP2a, guidance for structure-based design. The ligand used in probing the allostery-modulated conformations of PBP2a is ceftaroline **6** (Chart 1),[463] a newest generation cephalosporin whose structure was optimized empirically to inactivate PBP2a.[588–590] Comprehensive molecular-dynamics study substantiated the allostery-driven conformational change of PBP2a[591] but failed to validate occupancy of the active site by ceftaroline, as is seen crystallographically.[463] This failure may reflect the limitation of crystallographic PBP structure. Crystalline proteins are homogeneous solids. Yet the only time that PBPs are ever homogeneous is within crystals. Endogenous PBPs organize as components of the elongasome and divisome. Future structural study must focus on PBP structure within these complexes. The second question is the full mechanisms of the oxadiazole and quinazolinone structures. These structures have potent activity against MSSA, *S. pneumoniae*, *M. tuberculosis*, *C. difficile*, and other Gram-positive pathogens, none of which have PBP2a. Whether this dimensionality reflects multi-PBP inhibition, or additional mechanisms, is not known. The last question is whether these structures, although significant as probes that identify and exploit weakness in the *S. aureus* resistome, represent a meaningful addition to *S. aureus* chemotherapy. There is no reason to believe otherwise: the structures are drug-like, potent in vitro, and efficacious in in vivo pharmacological

models. Nonetheless, the barrier to moving structures from pharmacological models to the clinic is enormous, and especially so for antibiotics.

3.2.9. Additional Regulatory Control of the PBPs.—The bacterial cell envelope is an integrated assembly of lipids, proteins, enzymes, and polymers. As the viability of the bacterium depends on preserving the quality of this integration, it is of no surprise that the pathways toward this integration are tightly regulated. While placing the PBPs as the only foci (or even principle foci) of this regulation is myopic, given the central place of the β-lactams in chemotherapy, it is understandable. The preceding discussions identified undecaprenol partitioning, Lipid II complexation, WTA-peptidoglycan coordination, membrane-divisome coordination, and allosteric regulation of PBP2a are five processes where pathway coordination underlies the assembly of the cell envelope. In each of these five processes disruption (such as by a small-molecule antagonist) has shown decisive potential to abet the antibacterial activity of the β-lactam. These five are (emphatically) not the only such opportunities. Three additional deserve mention: the ClpXP protease system, FtsZ polymerization to form the Z-ring, and kinase-dependent regulation of cell-envelope assembly merit specific mention.

3.2.9.1. ClpXP.: Cells benefit from a clearance mechanism for imperfect proteins and for proteins no longer needed. A “machine” used by bacteria (also mitochondria and chloroplasts) for this task is ClpXP, wherein ClpX is an ATP-dependent enzyme catalyst of protein unfolding and ClpP is a protease. ClpX and ClpP oligomerize to form a cylindrical structure with an internal degradation chamber. While the ClpXP system is not essential for in vitro bacterial growth, its advantage to the virulent *S. aureus* is recognized.[592,593] The structures of three ClpXP machines (isolated from different Gram-negative bacteria) were disclosed recently.[594–596] Conceptualization of the role of ClpXP as housekeeping is incorrect. The breadth of ClpXP function is now recognized to be as expansive as our understanding of its function is limited. Much of this understanding derives from modulators —inactivators and allosteric activators—of ClpP activity. β-Lactone structures such as **16** (Chart 5) acylate irreversibly the active-site serine of ClpP.[597,598] Structure **17** (shown as the racemate) exemplifies a more potent ester class of ClpP inactivators. Both enantiomers of **17** are active, but with different effects on the protein structure of the ClpXP machine (upon inactivation by one enantiomer, the machine dissociates into smaller oligomers while the other enantiomer inactivates but does not induce dissociation).[599] In contrast to these inactivators, the antibacterial mechanism of a class of acyldepsipeptides (ADEPs) is allosteric activation of ClpP.[600,601] A structurally optimized semisynthetic ADEP is shown as structure **18**. Structure **19** (an analog of imipridone, an exploratory anticancer) represents a new class of ClpP activators having comparable activity to ADEPS but better drug-like character.[602] ClpXP activation is lethal to *S. aureus* persisters and in biofilm.[603]

While final judgment on the value of ClpP as an antibacterial target is not set, further studies are encouraging.[19,604,605] This discussion focuses on *S. aureus* to the exclusion of complementary studies with other Gram-positive bacteria, mycobacteria, and Gram-negative bacteria. Inactivation of *S. aureus* ClpP by β-lactones,[597,606] by peptidomimetic boronates,[607,608] by a new inhibitor class **20**,[609] and by genetic deletion attenuated

virulence.[593] Virulence attenuation was also seen by a competitive inhibitor (structure **21**) of ClpX.[606] However, irreversible inactivation of MRSA ClpP increased β-lactam resistance,[610] as a result of elevated levels of the Sle1 peptidoglycan amidase, providing a bypass of the bactericidal autolysis mechanism initiated by β-lactam inactivation of PBPs.[592,611] This amidase is an essential enzyme with respect to the β-lactam resistance of CA-MRSA, where it functions to accelerate daughter cell splitting (and leading to a reduction in cell size).[612] ClpXP localizes to the *S. aureus* septum[592] and functions to control the cellular concentration of FtsZ[613] by a ClpX-independent mechanism.[614–618] These observations are consistent with a direct interconnection among ClpXP, PBPs, and the cell-envelope synthesis. The obvious remaining question was whether activation of ClpP would synergize the bactericidal activity of the β-lactams. This question was answered in the affirmative using ADEPs in *S. aureus* and with an *N*-(3-chlorobenzoyl)-5-tetrazol-5-amine inhibitor of ClpP in vancomycin-resistant *Enterococci*.[619,620] Comprehensive analysis of the effects of ClpP activation on the antibiotic sensitivity of *S. aureus* was demonstrated using the imipridone ONC212 (MSSA, bactericidal MIC of 8–16 mg L^{-1}).[602] At either 2 μM or 4 μM concentration of ONC212 (depending on the antibiotic), ONC212 synergized with ampicillin (a β-lactam of the penicillin subclass), tetracycline, and ciprofloxacin and was additive with streptomycin and rifampin. ONC212 had no effect on the antibacterial activity of vancomycin.[602] As the imipridones have recognizable drug-like character and as yet are not structurally optimized for antibacterial potency, they have future promise.

3.2.9.2. FtsZ.: The FtsZ cytoskeletal protein is an essential protein of cytokinesis. The direct integration of FtsZ function with the catalytic functions of both the PBPs and ClpPX suggests that antagonists of the GTP-dependent polymerization of FtsZ might synergize with the cell-wall interacting antibacterials. FtsZ depletion in *S. aureus* dysregulates the ordered PBP assembly of the *S. aureus* septal peptidoglycan and results in the formation of enlarged and structurally unstable cells.[257] FtsZ is a promiscuous target and the number of identified small-molecule inhibitors of the function of this protein is large (Chart 6).[220–222] Among the most notable structures are the substituted 2,6-difluorobenzamides exemplified by PC190723 **22**.[621] Structure **22** binds to FtsZ, effects a cell morphology change identical to that described above, exerts bactericidal antistaphylococcal activity (including MRSA, MIC of 1 mg L^{-1}), and is efficacious at 30 mg kg^{-1} in a lethal *S. aureus* murine infection model (activity was comparable to vancomycin at 3 mg kg^{-1}). Spontaneous resistance mutation(s) (frequency of 2×10^{-8}) was high.[621] Resistance mutation to FtsZ inhibitors is observed commonly, although in many cases the relative virulence of the mutant(s) was not assessed. In the example of **22**, the resistant mutants showed reduced virulence.[223,622] Moreover, **22** was synergistic with imipenem (a carbapenem) against MRSA. Combination of **22** with imipenem markedly reduced (by 10×) the frequency of resistance mutation to **22**. The mechanism of **22** is stabilization of the FtsZ structure resulting in a deformed Z-ring.[623–626] As a consequence of the poor solubility (and lack of oral availability) of **22**,[627,628] extensive efforts were made toward the optimization (both as structures and as prodrugs) of the PC190723 class.[629] Among the former are the benzodioxane-containing structure (**23**),[630] the more potent PC190723-derived structure **24**,[631] and the imide pro-drug **25** of a second PC190723-derived structure (the active metabolite is TXA-707, structure **26**).[628] Pairing of **26** with each β-lactam within a panel of clinically used β-lactams confirmed a synergistic

interaction and further showed that the β-lactams that gave the best synergy targeted preferentially *S. aureus* PBP2 (imipenem and cefnidir).[226,632] Prodrug **25** (structure code TXA709) completed a phase 1 clinical trial.[633] A more general statement with respect to a relationship between FtsZ modulators and β-lactams is the observation that quinuclidine **27** (MRSA MIC 24 mg L^{-1}), a structure that impairs rather than stabilizes Z-ring formation, also showed broad-based β-lactam synergy (in the presence of 3–24 mg L^{-1} **27** a decrease is seen in the imipenem MIC from 16 mg L^{-1} to 4 mg L^{-1}).[224] Structure–activity optimization has given structures with improved, broad-spectrum antibacterial activity and lacking eukaryotic toxicity in cell structure (exemplified by structure **28**).[634] The best evidence of the promise of FtsZ inhibition is the consistency of positive results across several structural templates, by different medicinal chemistry teams. As necessary as positive consistency is for progression in drug development, it is not evident at this time whether there are unspoken formulation shortcomings (such as solubility) and/or pharmacological shortcomings (such as the uncertain identification of the entity optimally matched to a FtsZ inhibitor as an adjuvant, metabolism, safety, pharmacokinetics/pharmacodynamics). Until such studies are reported, the future impact of the FtsZ inhibitor is not known.

3.2.9.3. Two-Component Kinases.: Protein phosphorylation and dephosphorylation is a ubiquitous regulatory mechanism. In Gram-positive bacteria arginine phosphorylation identifies a protein for ClpP degradation.[635–637] Histidine phosphorylation is fundamental to the response of the bacterium to its environment by metabolic regulation, using two-component sensing (TCS, a kinase and its response regulator).[638–643] And cysteine/serine/threonine phosphorylation catalyzed by the "eSTK" eukaryotic-like serine threonine kinases (and countered by eSTP phosphatases) plays a myriad of roles. The genome of the *S. aureus* bacterium encodes 16 TCS (the *mecA* system adds a 17th)[644] and two eSTK kinases. Only one TCS, the WalKR (named for the its regulatory function with respect to the cell wall), is essential. WalKR is a prominent regulator of cell-wall homeostasis (especially autolysis) and of resistance to cell-wall-acting antibacterials.[645,646] Two-component systems combine a sensor kinase (here, the WalK homodimer) which activates its response regulator (WalR) by phosphorylation. The two eSTK kinases are Stk1 (previously called PknB, and catalyzing cysteine/serine/threonine phosphorylation) and the CapAB heterodimer (a bacterial-tyrosine or BY-kinase). BY-kinases are closely associated with regulation of the capsular polysaccharide of the cell envelope.[80] STK1 is a prominent regulator of itself (by autophosphorylation),[647] metabolism, virulence, peptidoglycan biosynthesis, resistance to cell-wall-acting antibacterials, and several TCS.[648] A notable feature of Stk1 is the presence of three PBP-serine-threonine-kinase-associated (PASTA) domains that contact directly the peptidoglycan.[649–654] Stk1 is recruited to the *S. aureus* septum in response to the presence of Lipid II and concurrent PASTA-domain recognition of the peptidoglycan.[655] The compelling sense of this terse summary is a regulatory labyrinth of kinases as interdependent networks within *S. aureus*. Their complexity cannot be overstated. Regulatory interdependency occurs by cross-talk and by protein–protein interaction: in the Gram-positive pathogen *Streptococcus pneumoniae*, its WalKR and its PASTA domain-containing eSTK (StkP) associate.[656] While *S. aureus* WalKR and Stk1 crosstalk, there is no evidence as yet of a protein–protein interaction.[655] The kinase substrates for *S. aureus* include transcription factors, TCS proteins, and enzymes. The hypotheses that this interdependency could identify

targets for direct antibacterial intervention, or alternatively targets whose loss of function would synergize with the β-lactams, are evident.[652] A breadth of experimental efforts with natural product and synthetic kinase inhibitors, mostly reported within the past few years, has explored these possibilities.

The outstanding characteristic of the WalKR system is its role in initiating, frequently by mutation, a thickening of the cell wall as a resistance response to cell-wall-active antibiotics. This phenomenon was first observed for vancomycin but is now recognized as a general response to many cell-wall-active antibiotics (including daptomycin and the Lipid II-binding peptide siamycin).[174,646,657–659] ClpP with WalKR cooperates toward vancomycin resistance.[660] Although the customary interpretation of the thicker cell wall is reduced antibiotic access to the inner-wall zone due in part to greater competitive binding to the peptidoglycan, the WalKR response is more versatile. For example, exposure of *S. aureus* to sub-MIC β-lactams increases the MIC for vancomycin.[661] The signalling entity for the WalKR system in *Bacillus subtilis* is altered peptidoglycan structure in response to the autolysin activities controlled by this system.[662,663] While the signaling entity for *S. aureus* is not known, here too the WalKR TCS controls autolysis activity,[664,665] possibly in response to altered wall teichoic acid structure rather than peptidoglycan.[666] The effect of small-molecule modulation of the WalKR TCS in *S. aureus* is less studied than that of its eSTK system.[667] Nonetheless, experimental observations confirm antibacterial relevance for this system. Three natural products, each isolated from different *Streptomyces* strains,[668] target the *S. aureus* WalKR system (Chart 7). Walkmycin B (**29**) inhibits WalK autophosphorylation (IC_{50} 6 μM) with an MIC (both MSSA and a MRSA strain) of 0.25 mg L^{-1}.[669] Waldiomycin (**30**) has a comparable affinity for WalK but a poorer MIC of 4–8 mg L^{-1}.[670–672] Signermycin (**31**) (MIC of 3 mg L^{-1} both MSSA and MRSA) binds to the interface domain of the WalK homodimer and prevents autophosphorylation, resulting in inhibition of cell division.[673] A class of synthetic thiazolo[3,2-*a*]pyrimidin-3-one structures (exemplified by **32**) showed comparable MIC values (2–6 mg L^{-1}) against *S. aureus*.[674] Screening of an 82, 000-membered compound library for efficacy in an MRSA-infected *Caenorhabditis elegans* assay identified the eukaryotic kinase inhibitor IMD0354 (**33**).[675] IMD0354 demonstrated potent bacteriostatic activity across a panel of strains, including vancomycin-resistant strains (representative MIC values of 0.06–0.25 mg L^{-1}). Its mechanism was suggested as membrane permeabilization, however, and not that of inhibition of bacterial kinases.[675] IMD0354 is weakly active against Gram-negative bacteria but was identified independently as having potent activity as an adjuvant of the cell-wall-targeting polymyxin antibiotics.[676] The *S. aureus* GraXRS (also called GraRS with GraR the sensor protein, GraS the sensor kinase GraS, and GraX the signal transduction accessory protein associated with the transporter VraFG) two-component stress-response and cell-wall system is critical to both *S. aureus* resistance and virulence pathways.[677,678] GraR-regulated genes include *mprF* and the *dltABCD* system for D-Ala decoration of the cell-wall teichoic acids.[333] MprF is a bifunctional catalyst of both lysyl modification of the membrane phospholipids and of their translocation. Its activity correlates with resistance to antimicrobial peptides, vancomycin, and daptomycin.[679–681] Cell-based screening of a 45, 000-membered compound library for β-lactam adjuvants activity against MRSA identified structure MAC-545496 (**34**).[682] Structure **34** was active also as a single agent in the *Galleria*

mellonella larvae assay. Its mechanism is inhibition, at nM concentration, of GraR.[682] Moreover, an independent screen of a 1, 280-membered library of off-patent approved drugs as inhibitors of the GraXRS MRSA system identified the ability of the porphyrin verteporfin (**35**) to enhance PMN-mediated bacterial killing and with efficacy in a murine model of MRSA wound infection. Preliminary mechanistic study implicated redox modulation of the cysteine-227 of GraS.[683] All of the compounds of Chart 7 represent structural opportunity, especially given the availability of the structure of the extracellular domain of the WalK protein.[684–686]

