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# Bacterial Resistance to $\beta$ -Lactam Antibiotics: Compelling Opportunism, Compelling Opportunity<sup>†</sup>

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Received June 3, 2004

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## 1. Introduction

The simplistic image of the bacterium as an isolated, planktonic, self-cloning automaton is refuted. We now recognize bacteria as microorganisms of enormous diversity and adaptability. They can thrive under conditions that we regard as extreme—in the absence of oxygen and at high temperatures, to choose but two examples—and they can adjust with surprising alacrity to their environment, and to their circumstance, so as to improve their fitness for survival.

The focus of this review is that of bacterial biochemical adaptation to a particular circumstance of profound concern to the human species: that of bacterial tolerance and resistance to the  $\beta$ -lactam antibiotics.<sup>1</sup> The  $\beta$ -lactam antibiotic family originally was limited to the penicillin (sulfur-containing penams) and then the cephalosporin (sulfur-containing

cephems)  $\beta$ -lactams but now includes natural and synthetic monocyclic  $\beta$ -lactams, carbapenems, oxapenams, carbacephems, and oxacephems (Scheme 1). The  $\beta$ -lactams are one of the three largest antibiotic classes (the others are the macrolides and fluoroquinolones).<sup>2,3</sup> Mere words cannot properly emphasize the role that these antibiotics have to the preservation of human health. Nor do words adequately emphasize the disquieting reality that at the same moment we profit from the use of antibiotics, that there is cost and that this cost is inexorable bacterial resistance.<sup>4–8</sup>

Bacterial resistance is not a new phenomenon. We now recognize that resistance is the inevitable consequence of organisms competing for the same ecological niche. Yet it is only during the past 60 years that resistance has been transformed by man (as the driving evolutionary force)<sup>9</sup> from what might be reasonably described as stasis—bacteria competing against bacteria—to that of a disequilibrium of chemical warfare<sup>2,10–14</sup> where bacteria additionally compete directly with us. Assuredly, this is a competition with uncertain outcome. While the phenomenon of bacterial resistance is evolutionarily ancient,<sup>15,16</sup> the consequence of this (so very recent) warfare is that of accelerated dispersion of the mechanisms for resistance across the bacterial kingdom, increasing selection for bacteria that have acquired these mechanisms, and devaluation of our antibiotic armamentarium.

Bacterial resistance mechanisms with respect to the  $\beta$ -lactam antibiotics are divided between those that occur at the level of primary metabolism (altered and acquired proteins and enzymes) and those that occur at the level of secondary metabolism (the biosynthesis of modified  $\beta$ -lactams that are better antagonists of the altered proteins). While the realization of the outstanding importance of secondary metabolites as drug templates dates to the moment of their very discovery by man,<sup>17,18</sup> the recognition that these secondary metabolites occupy a logical place in the evolution of bacterial resistance is a more recent consensus, as discussed in several lucid reviews<sup>13,19–23</sup> The parallel medicinal chemistry development of  $\beta$ -lactam structure is presented by Dalhoff and Thomson<sup>24</sup> and is not further discussed here. Our focus is the bacterial response to the selection pressure exerted by the  $\beta$ -lactam antibiotics. Among the changes that are accomplished are alterations (mutations) in the molecular target of the

<sup>†</sup> This review is dedicated to our Ph.D. mentors: To Chris Walsh, on the occasion of his 60th birthday and with appreciation for his unique ability to inspire and guide self-discovery. To Bill Hase, for his guidance and creating an environment that expected perfection in every aspect of scientific investigation. To Michael Johnston, for his creativity, passionate engagement, and single-minded ability to contribute to the many things that he has tackled in life.

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Jed F. Fisher is an alumnus of S.U.N.Y. Stony Brook (B.S., under Bill Fowler) and M.I.T. (Ph.D., under Chris Walsh). Following a postdoctoral stay at Harvard (with Jeremy Knowles), a faculty position at the University of Minnesota, and nearly two decades in the pharmaceutical industry (at Upjohn and Pharmacia), he joined the Mobashery group at the University of Notre Dame in 2004. His scientific accomplishments include the collaborative discovery of the utility of coenzyme mimetics to discern flavoenzyme mechanism, the acyl–enzyme pathway for  $\beta$ -lactamase substrate hydrolysis and inhibitor inactivation, the autocatalytic pathway for anthracycline and mitomycin reductive nucleophilic substitution, the first rational design of an inhibitor of protein–protein association, and the virtue of glycosylation to alter peptidomimetic pharmacodynamics.



Samy Meroueh studied chemistry at Wayne State University, where he received his Ph.D. degree in Physical Chemistry, under the supervision of William L. Hase. He then joined the group of Shahriar Mobashery, where he is currently a Walther Cancer Institute Fellow at the University of Notre Dame in South Bend, IN. He is using a variety of tools, including computational, calorimetry, and X-ray crystallography, toward understanding the catalytic machinery of enzymes that are involved in antimicrobial resistance and the design and discovery of molecules that would target those enzymes as potential antimicrobial agents.

$\beta$ -lactams, the (ancient) transformation of these enzymes into families of  $\beta$ -lactam hydrolytic deactivating enzymes (the  $\beta$ -lactamases), the expression