3.2.9.4. Eukaryotic-like Kinases.: Inhibitors of the eSTK kinases also show broad antibiotic synergy, not just against MRSA but also including other Gram-positive bacteria (such as the enterococci),[654,687] mycobacteria,[688–692] and Gram-negative bacteria.[693] Although the eSTK network of mycobacteria is more complex than that of *S. aureus*,[688,694] *S. aureus* has the eSTK, Stk1 (also called PknB in the earlier literature). STK1 recognizes Lipid II,[691] it interacts with peptidoglycan through PASTA domain recognition,[695] and its inhibition by small molecule potentiators improves the efficacy of β-lactam antibiotics.[696] The genes for Stk1 of *S. aureus* (SA1063) and its complementary phosphatase Stp1 (SA1062) are adjacent. Neither is an essential enzyme. Genetic deletion of Stp1 gives a thickened-cell-wall *S. aureus* phenotype. Genetic deletion of both Stp1 and Stk1 gave a phenotype sensitized to β-lactams, as also seen when the Stk1 gene alone was deleted.[697,698] No change in sensitivity was seen, however, with respect to vancomycin. The β-lactam sensitivity of two MRSA strains (MW2 and LAC) was compared upon *stk1* deletion. Using nafcillin (a penicillin having a structure closely related to oxacillin) and imipenem as representative β-lactams, the nafcillin MIC change for MW2 was from 32 mg L^{-1} to 2 mg L^{-1} (breakpoint value) and for LAC was from 16 mg L^{-1} to 4 mg L^{-1}. The imipenem MIC change for MW2 was from 1 mg L^{-1} to 0.12 mg L^{-1} and for LAC was from 0.75 mg L^{-1} to 0.06 mg L^{-1}.[699] Screening a small library of drug-like structures for inhibition of Stk1 autophosphorylation[647] identified four arylsulfonamides (representative structure is **36** of Chart 8) active at 2 μM concentration.[699] Staurosporine, the paneukaryotic kinase inhibitor, was equally active. None of the four sulfonamides was antibacterial, and none showed toxicity (in limited assay) to mice. In in vitro growth assay neither MRSA strain was impeded by the presence of 4 mg L^{-1} nafcillin. In contrast, at this same nafcillin concentration and in the presence of 13 μM sulfonamide (or staurosporine), bacterial growth was inhibited by 50%. Stk1 inhibitors are adjuvants of the bactericidal activity of the β-lactams.

This conclusion was validated concurrently using the GSK690693 kinase inhibitor **37** to inhibit the Stk enzyme of the Gram-positive bacterium *Listeria monocytogenes*.[700,701] GSK690093was inactive against *S. aureus* but was active against mycobacteria.[696] A series of other structures, representing different chemotypes, were active. Triarylimidazole structures inhibited Stk1 and synergized with β-lactams (at a concentration of 7 mg L^{-1} for inhibitor **38** the MIC of oxacillin was reduced from 256 mg L^{-1} to 4 mg L^{-1} for the MRSA252 strain; from 16 mg L^{-1} to 4 mg L^{-1} for the MRSA NRS123 strain; and from 32 mg L^{-1} to 0.5 mg L^{-1} for the MRSA NRS70 strain).[702] Although a relatively weak inhibitor (IC_{50} 50 μM) of Stk1 autophosphorylation, the 4,5-dihydro-5-oxo-1-thioxo-1*H*-

thiazolo[3,4-*a*]-quinazoline (Inh2-B1, **39**) in combination with a β-lactam protected mice from a lethal MRSA challenge.[703] β-Lactam alone and **39** alone were ineffective. Inh2-B1 alone, however, inhibited biofilm formation. The eukaryotic kinase inhibitor GW779439X (**40**) potentiated β-lactams, notably including the MRSA-active ceftaroline, against multiple MSSA and MRSA strains.[704] At 5 μM **40** the MIC of ceftaroline decreased 2-fold for the MRSA USA 300-LAC strain. The decrease was also 2-fold for Meropenem; 8-fold for nafcillin; and 16-fold for oxacillin (identical potentiation ratios were seen for the Δ*stk1* strain). The MIC for vancomycin was unaltered. A pose for the Stk1·**40** complex was validated by structure-based design. As a follow-up to studies on tricyclic amine antidepressants as β-lactam adjuvants against MRSA,[705] the FDA-approved and nontoxic antihistamine loratadine **41** was identified as an Stk1 inhibitor that inhibited biofilm formation and synergized β-lactam activity (MRSA USA300 MIC of oxacillin 32 mg L^{-1} falls to 1 mg L^{-1} in the presence of 50 μM loratadine). Although the MIC for vancomycin (1 mg L^{-1}) for this strain was unaltered, in the presence of 50 μM loratadine the MIC for vancomycin-resistant *S. aureus* was reduced from 512 mg L^{-1} to 32 mg L^{-1}. The inhibitory effect of loratadine on biofilm, and interestingly its additional ability to antagonize BlaZ expression, was strain-dependent. These studies have self-consistency. Pharmacological activity of β-lactam-inhibitor pairs was seen even with structurally unoptimized Stk1 inhibitors. The inability of Stk1 inhibitors to synergize the activity of vancomycin, a cell-wall-targeting antibiotic that binds to peptidoglycan but does not alter its structure, is consistent with Stk1 as responsive to alteration of peptidoglycan structure (an inevitable consequence of PBP inactivation). Likewise, the different magnitudes of synergy that are seen for different β-lactam-inhibitor pairs is understandable. Different β-lactams have very different relative abilities to inactivate PBPs. Given that Stk1 has a septal location in *S. aureus*, the optimal β-lactam for pairing with an Stk1 inhibitor is likely a β-lactam with selectivity for PBP1 or for PBP2 (or PBP2a).

A final set of structures underscores the breadth of promise to kinase inhibition as a focus, and as a starting point, for *S. aureus*-targeted antibacterial discovery. The eukaryotic tyrosine kinase inhibitor sorafenib (diarylurea **42**) showed intrinsic Gram-positive antibacterial activity (MSSA, MIC 4 mg L^{-1}; poorer activity against MRSA; *S. epidermidis* 32 mg L^{-1}) upon screening a eukaryotic kinase-inhibitor library. Sorafenib was active as a single agent against several MRSA strains (MIC 15–45 mg L^{-1}). Its target was not identified. An activity-guided synthetic effort gave the bactericidal 2-chloroethyl-*N,N′*-diphenylmalondiamide structure SC5005 (**43**, MIC_{90} of ≤0.5 mg L^{-1}).[706] SC5005 showed a low frequency of resistance, and as a single agent (10 mg kg^{-1} i.p.) was active in a lethal MRSA infection mouse model. Although the mechanism of SC5005 was presumed to be eSTK inhibition, this mechanism was not proved. A separate activity-guided effort stimulated by the sorafenib structure (**42**) gave the orally available *N,N′*-diphenylurea PK150 (**44**, MRSA NCTC8325 MIC 0.12 mg L^{-1}).[707] It showed a low frequency of resistance, potent antibiofilm activity, and eradicated persister *S. aureus*. However, **44** lacked eukaryotic kinase inhibition activity. Photoaffinity proteomics with *S. aureus* identified two targets, SpsB (signal peptidase IB) and MenG (demethylmenaquinone methyltransferase, the final enzyme of the menaquinone biosynthetic pathway). Analysis of the mechanism of **44** by scanning and by transmission electron microscopy showed blebbing

defects at the division septum, consistent with dysregulation of the autolysin activities required for cell separation of *S. aureus*.[707] It is additionally noted here that a different *N,N′*-diarylurea, PQ401 (**45**, MRSA MIC of 4 mg L^{-1} across a panel of strains), was discovered independently and with experimental data consistent with a membrane-disruption mechanism.[708] A possible contribution of Stk1 inhibition to the activities of these ureas and the possibility of their synergism with β-lactams (PQ401 is shown already to be synergistic with aminoglycosides) remain to be determined.

The phosphorylation status of the eSTK enzyme is regulated by a separate kinase and phosphatase. Given the observation that Stk1 inhibition synergizes the activity of the β-lactams, loss of function of the StkP phosphatase (Stp1) could represent a resistance mechanism. This mechanism is observed. Serial sub-MIC laboratory passage of a MSSA strain lacking both *blaZ* and *mecA* gave β-lactam resistance as a result of a point mutation in the *stp1* gene.[709] Complementary studies of Stp1 using the anionic diphenylmethane derivative MDSA **46** as an inhibitor (IC_{50} = 10 μM), however, underscore caution with respect to modulation of the Stk1/Stp1 system. Inhibition of Stp1 (or *stp1* deletion) suppresses the virulence of *S. aureus* as a result of preservation of the phosphorylated state of the SarA/MgtA TCS.[710,711] Given this seemingly paradoxical result, the pharmacological response from kinase inhibition must be anticipated to show complexity with respect to inhibitor selectivity and the infection model.

3.2.9.5. Kinase Inhibitors as Adjuvants against *S. aureus* β-Lactam Resistance.: Although this summary of kinase inhibitors as β-lactam adjuvants against MRSA is organized into the separate sections of 3.2.9.3 (two-component kinase inhibition) and 3.2.9.4 (eSTK kinase inhibition), this separation is artificial. The two-component kinases and the eSTK kinase of *S. aureus* are interacting regulatory components tasked mutually with monitoring and responding to cell-envelope-targeting antibiotics.[80,656,712,713] While the overall organization of this interaction is not known, we know that in *S. pneumoniae* two of these components (StkP and WalK) have a protein–protein interaction.[656] While the cognate experiment in *S. aureus* (its Stk1 with WalK) has not yet been done, it is certain that its kinase systems respond interdependently to not just pathway metabolites but to the conformational status of their proteins. Beyond this truism, further explanation is not possible as to how these kinase systems contribute (for example) to the phenomenon of exposure of *S. aureus* to subinhibitory β-lactam concentrations elevating the MIC of vancomycin.[661] In key respects it may suffice to know that no such MIC increase for vancomycin is seen when the exposure is concurrent β-lactam and vancomycin.[714] An identical phenomenon is seen in the clinical use of vancomycin for MRSA infection. When vancomycin clinical failure occurs it coincides with MRSA strains showing diminished daptomycin susceptibility.[715] This result is understood as a common resistance mechanism: membrane alteration as a result of elevated MprF activity.[679,681,716–718] As noted previously, the *mprF* gene is regulated by the two-component kinase systems.[682,719] This observation exemplifies both the path and the challenge of future antibacterial chemotherapy. The path is multiagent chemotherapy. The challenge is devising an experimental path toward the identification of the most efficacious combination of entities. While conceptualizing the challenge in terms of identifying kinase-inhibitor adjuvants of β-lactam efficacy is sensible

within the framework of the β-lactams as known and proven clinical entities, outside of this framework the suggestion of (for example) pairing an inhibitor of a two-component kinase with an inhibitor of an eSTK kinase, to achieve virulence suppression, is plausible. Initial analyses suggest that the creation of kinase inhibitors that show selectivity not just against eukaryotic kinases, but also among bacterial kinases, is possible.[641] The exploration of kinase inhibition—as is also the case for ClpXP and FtsZ inhibition—as an approach to *S. aureus* adjuvant chemotherapy has only just begun. Nonetheless, the preliminary data indicate that all three represent promise both as antibacterial targets and as antibacterial adjuvants.

4. AGAINST THE FORTRESS

4.1. β-Lactams against Bacterial Fortresses

The persistence of the bacteria across the eons reflects in large part the near perfection of their protective cell envelope. Here, “perfection” describes the intricate organization for the assembly in place of the individual components of its envelope, synchronized with the duplication and separation of its genome, as a protective barrier. Here also, “near perfection” underscores that this same intricacy leaves the bacterium vulnerable to the disruption of the orchestration. The metaphorical giant of the bacterium trips and stumbles over a pebble. Of all such pebbles the β-lactam was, is, and will remain paramount. The half-century study of just the ternary relationship among the β-lactam, the PBP, and the peptidoglycan has given way to their place within a much more complicated matrix of a dynamic bacterial envelope. Over the past decade many of the dramatis personae of the envelope—proteins, enzymes, and structures—have been identified. These actors are now named, and in many cases both the act that they appear and portions of the dialogue that they are called to voice have come into clearer focus. While we remain decades away (if that) from the full text of the play, among the clearer foci is a better understanding of the bactericidal mechanism of the β-lactams. The elegance with which they meld structural mimicry of the D-Ala-D-Ala stem terminus with decisively different acylation chemistry, thus trapping the PBPs as inert acyl-enzymes incapable of completing the synthesis of the peptidoglycan of the cell wall, was among the earliest mechanistic discernments. This discernment coalesced subsequently with recognition that net peptidoglycan biosynthesis was the difference between acylation-dependent accretion against hydrolase-dependent removal, repair, and remodeling. Loss of PBP function was understood to disrupt the balance between accretion and removal, with removal—catalyzed by mis-regulation of the very same autolysins required for cell division—eventually resulting in the structural failure of the peptidoglycan, and hence the bacterium.[720] The many studies that confirm adjuvant structures that synergize with β-lactams indicate that the question of how and where to synergize with PBP inactivation has multiple answers. This conclusion is not a surprise. Bacteria have different ecological niches, different shapes, different cell-envelope structures, different regulatory pathways, different metabolic requirements, multiple peptidoglycan structures, and different ensembles of PBP. The expectation of a common answer is naive. Our focus here is *S. aureus*.

4.2. β-Lactams against the *S. aureus* Fortress: In the Laboratory

The focus of the in vitro study of a pathogen is the identification of structures and mechanisms as a first step to its possible future control. The patient today with bacteremia as a result of infection by extensively resistant *S. aureus*—to β-lactams, vancomycin, daptomycin, and linezolid—is the exception.[721] How may in vitro studies suggest answers, should the exception become the rule? This review enumerates opportunity for structures and mechanisms within wall teichoic acid biosynthesis, within interference with undecaprenol phosphate/Lipid II recycling, within PBP allosteric regulation, within the coordination of the cytoskeleton to the envelope, and within kinase regulation of pathways. This list is a beginning. Some of these new structures have potential as single agents, and many synergize the in vitro activity of β-lactams. The uncertain predictive value of in vitro synergy for in vivo synergy is understood. Persistent curiosity with respect to the important chemical intricacy of bacterial function will refine our ability to use the former to focus on the latter. For example, the straightforward task of correlating β-lactam selectivity to PBP function has been done for only a small number of pathogenic bacteria,[197] notwithstanding the clinical importance of this correlation. The sensitivity of PBP2a of *S. aureus* to different β-lactams varies significantly.[722] Comprehensive in vitro synergy evaluations can detect important patterns, such as the observation that the synergy of β-lactams with the lipoglycopeptides dalbavancin, oritavancin, and telavancin was superior to that of vancomycin and teicoplanin.[723] Regardless of mechanism, examining the effect of the antibacterial on the metabolism of the bacterium is more important than its effect on the growth of the bacterium.[724–726] The discovery of new antibacterial structure—whether from Nature or from synthetic libraries—is by no means exhausted.[727–729]

Nor is value from the study of the β-lactams exhausted. *S. pneumoniae* is the Gram-positive ovococcus with similarity (and also, important difference) to the *S. aureus* coccus with respect to the structure of its cell envelope.[201,730,731] In both bacteria peptidoglycan biosynthesis involves coordination of initial bPBP (PBP1 in *S. aureus*) activity with subsequent aPBP activity. Persuasive evidence suggests a peptidoglycan sizing mechanism, whereby the peptidoglycan strand from the bPBP is measured and then terminated by a task-specific peptidoglycan-cleaving enzyme called a lytic transglycosylase.[732] The strand is then suggested to transfer to the aPBP for incorporation into the peptidoglycan polymer.[733] Failure to complete this transfer is toxic to the bacterium. Since exploratory inhibitors of the lytic transglycosylases are known, if *S. aureus* uses a similar mechanism its disruption could represent another point for β-lactam synergy. The bactericidal event that culminates the activity of the β-lactams in *S. pneumoniae* (and other bacteria, including *S. aureus*) is suggested as disregulation of their autolysin activity.[64] Transfer of the primary autolysin LytA of *S. pneumoniae* to the inner-wall zone is suggested to occur in the form of a catalytically suppressed LytA·lipoteichoic acid complex. The transition from LTA biosynthesis to wall teichoic acid synthesis is proposed to effect a change from LTA biosynthesis to WTA biosynthesis as a regulatory event contributing to the unmasking of the LytA activity. Inactivation of the PBPs by β-lactams disrupts teichoic acid biosynthesis so as to effect the premature activation of LytA culminating in the structural failure of the wall.[64] A conceptually similar process may operate for *S. aureus*. However, the LTA and WTA biosynthetic pathways in *S. aureus* are separate (these pathways overlap in *S.*

pneumoniae). While the mechanism in *S. aureus* may have similarity, it must also have a key point of difference. An important observation with respect to a difference is the observation that small-molecule inhibition of the TarG transferase blocks the transfer to the inner-wall zone of *both* the WTA and the primary *S. aureus* autolysin Atl.[386] Atl normally translocates to the septal perimeter of the dividing *S. aureus* cell.[734] Atl is proteolytically activated to release, in a spatially defined manner, its two enzymatic domains: the amidase AmiA and the glycosylase GlcA.[735–737] Upon activation peptidoglycan degradation occurs as a result of initial cleavage of the stem peptide (at the MurNAc-L-ala amide bond) by AmiA, followed by the release of GlcNAc-MurNac disaccharides as a result of AmiA acting as an exoglycosylase.[738] Their cooperative catalysis enables, after cell division, the separation from each other of the daughter cells as a result of the degradation of the interfacial peptidoglycan. As Atl complexes with teichoic acids,[739] the teichoic acids are suggested to have a role in Atl localization to nascent WTA. A structural distinction that may explain Atl binding to nascent WTA and exclusion from mature WTA is LTA-dependent tailoring, by D-Ala transacylation. The altered electrostatic character of WTA as a result of D-Ala decoration may represent the basis for Atl localization.[349,359,360] A conceptual proposal for WTA·Atl complexation is given in Figure 9. Proper tailoring of the teichoic acids is critical to MRSA virulence.[45,303,333,346,358,740] Interference with D-Ala tailoring of the teichoic acids of both *Enterococci* and *S. aureus* strains sensitizes these bacteria to β-lactams.[361,741] Interference with D-Ala (and glycosylation)[400] tailoring of the teichoic acids represents opportunity for antibacterial discovery.[333,411]

Because our understanding of the matrix behind the cell envelope is so primitive, these explorations will need to transcend the orthodox. One example involves the β-lactamase inhibitor, clavulanate, as adjuvant for the β-lactams in the therapy of MRSA infection.[743] The orthodox consensus is that the contribution of the BlaZ β-lactamase to MRSA resistance is secondary to that of PBP2a. Yet the combination of clavulanate with β-lactams is synergistic against both *mecA* MRSA strains[744] and *mecC* MRSA.[745] The less orthodox hypothesis is that clavulanate sensitizes PBP2a to penicillin inactivation (by an uncertain mechanism, possibly by allostery) characterized by collateral sensitivity: two agents, where resistance to one agent sensitizes the bacterium to the other.[746,747] The penicillin–clavulanate combination is not the only collaterally sensitive β-lactam combination. Combination of two β-lactams (meropenem and piperacillin) with a β-lactamase inhibitor (tazobactam, also a β-lactam and mechanistically related to clavulanate) is active against MRSA and suppresses resistance development.[748] Given the increasing likelihood that the most challenging bacterial pathogens will require multiagent chemotherapy—as is already the case for *Mycobacterium tuberculosis*—the search for collateral sensitivity with respect to the bactericidal mechanism[749,750] *and* with respect to suppression of resistance development[751,752] increasingly will represent the focus of in vitro antibacterial discovery.

4.3. β-Lactams against the *S. aureus* Fortress: In the Clinic

The clinic is today and not tomorrow. Time has changed but not abolished the preeminent role for the β-lactams as chemotherapy against *S. aureus* infection.[24,42] Cefazolin as an older cephalosporin remains effective against MSSA, and the combination of cefazolin with ertapenem (a carbapenem) was effective against persistent MSSA bacteremia.[753] First-

line therapeutic agents for MRSA bacteremia are vancomycin and daptomycin. Current practice in the case of clinical failure of these agents is the addition of another antibacterial (combination therapy).[754] Depending on the infection circumstance the added antibacterial is selected from among clinically established non-β-lactam Gram-positive antibacterials (such as linezolid, trimethoprim-sulfamethazole, and fosfomycin), clinically established β-lactams (such as imipenem and ertapenem), the newest-generation cephalosporins ceftobiprole (approved in Europe) and ceftaroline fosamil (approved in the US),[755–759] and from among six other newly approved agents (oritavancin, dalbavancin, telavancin, tedizolid, delafloxacin, and omadacycline).[41,760–763] The circumstances of frequent first-line agent failure, and a breadth of agent options for combination, is the basis for two current debates: whether it is sensible to wait until clinical failure of the first-line agents to progress to combination therapy,[764] and then following the decision in favor of combination therapy, the appropriate agent for the combination.[765] While the potential therapeutic benefit of β-lactam combination therapy in Gram-negative infection is beyond doubt (decades of favorable outcome with β-lactam–β-lactamase inhibitor combinations, now even further expanded by the newest β-lactamase inhibitor structures),[766] the challenge of developing a rational experimental path toward the identification of favorable combinations with respect to efficacy and safety, is daunting. The selection of the cefazolin-ertapenem pairing for persistent MSSA bacteremia was made on the basis of complementary PBP targeting: cefazolin for PBP1 and ertapenem for PBP2, and was supported by in vitro synergy.[753] Notwithstanding the sensibility of this basis and progress toward more effective methods for in vitro validation of synergy,[767] the translation of in vitro synergy to the clinic is not predictable. The observation of unexpected clinical efficacy for the cefazolin-ertapenem pair against MSSA may be argued as balanced by observations with the synergistic combination of daptomycin and fosfomycin.[768,769] Daptomycin (as a calcium complex) is a cell-wall-targeting antibiotic with complex mechanisms of action, including membrane disruption and membrane-dependent interference in undecaprenol phosphate recycling.[351,412,770] Fosfomycin is an inhibitor of the MurA, an early enzyme of Lipid II biosynthesis. The daptomycin–fosfomycin combination showed modest (not significant) improvement in efficacy with greater (not significant) incidence of adverse events, compared to daptomycin alone.[771] The combination of vancomycin and penicillin also is synergistic against MRSA. Nonetheless, this combination was a clinical failure: the benefit of the combination was countered by an increased risk of nephrotoxicty.[764,772] Combination of daptomycin with β-lactams also shows pronounced collateral synergy against MRSA, as a phenomenon known as the seesaw effect.[418–420,680,773] Cephalosporin–daptomycin pairing shows clinical promise[774,775] with in vitro study identifying ceftaroline, a cephalosporin optimized for PBP2a affinity, as a particularly favorable choice.[413,414] The magnitude of the challenge is exemplified by the in vitro observation that the resistance response of MRSA to a carbapenem (Meropenem) included *mecA* mutation and mutation of PBP1 and PBP2, established collateral resistance to ceftaroline.[776] However, neither initial use of noncarbapenem β-lactams alone nor concurrent carbapenem-ceftaroline combination, gave comparable resistance mutation. Notwithstanding the important (if not essential) value of clinical evaluation,[765] the immediate future for the progression of candidates for MRSA chemotherapy will largely be empirical prioritization. This empirical exploration surely will include further evaluation of β-lactam synergy— known additional synergistic pairings

identified by in vitro study against multistrain MRSA include ceftaroline-dalbavancin[415] and imipenem-linezolid[777]—as well as answers as to whether the lipoglycopeptides (such as dalbavancin and oritavancin) are intrinsically superior to vancomycin[416,778–780] or whether fundamentally different approaches to MRSA therapy, such as the use of "metabolism" adjuvants[453,781–784] (some as simple as bicarbonate)[785] or lysin (enzymatic) adjuvants.[786–789]

Yet the forward path—whether that of adjuvants or innovative single agents for MRSA—is poorly lit. The diversity of MRSA strains is expanding. Clinical treatment of MRSA infection will still invariably begin with older, less expensive, and less potent agents (such as vancomycin), rather than the newer and more potent (but also more expensive) agents. Even simple change, such as an early intravenous to oral β-lactam transition in uncomplicated *S. aureus* bacteremia,[775,790] is recent innovation. Compelling in vitro discovery of combinations must pass the daunting barrier, in its stringency and its unpredictability, of matched pharmacokinetics.[791,792] The pragmatics of treating bacterial infection require that antibacterial combinations be formulated as a fixed dose,[793] with the attendant requirement of a business framework to support the choices for the combination. Journeys begin with a first step, and drug discovery begins with the integration of promising structure into inchoate mechanistic understanding.[456] Although *S. aureus* remains a fortress, its fortress is pregnable. This review is a narrative of promising structure, of compelling yet an incomplete understanding of the interplay among its targets, and last of promise as to where entry into the fortress is possible.

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Biographies

Jed F. Fisher purified his first enzyme 47 years ago, at a time when the sharp ammonium sulfate cut was an essential laboratory skill. His transformation into a biological chemist was shaped by the generosity of many scientists, given across the breadth of teachable moments to decades of friendship. His biographical statement is in the form of grateful acknowledgement to these scientists: Bill Fowler, Bob Kerber, George Whitesides, Jack Baldwin, Dan Kemp, Yak Cheung, Rob Spencer, Greg Kaczorowski, Pat Marcotte, Paula Olsiewski, Tom Cromartie, Michael Johnston, Mike Marletta, Vince Massey, Jim Becvar, Konrad Bloch, Bob Woodward, Frank Westheimer, Dwight Peterson, Yong Tae Lee, Dave Brand, Beth Abdella, Kim Clark-Ferris, K. Ramakrishnan, George Barany, Francis Barany, Paul Gassman, Al Moscowitz, Maurie Kreevoy, Allen Harrison, Roy Johnson, Steve Tanis, Al Chrusciel, Julia Clay, Will McWhorter, Joe Strohbach, Bruce Pearlman, Peter Wuts, Dennis Epps, Tomi Sawyer, Imadul Islam, Ed Vedejs, Scott Denmark, Lydia Hines, Jake Szmuszkovicz, Mary Woolley, Marta Toth, Sergei Vakulenko, Mayland Chang, Juan Hermoso, Karen Bush, Samy Meroueh, Mijoon Lee, Dusan Hesek, Chris Forbes, Peter O'Daniel, Marc Boudreau, Leticia Llarrull, Sebastian Testero, Malika Kumarasiri, Jarrod Johnson, Kiran Mahasenan, David Dik, Enrico Speri, Stefania De Benedetti, Choon Kim,

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ABBREVIATIONS USED

ADEP	acyldepsipeptide inhibitor of ClpXP
aPBP	Class A PBP, a HMM-PBP that is bifunctional with both transglycosylase and transpeptidase catalytic activities, and in *S. aureus* is PBP2
bPBP	Class B PBP, a HMM-PBP that is monofunctional as a transpeptidase, and in *S. aureus* is PBP1 and PBP3
cPBP	Class C PBP, a low-molecular-mass PBP, and in *S. aureus* is the PBP4 transpeptidase
CA-MRSA	community-acquired MRSA
eSTK	eukaryotic-type bacterial serine-threonine kinase
HA-MRSA	hospital-acquired MRSA
HMM-PBP	high molecular mass-penicillin binding protein
IWZ	inner wall zone
LCP	LytR-CpsA-Psr protein family
LMM-PBP	low molecular mass-penicillin binding protein
LTA	lipoteichoic acid
MSSA	methicillin-sensitive *S. aureus*
MRSA	methicillin-resistant *S. aureus*
NAG	GlcNAc, *N*-acetylglycosamine
NAM	MurNAc, *N*-acetylmuramic acid
PBP	penicillin binding protein
TCS	bacterial two-component kinase system
WTA	wall teichoic acid

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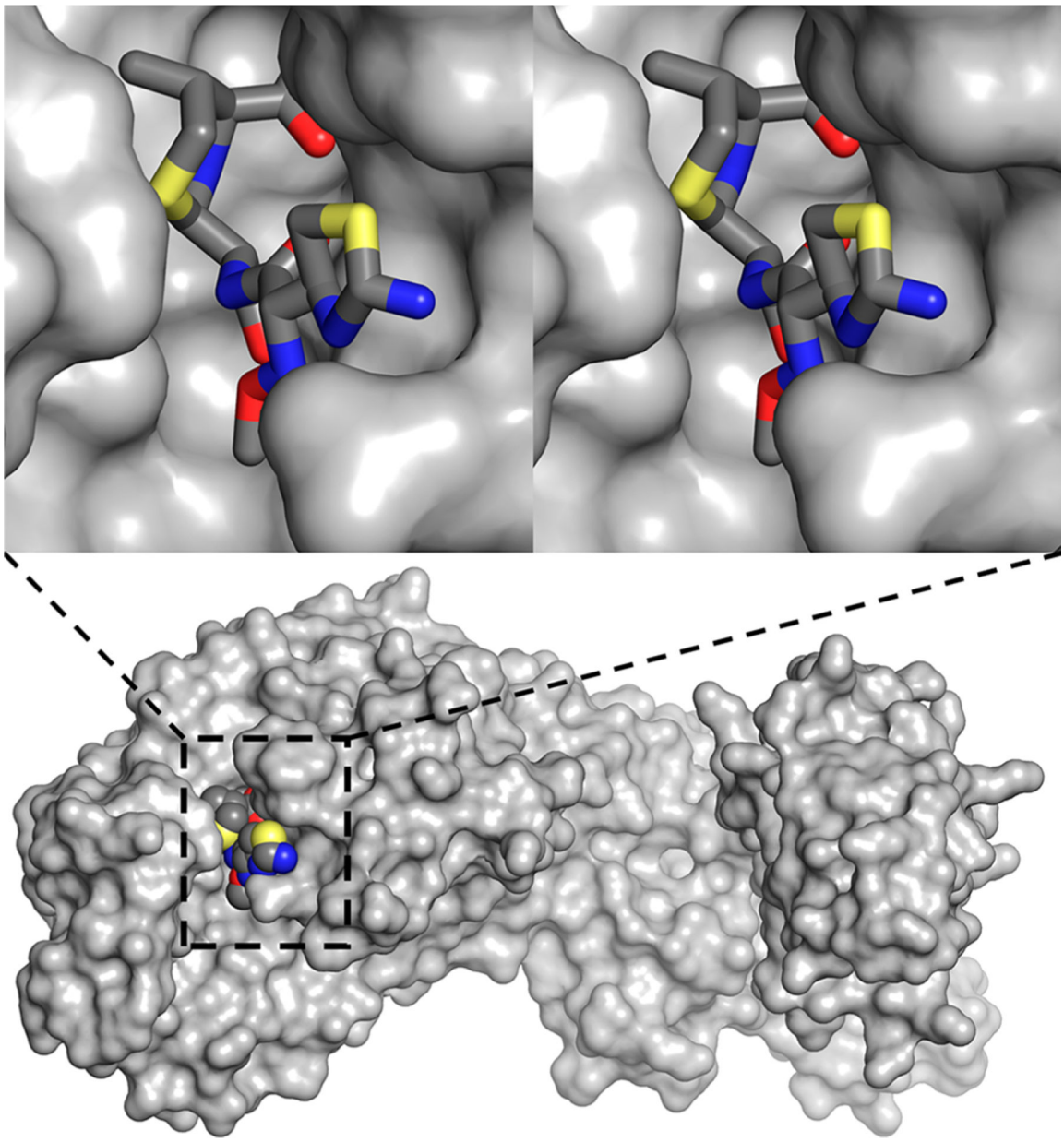


Figure 1.

Top, the stereoview of *S. aureus* PBP3 acylated within the active site by the cephalosporin cefotaxime (PDB 3VSL).[34] Activation of the active-site serine nucleophile (Ser392) is accomplished by a lysine general base. The perspective shown in Chart 2 for the cefotaxime-derived acyl-enzyme of PBP3 corresponds to this stereoview. The thiazolamine segment is in the foreground. The carbonyl of the acyl-enzyme is in the background. The nucleophilic oxygen of the serine is not visible (hidden behind the protein). Bottom, structure of the PBP3 cefotaxime-derived acyl-enzyme represented as a solvent-accessible surface with the bound antibiotic depicted space-filled and color-coded by atom types (blue for nitrogen, red for oxygen, yellow for sulfur, and gray for carbon). In this perspective the transpeptidase active site (to the left), here occupied by this acyl-enzyme, projects into the inner wall zone.

The membrane-binding segment of PBP3 is not shown. Its location would be to the right of the protein.

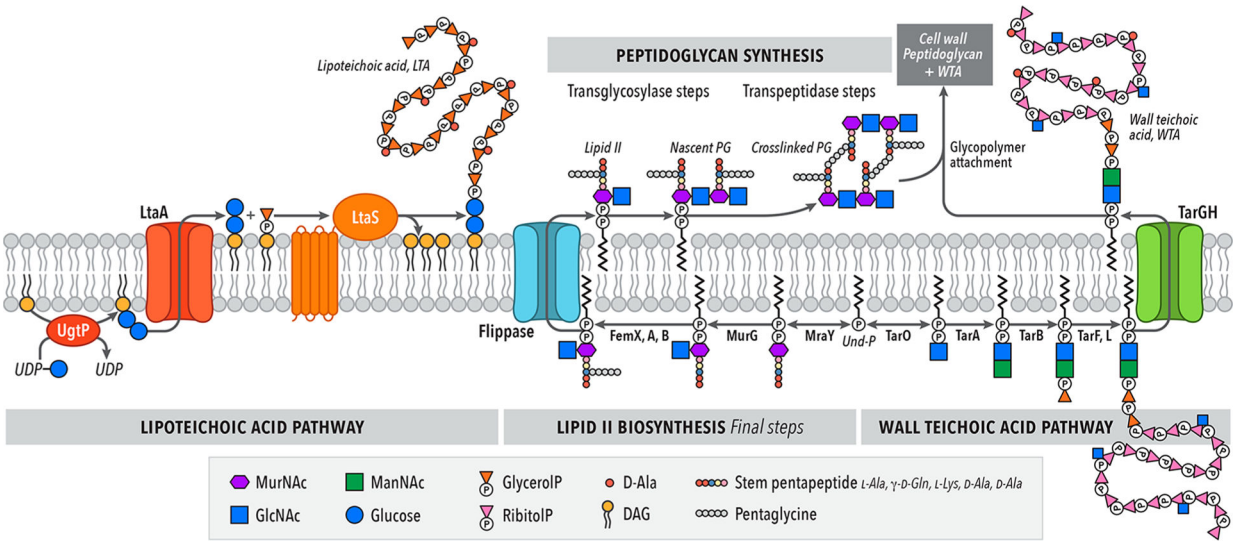


Figure 2.

This figure serves as an organizational guide to the three key structural entities of the Gram-positive cell envelope and thus gives context to many of the topics within this review. The horizontal center of the figure is the lipid bilayer of the single membrane of the Gram-positive bacterium. Above this membrane is the inner wall zone, above which the peptidoglycan cell wall (peptidoglycan synthesis by Lipid II polymerization and WTA glycopolymer attachment) is assembled. Within this membrane are (from left to right) the integral membrane transporters for the lipoteichoic acids (LTAs), for Lipid II, and for the wall teichoic acids (WTAs). Within the inner leaflet of this membrane are the membrane enzymes of the final biosynthetic steps of the lipid anchor of the lipoteichoic acids, of Lipid II, and of the wall teichoic acids. Lipid II and the WTAs share the common membrane carrier, undecaprenyl phosphate (Und-P). After their translocation this carrier is released, as the diphosphate, in the outer leaflet of the membrane. Efficient recycling of the Und-P carrier (not illustrated in this figure) is critical to balancing Lipid II and WTA availability.[89–93] The combination of the lipid segment of the LTAs within the outer leaflet of the membrane, and the interdigitation of the glycopolymer of the LTA into the cell wall, conjoin the two and thus are essential to the structural integrity of the overall cell envelope. Covalent WTA attachment to the peptidoglycan creates a formidable exterior polymeric barrier for controlling solute access to the bacterium.

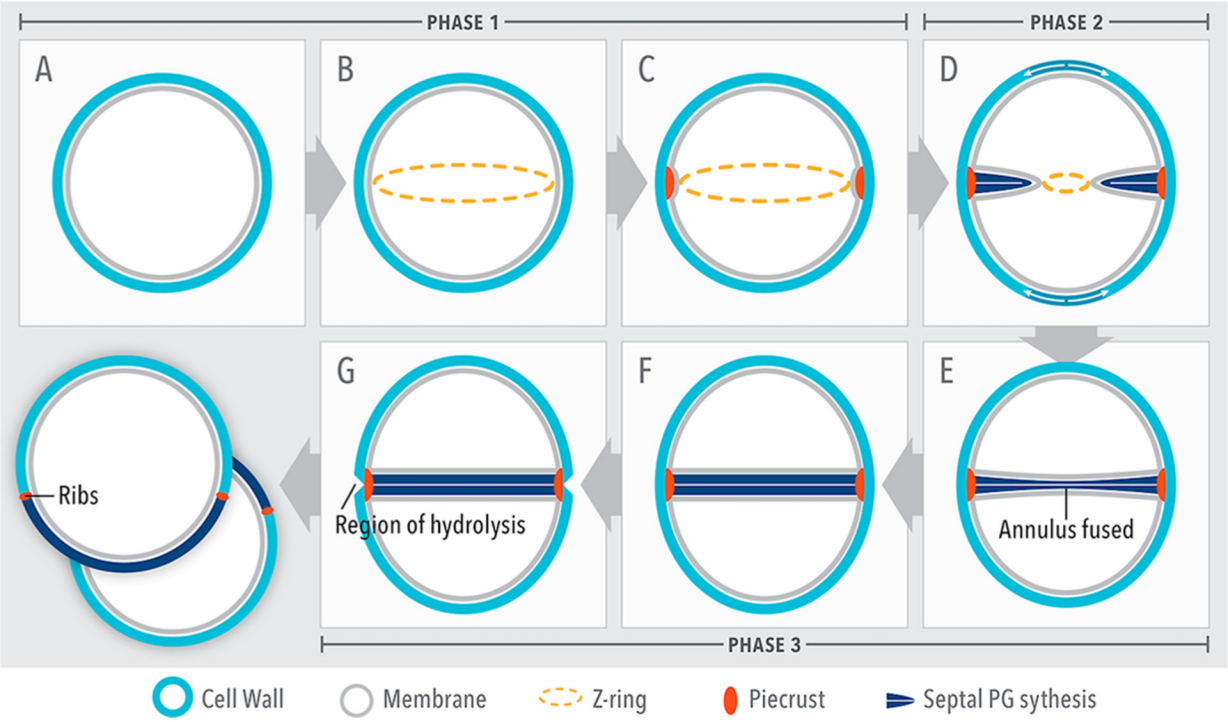


Figure 3.

Cartoon schematic of the dividing *S. aureus* coccus. The gray spherical shell is the bacterial membrane. The turquoise spherical shell is the peptidoglycan. (A) The near-spherical coccus. (B) Midcell formation of a Z-ring (dashed-yellow circle) by *inter alia* GTP-dependent polymerization of the FtsZ protein. (C) Synthesis of new peptidoglycan (red) where the Z-ring is in contact with the old peptidoglycan, as a prelude for the invagination process of the cell envelope to enable cell division. The red peptidoglycan appears ultimately on the surface of the daughter cells as surface ribs (bottom left panel). These ribs (from previous cell division) are present in the bacterium of panels (A) and (B) but are not shown. (D) Progressive Z-ring constriction guides the synthesis of the septal peptidoglycan (dark-blue) built upon the red “rib” peptidoglycan. The blue peptidoglycan grows inward in a concentric motion of a leading edge, behind which the leading-edge peptidoglycan is progressively “thickened”. The different red-blue peptidoglycan coloration reflects both that different PBPs are used for the synthesis of two and the likely possibility that the polymeric structure of the two peptidoglycans is different. The white line centered in the blue peptidoglycan indicates a structural gap (of unknown structure or separation nature) created in the inward-growing peptidoglycan. (E) Septum formation is completed as an annulus fusion followed by (F) completion of the septal peptidoglycan. (G) Controlled degradation of the peptidoglycan external to red rib, and within the gap of the septum, prepares the cells for their final separation. This separation is driven by the internal osmotic pressure of the cells. The “popping” transition to give initially two hemispherically shaped daughter bacteria occurs on a millisecond time scale. Structural reshaping of the hemispherical bacteria to the near-spherical bacteria of the panel is likewise fast. Following division, the blue peptidoglycan is remodeled to give the uniformity of polymeric structure as indicated by the turquoise coloration of panel (A).

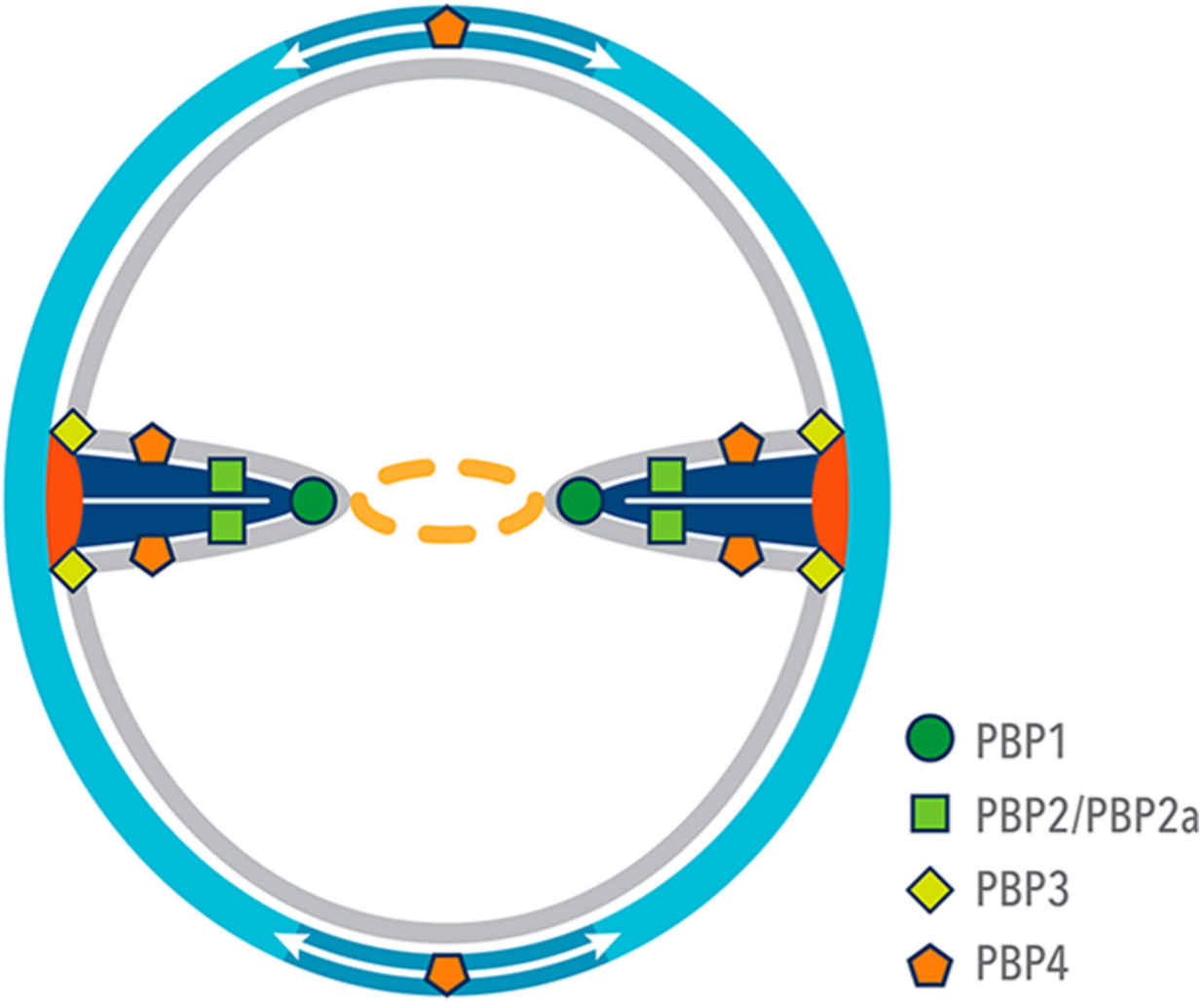


Figure 4.

Peptidoglycan biosynthesis in the methicillin-susceptible *S. aureus* is accomplished by four PBP enzymes (PBPs 1–4). Although there is functional redundancy within the four and only PBP1 and PBP2 are essential, the pathogenic *S. aureus* bacterium requires all four PBP activities. Current mechanistic understanding suggests that synthesis of the red “rib” peptidoglycan (see Figure 3) is a primary task of PBP3; synthesis of the leading-edge septal peptidoglycan by the progressive concentric motion of the divisome is the primary task of PBP1 within the divisome complex; and the task of thickening the peptidoglycan toward structural strength, upon the leading-edge peptidoglycan, is the task of PBP2. PBP4 engages in the remodeling of the septal peptidoglycan and the wall peptidoglycan. In methicillin-resistant *S. aureus*, the essential transpeptidase-catalyzed cross-linking function of PBP2 is compromised by inactivation by the clinically achieved concentrations of the β-lactam antibiotics. Acquisition by these bacteria of the *mec* gene enables expression of a fifth PBP, that of PBP2a, that functions in complex with PBP2 to complete septal peptidoglycan synthesis. The transglycosylase activity of PBP2 coordinates with the transpeptidase activity of PBP2a for this completion. As inactivation of PBP2a requires higher β-lactam

concentrations than can be achieved with almost all β-lactams, the PBP2·PBP2a pair continues to function, and the MRSA bacterium shows β-lactam resistance.

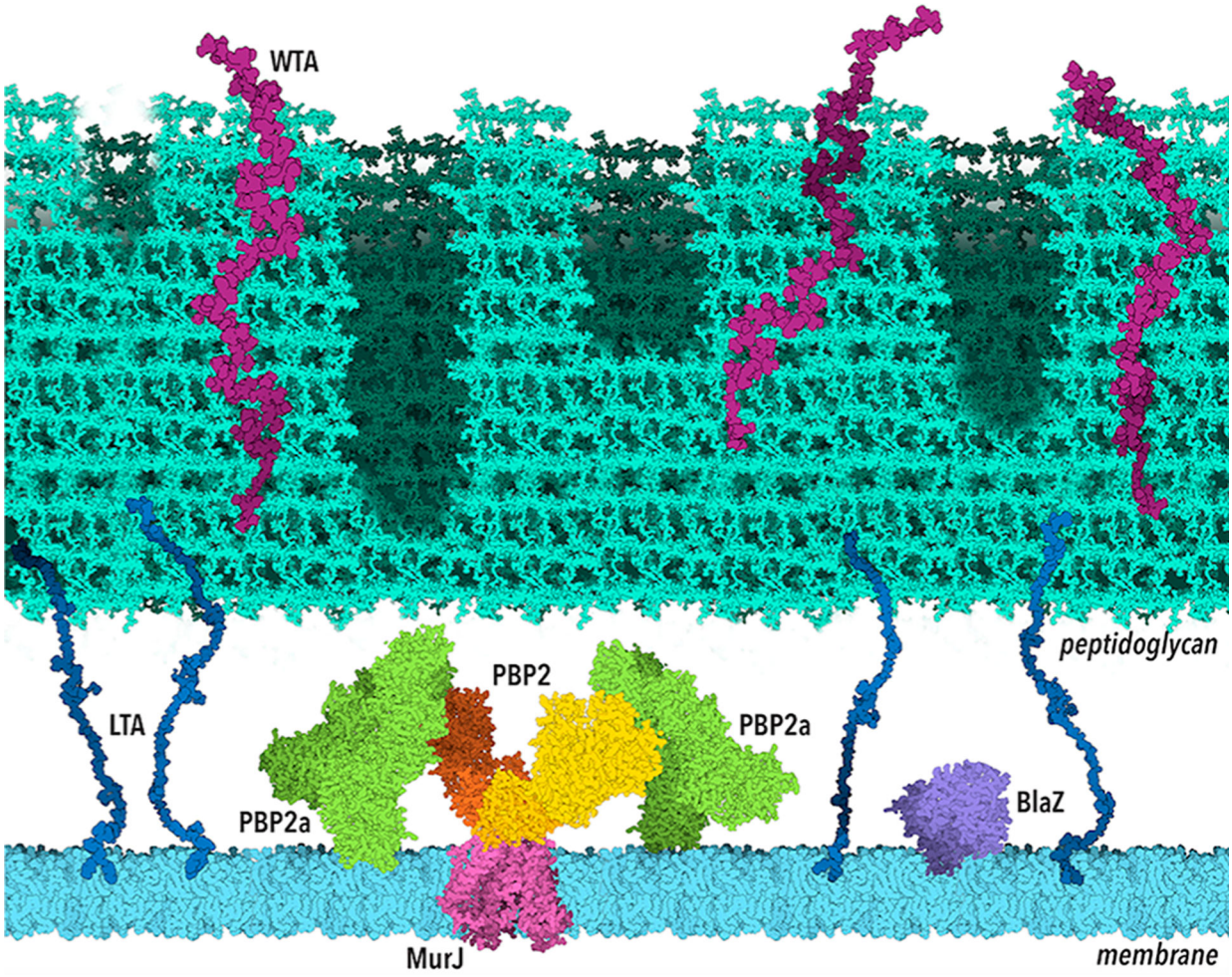


Figure 5.

Cross-section cartoon perspective of the MRSA *S. aureus* cell envelope. This cartoon complements the structures shown in Scheme 2. This cartoon is suggestive of the structural organization of the envelope and is not intended to indicate a realism for that organization. Here the multiprotein, multienzyme divisome complex is represented by the integral membrane "flippase" MurJ (magenta) that delivers Lipid II to a PBP2 homodimer (monomers are colored in yellow and orange) in respective complex with two PBP2a enzymes (lime-green). The BlaZ β-lactamase resistance enzyme (light-purple) is a lipoprotein of the outer leaflet of the membrane. The membrane-anchored and structurally essential LTA molecules (dark-blue) interconnect the membrane (sky-blue) to the peptidoglycan. The molecular basis for the interaction between the LTAs and the peptidoglycan is not known. The LTAs do not project to the surface of the bacterium. The surface of the bacterium comprises the WTA molecules (purple) covalently attached to the peptidoglycan polymer (sea-green). The forest-green shadowing shown for the peptidoglycan indicates that the peptidoglycan is not a uniform polymer but has gaps and cavities. The density of both the LTAs and WTAs with respect to the peptidoglycan is greater than is suggested by the cartoon.

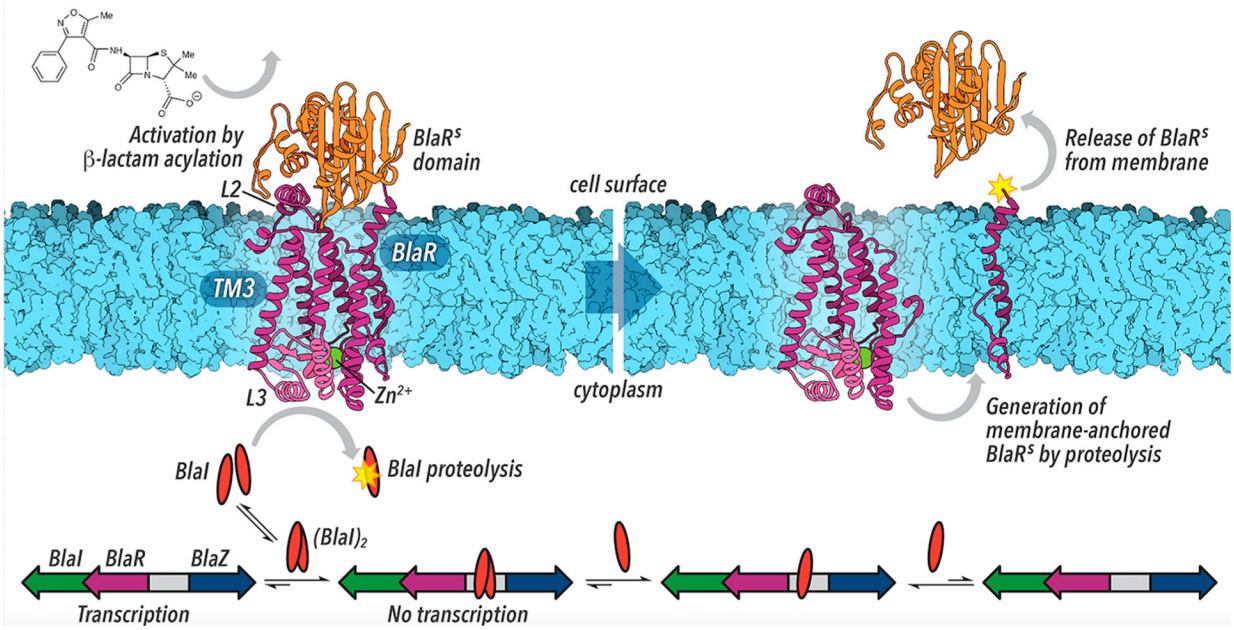


Figure 6.

Schematic for the activation and turnover of BlaR. Antibiotic recognition on the cell surface by BlaR (left panel) leads to activation of its zinc-protease domain at the inner membrane-cytoplasm interface of this transmembrane protein. This protease activity degrades BlaI. As a result of the loss of BlaI the antibiotic-resistance genes of its operon, including that for BlaR1 itself, are derepressed. BlaR1 eventually experiences fragmentation at two sites, with cleavage at one shedding the sensor domain ($BlaR^S$) from the membrane (right panel). This model of the BlaR protein is based on the corresponding model for the MecR protein as proposed by Belluzo et al.[497]

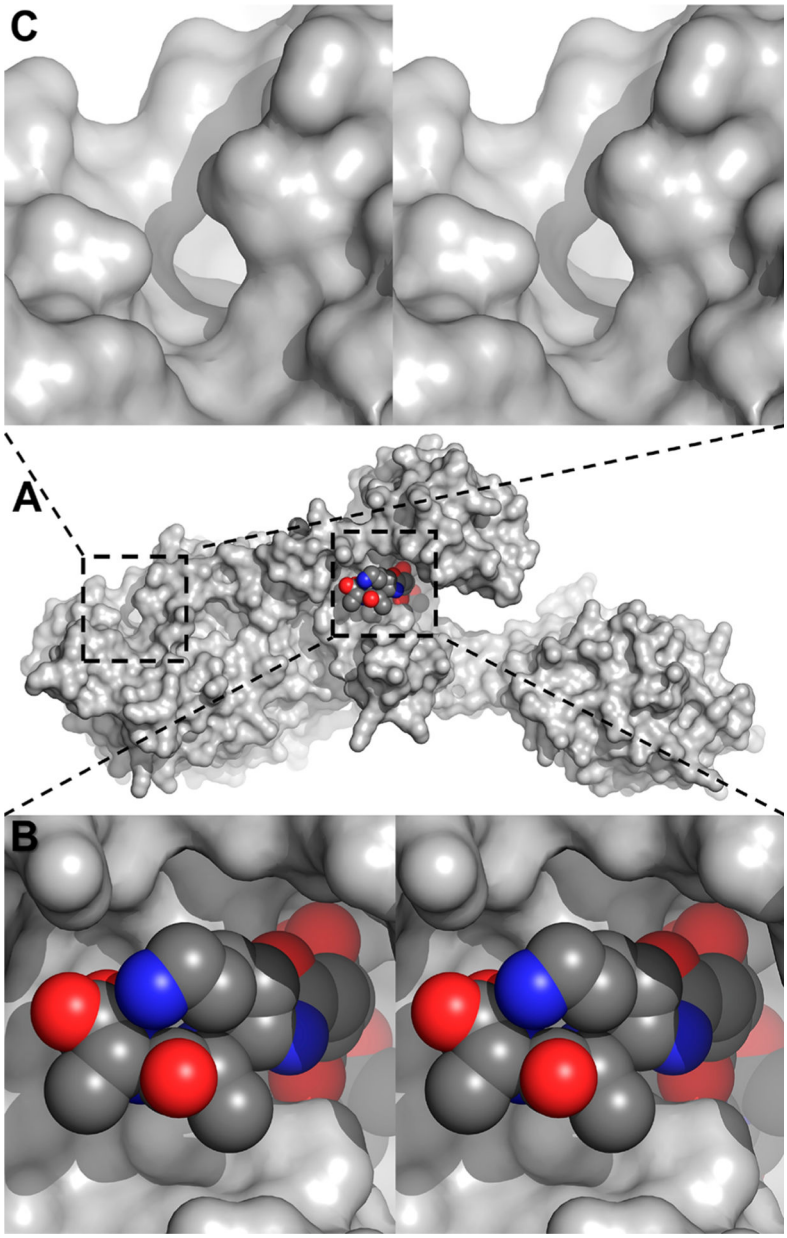


Figure 7.

(A) X-ray structure of the *S. aureus* PBP2a shown as a light gray solvent-accessible surface with a synthetic peptidoglycan fragment, depicted in space-filled presentation (carbons in dark gray, oxygens in red, and nitrogens in blue), bound to the allosteric site. (B) Stereoview of the allosteric site with the bound peptidoglycan and (C) of the unoccupied active site. The active site is approximately 60 Å distant from the allosteric site. The structural changes in the allosteric transformation that controls substrate access to the active-site serine, spanning the two sites, is understood by crystallographic evidence, computational simulations, and kinetic data.

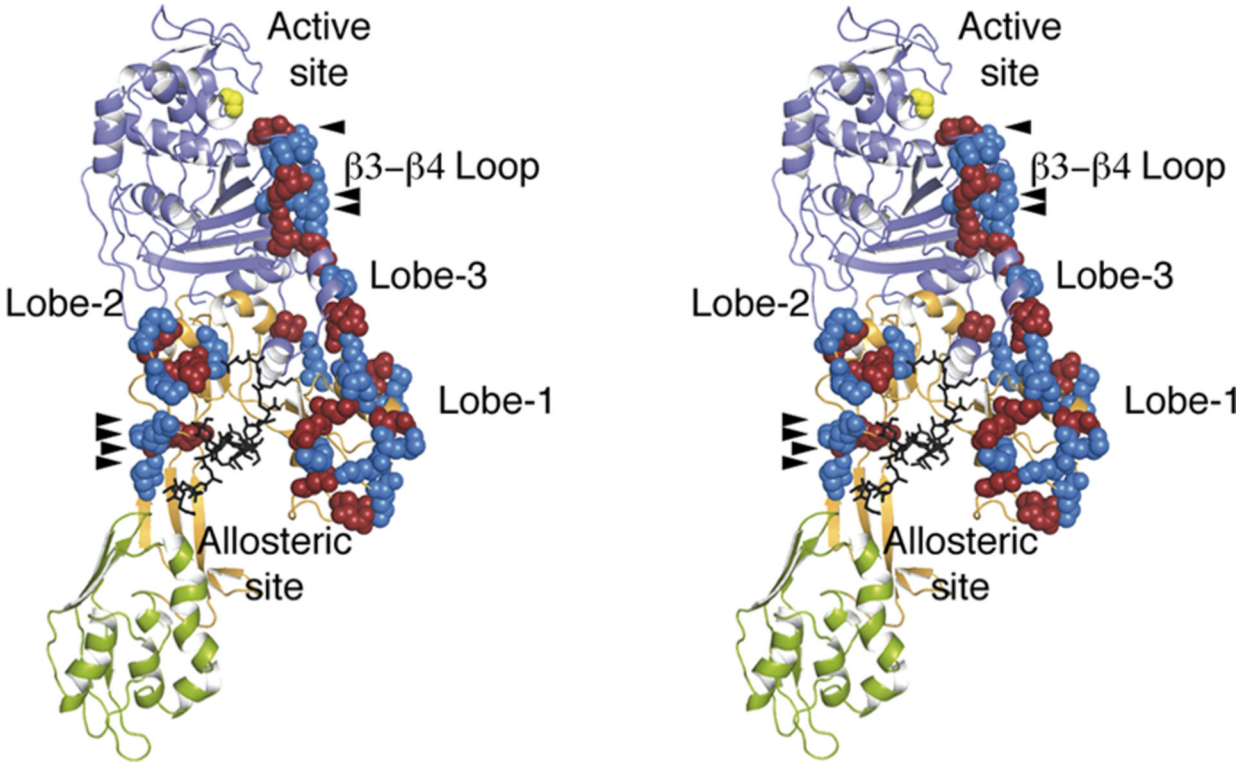


Figure 8.

Stereoview of the allosteric signal propagation in *S. aureus* PBP2a.[463] Binding of the peptidoglycan (black structure at the allosteric site (between Lobe-1 and Lobe-2) propagates a network of salt-bridge interactions extending between the allosteric and catalytic domains (the transpeptidase active site is at the top of the enzyme). The seven salt-bridge interactions seen by crystallography are identified with arrowheads. The catalytic serine (yellow at 12 o'clock) and the acidic (red) and basic (blue) residues of the salt-bridge interactions are shown as spheres. Peptidoglycan (or small molecule) binding at the allosteric site stimulates a domino motion from the allosteric site (intersection of Lobe-1 and Lobe-2), through Lobe-3, and onto the β3–β4 loop that controls access to the active site.

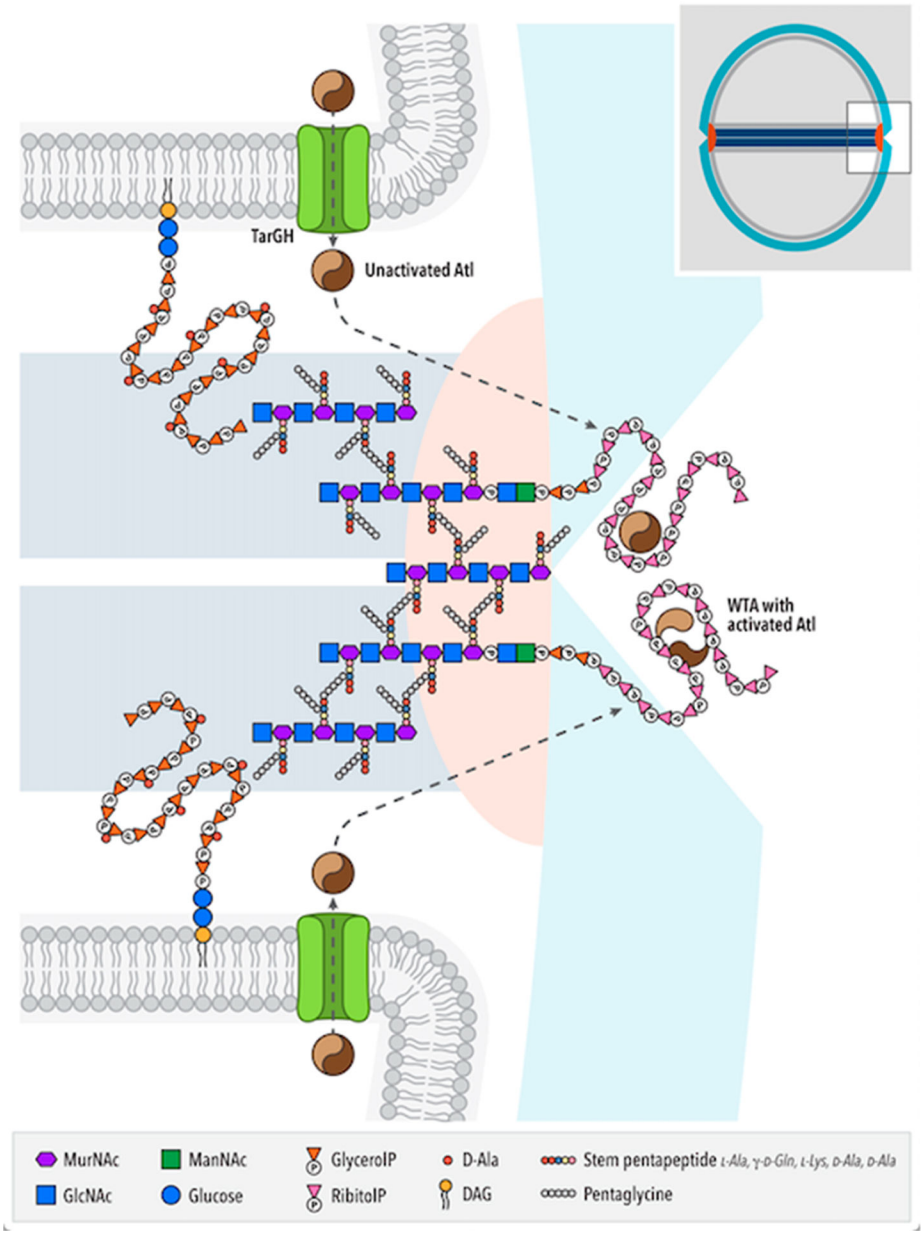
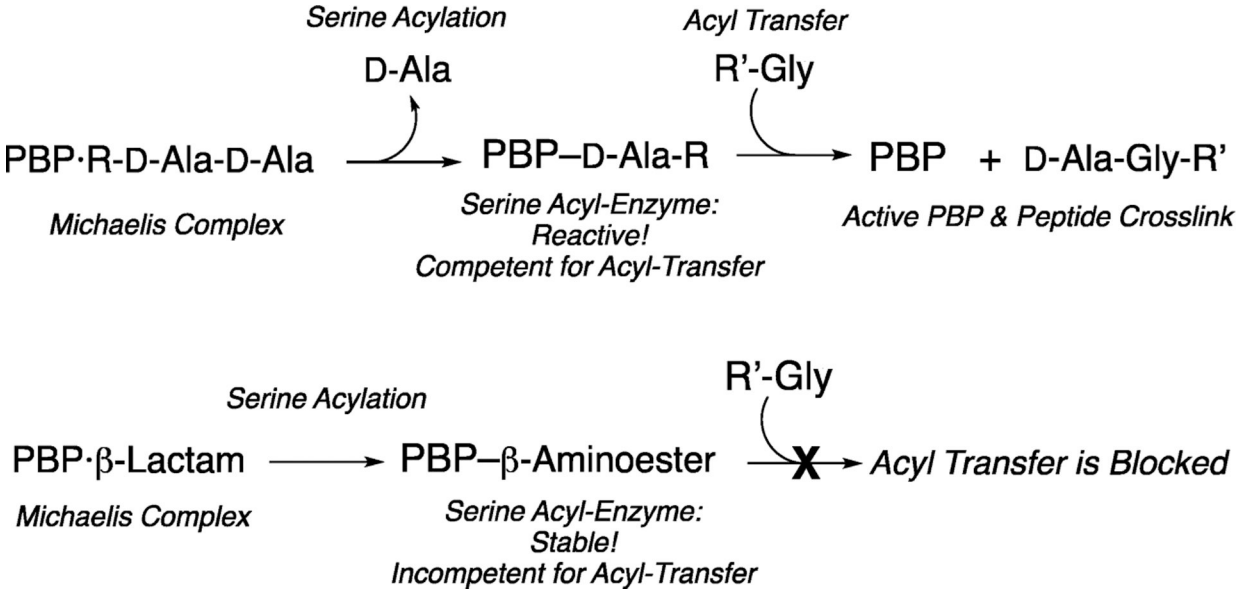


Figure 9.

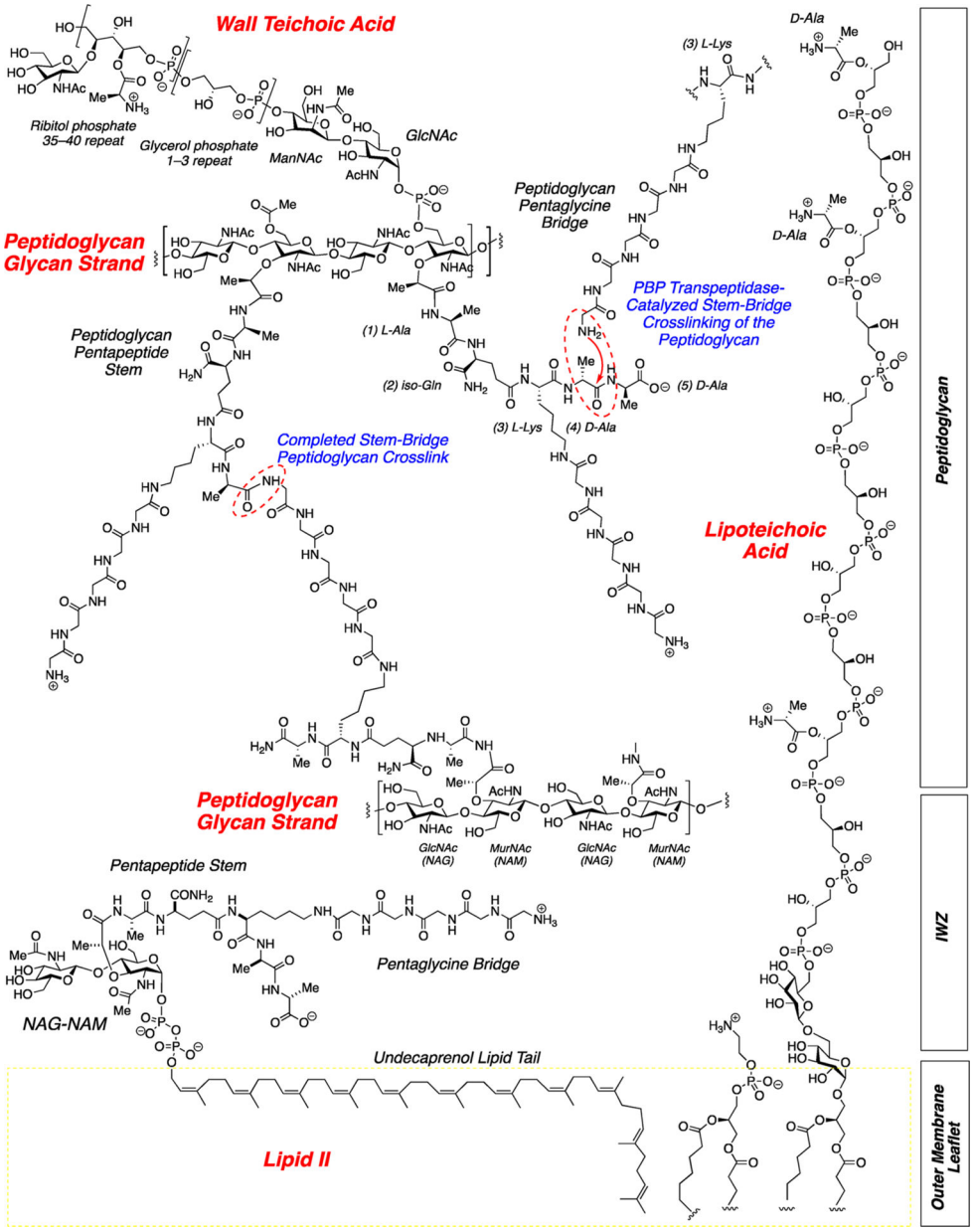
Suggested integration of the structural components of the *S. aureus* cell envelope with respect to spatial control of the Atl autolysin in *S. aureus* cell division. The structural components are rendered in cartoon form and placed with reference to Panel G of Figure 3 (duplicated as the top right inset). The structural components are (7 o'clock to 3 o'clock) the LTA (decorated with D-Ala residues), the peptidoglycan (bifurcated to indicate growth of the dual septa of the daughter cells), and nascent WTA at the septal perimeter. The icons used for the saccharides follow glycan icon nomenclature (Glc, blue circle; GlcNAc, blue square; ManNAc, green square; MurNAc, purple hexagon).[742] Nascent WTA is not decorated with D-Ala residues. The Atl pro-bifunctional autolysin enzyme, represented by yin and yang (light brown/dark brown) circle symbol, is transported to engage the nascent

WTA either through or in coordination with the TarGH transporter (the arrows of the figure are meant to represent either possibility). Atl is held in place by electrostatic interaction with the nascent WTA. The mature WTA found elsewhere on the cell envelope is suggested to be decorated by D-Ala residues (by transacylation of the D-Ala residues of the LTA) and thus incapable of binding Atl. Accordingly, Atl is held to the septal perimeter. Atl activation is tightly regulated (by an unknown mechanism) to the final stage of cytokinesis. Based on observations made with *S. pneumoniae*, inactivation of PBPs by the β-lactams disrupts this regulation, leading to premature activation of Atl autolysin and disregulated peptidoglycan degradation. This degradation is suggested as the culminating event of the bactericidal mechanism of the β-lactams.



Scheme 1. Mechanism of the β-Lactams Is PBP Inactivation by the Formation of a Stable Acyl-Enzyme Derived from the β-Lactam[a]

[a]This scheme provides spare kinetic summaries for PBP turnover of substrates and inactivation by β-lactams. The upper kinetic equation is substrate turnover. The PBP recognizes the R-D-Ala-D-Ala terminus of the peptidoglycan stem (see Scheme 2). From this Michaelis complex, an active-site lysine catalyzes opening of the β-lactam ring by a nucleophilic serine to give an acyl-enzyme intermediate. The PBP family divides between PBPs that catalyze peptidoglycan polymerization and peptidoglycan remodeling. In *S. aureus* the polymerizing PBPs transfer the acyl moiety, achieving a crosslinking reaction, to the amine of the terminal glycine residue of the bridge peptide of an adjacent peptidoglycan strand. In this scheme the bridge acyl-acceptor is abbreviated as R′-Gly. Note that the use of R′-Gly is not general, as different bacteria have different bridge structures. This kinetic sequence is contrasted with PBP inactivation by β-lactams. Here, the β-lactam is recognized as an R-D-Ala-D-Ala structural mimetic, and the active-site serine is acylated efficiently (lower kinetic equation). In contrast to PBP turnover, where there is departure of the terminal D-Ala as a leaving group, no leaving group departs upon β-lactam acylation of the active site serine. As a consequence, the β-lactam-derived acyl-enzyme (representative structure given in Chart 2) is incompetent for acyl-transfer. It is stable for multiple hours, far too long to sustain viability to the bacterium. The structural basis for the stability of the β-lactam-derived acyl-enzyme is steric interference with the acyl-acceptor (R′-Gly in polymerization reaction of *S. aureus*).[30,32,33]



Scheme 2. Principle Structures of the *S. aureus* Cell Envelope[a]

[a]The cell envelope surrounds the cytoplasm of the bacterium in the following order: membrane (adjacent to the cytoplasm: here showing only the outer leaflet and with abbreviated acyl structures for the diacylglycerol); the inner-wall zone (contains many of the enzymes used in cell-envelope creation, not shown here); a wall teichoic acid (WTA, top left) attached covalently to the polymeric peptidoglycan (top left, below the wall teichoic acid). The wall teichoic acid–peptidoglycan is the surface structure of some pathogenic *S. aureus* strains. Many other *S. aureus* strains have polysaccharides (not shown) attached to the peptidoglycan. The lipoteichoic acid (LTA, right structure) extends from the membrane through the inner-wall zone and intercalates the peptidoglycan. LTAs are essential to the structural integrity of the envelope. Lipid II (bottom left) is assembled in the cytoplasm and

translocated from the inner leaflet of the membrane to the outer leaflet of the membrane, with its disaccharide glycopeptide segment projecting into the inner-wall zone. Lipid II is the membrane-bound biosynthetic entity assembled into the peptidoglycan polymer. The Lipid II structure is parsed into four segments: an *undecaprenol diphosphate* membrane lipid, the NAG-NAM *disaccharide*, a pentapeptide *stem* whose last two amino acids are D-Ala-D-Ala, and a pentaglycine *bridge* attached to the *ε*-amine of the third amino acid (L-Lys) of the stem. Above the pentaglycine bridge of Lipid II is a nascent peptidoglycan strand (shown as a tetrasaccharide, formed from a transglycosylation reaction using Lipid II as the glycosyl donor adding to the terminal GlcNAc saccharide of a nascent peptidoglycan strand) that has been cross-linked (bridge-stem-bridge) to a second peptidoglycan strand. The dashed red oval to the left shows the functional group resulting from the cross-linking: and amide formed from the terminal glycine of the bridge to the carbonyl of the fourth amino acid (the penultimate D-Ala) of the stem. The second dashed red oval (top center) shows the reaction that forms this amide. The amine of the terminal glycine adds to the carbonyl of the (fourth amino acid of the stem) D-Ala, displacing the terminal D-Ala as the leaving group. This reaction is catalyzed by the Penicillin Binding Protein (PBP) enzymes, by a sequence of acyl-transfer to the active-site serine of the PBP, followed by acyl-transfer from this serine acyl-enzyme to the terminal amine of the Gly_5 bridge.

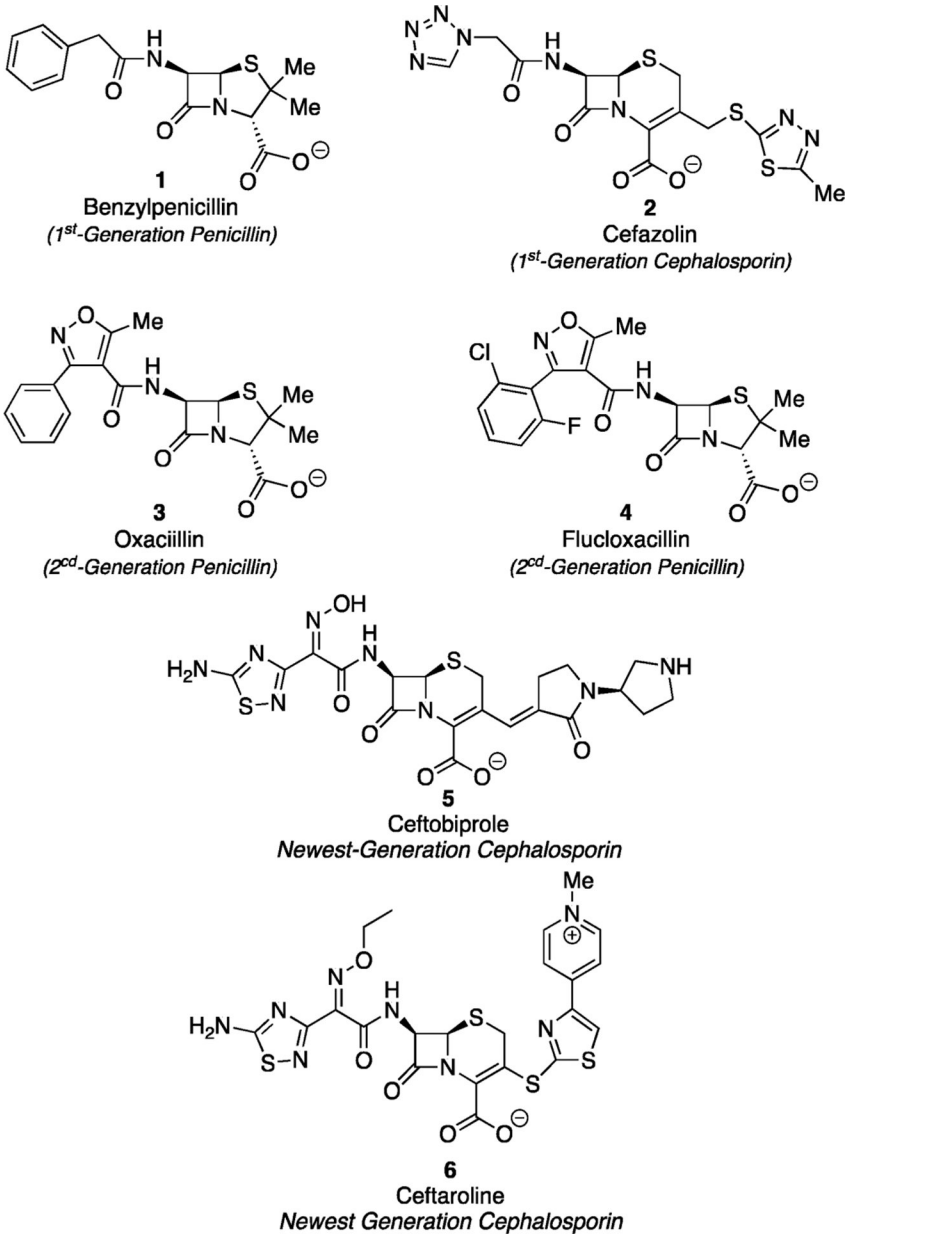


Chart 1. Six Representative Structures of the β-Lactams Used in *S. aureus* Chemotherapy[a]

[a]Benzylpenicillin **1** is a first-generation penicillin that lost quickly its clinical efficacy due to the acquisition by *S. aureus* of an enzyme, the BlaZ β-lactamase, which deactivated the penicillin by catalytic hydrolysis of its β-lactam ring to give the inactive β-amino acid metabolite. Cefazolin **2** is a first-generation cephalosporin that is a poor BlaZ substrate and thus is active against methicillin-susceptible *S. aureus* (MSSA). Oxacillin **3** and flucloxacillin **4** are second-generation penicillins of the methicillin class. They are poor BlaZ substrates and are still used in MSSA therapy. Ceftobiprole **5** and Ceftaroline **6** are the newest-generation cephalosporins with both Gram-positive and Gram-negative efficacy. In particular with respect to *S. aureus*, both structures have an enhanced ability to be recognized by and to inactivate the resistance penicillin-binding protein PBP2a

of methicillin-resistant *S. aureus* (MRSA). Both drugs are used clinically as prodrug formulations.

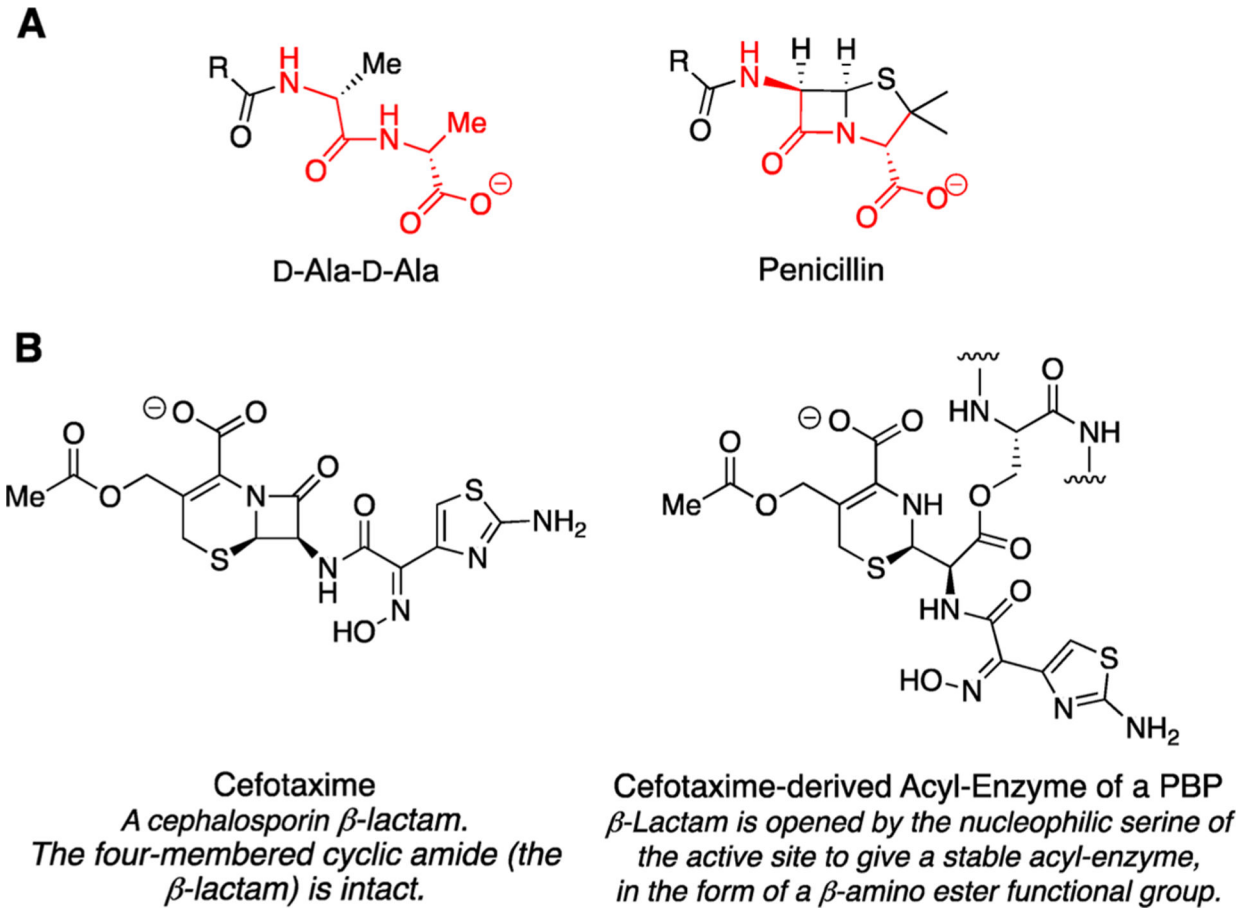


Chart 2. The β-Lactams as Structural Mimetics of the D-Ala-D-Ala Stem Dipeptide Terminus of the Peptidoglycan

The **A** structures compare (left) the R-D-Ala-D-Ala dipeptide terminus of the stem peptide of the peptidoglycan to the structure (right) of a penicillin. The red color identifies the structure commonality as proposed by Tipper and Strominger. The left of the **B** structures is that of the cephalosporin cefotaxime. To its right is the acyl-enzyme structure of a PBP inactivated by cefotaxime. The mechanism of the inactivation is ring-opening of the β-lactam by the active-site serine nucleophile to give the stable acyl-enzyme. This acyl-enzyme is stable as it is unreactive for acyl-transfer. In normal PBP catalysis, the acyl moiety of a peptidoglycan-derived acyl-enzyme is transferred, as a crosslinking reaction, to the terminal amine of the bridge peptide of an adjacent peptidoglycan strand. The correlation between the irreversible incorporation of penicillins into the bacterial PBPs, and the bactericidal mechanism of the penicillins, by Strominger was a milestone both for mechanistic enzymology and for the determination of antibiotic mechanism.[31]

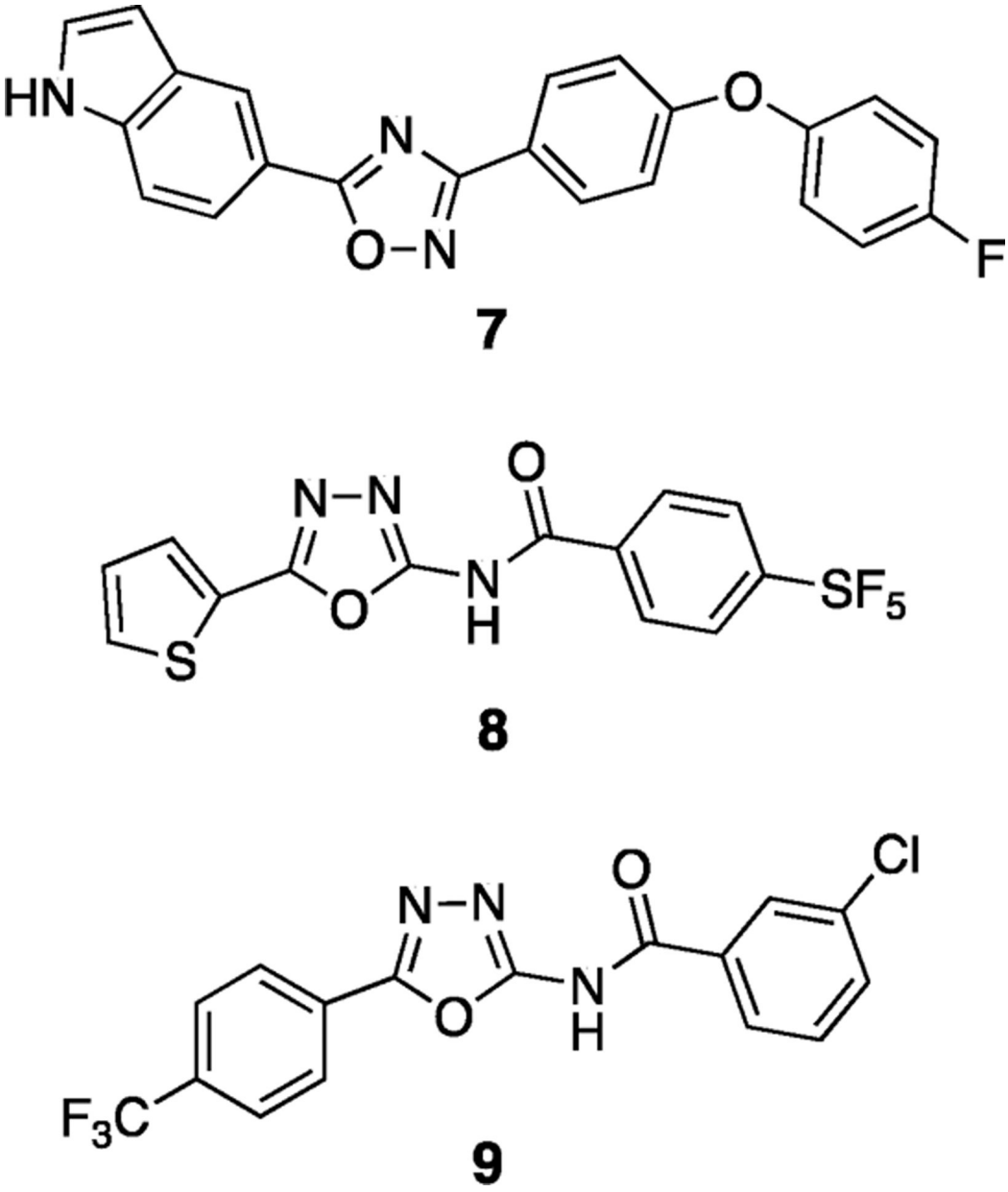


Chart 3.
MRSA-Acting Oxadiazole Structures

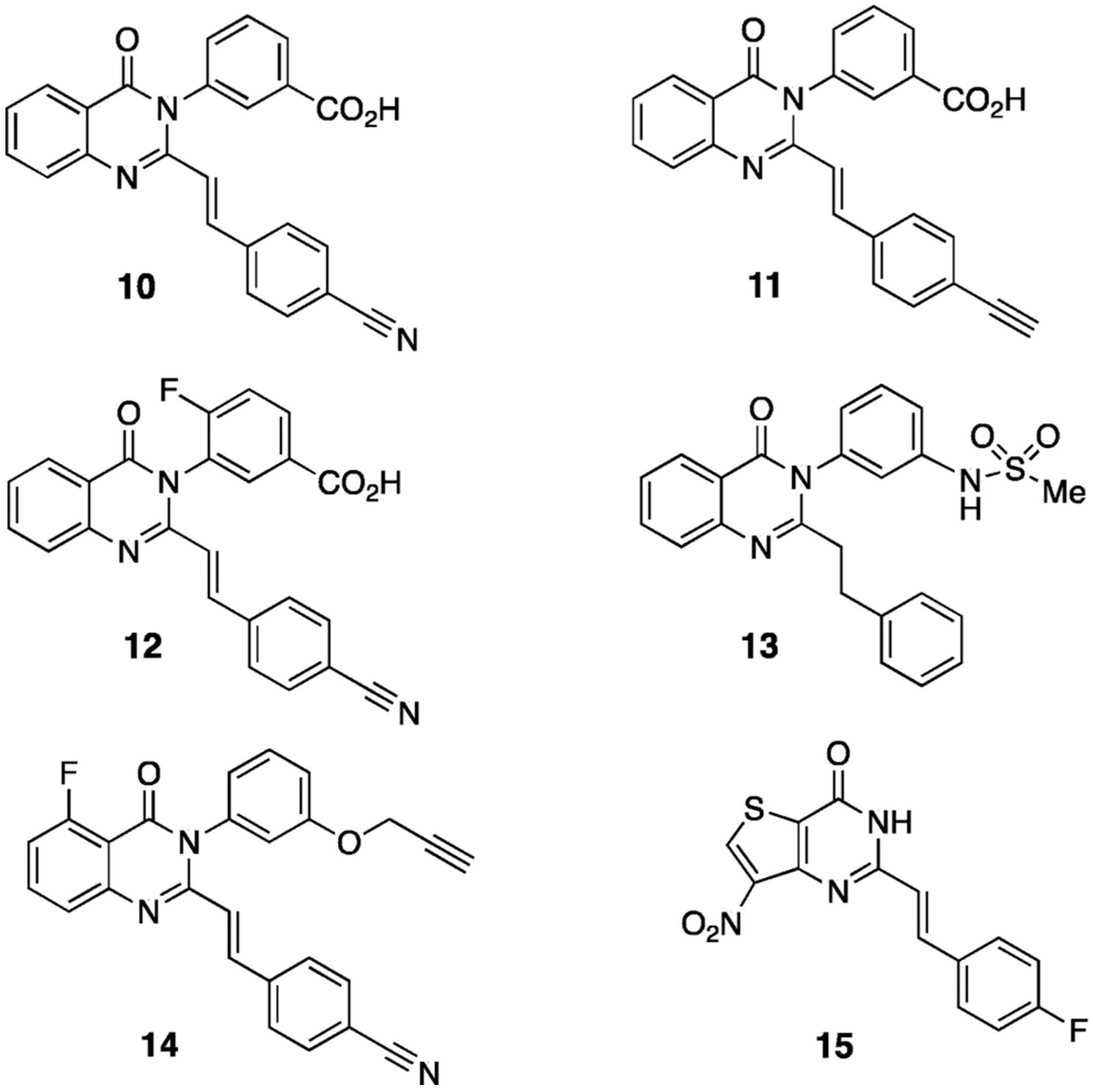


Chart 4.
MRSA-Acting Quinazolin-4-one Structures

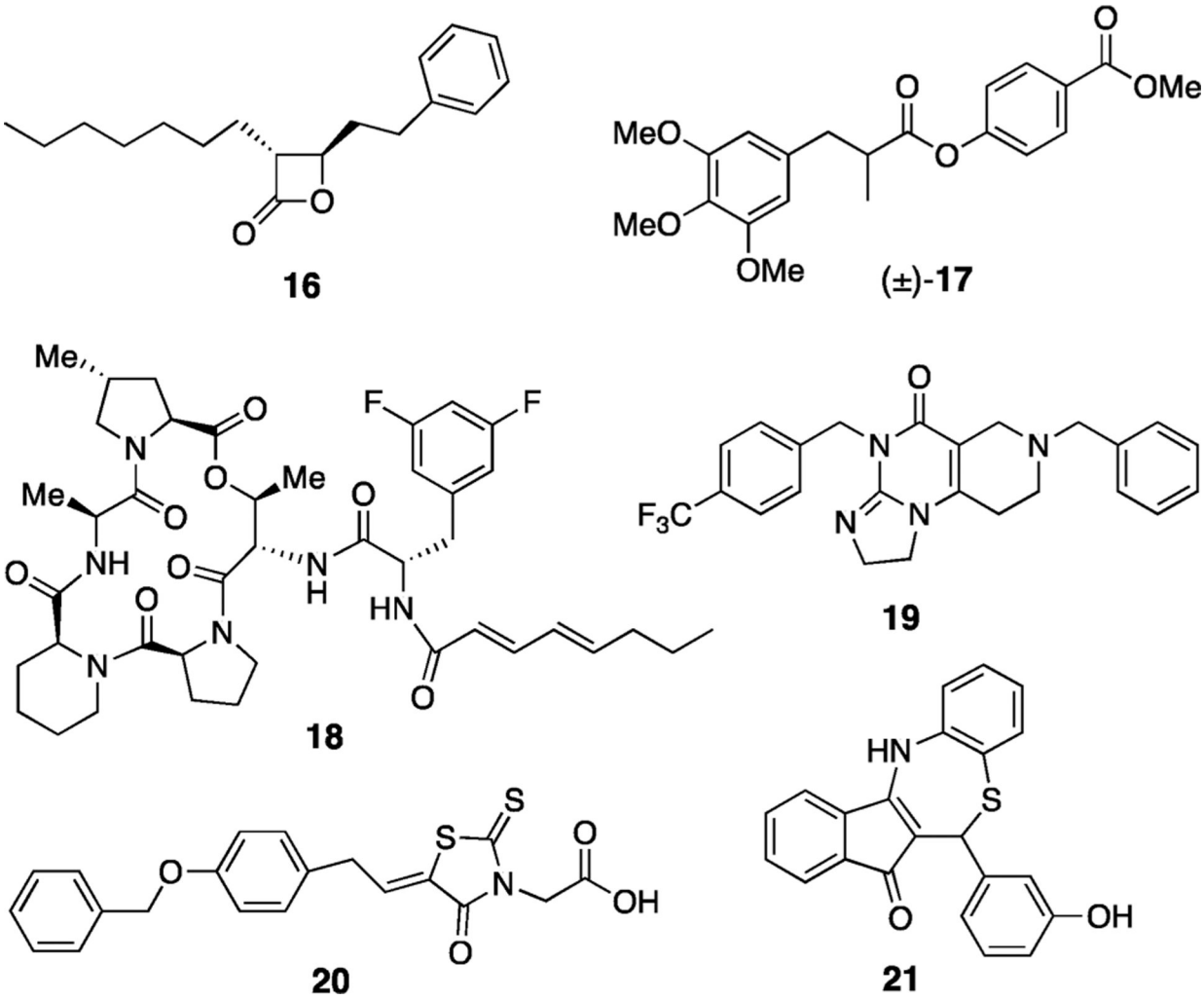


Chart 5.
MRSA-Acting ClpP Inhibitors

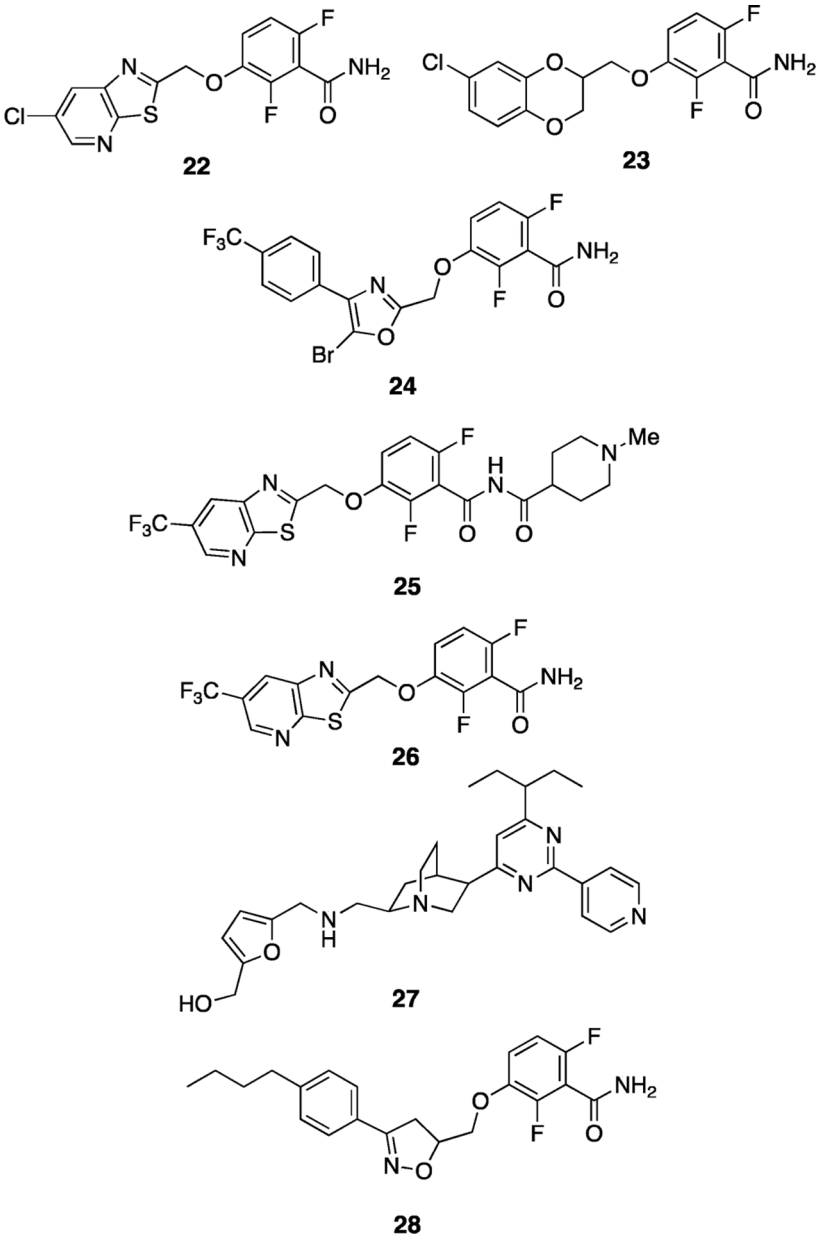


Chart 6.
MRSA-Acting FtsZ Inhibitors

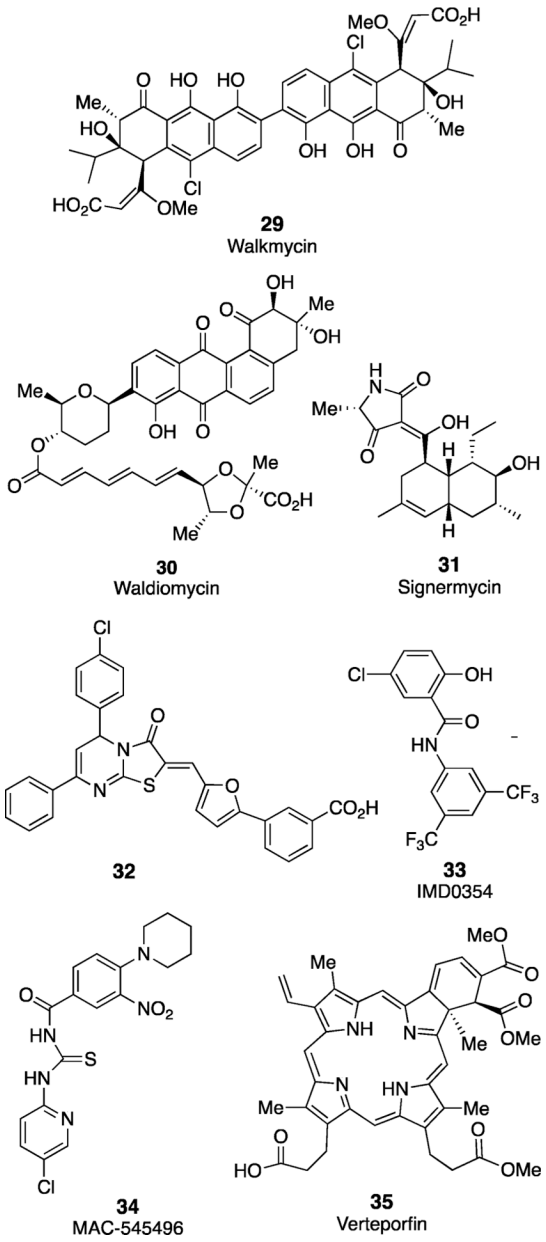


Chart 7.
MRSA-Acting Two-Component Kinase Inhibitors

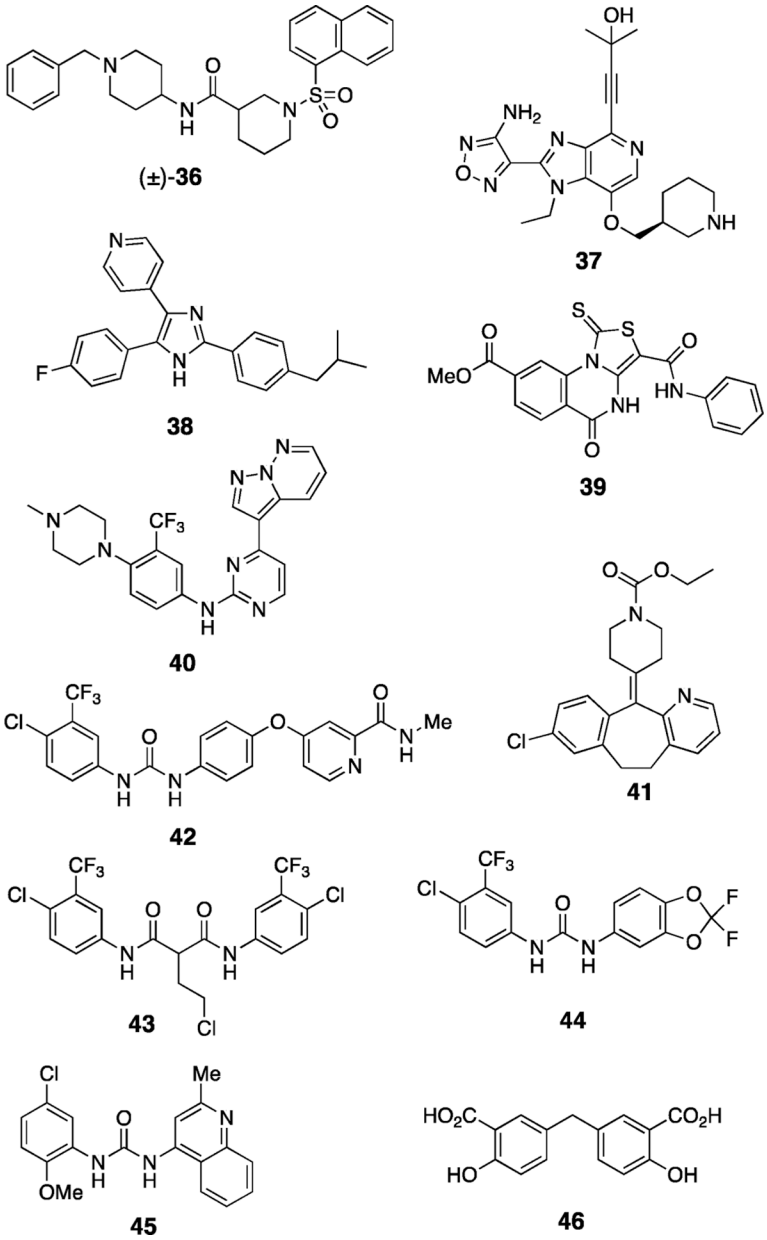


Chart 8.
MRSA-Acting Serine–Threonine Kinase Inhibitors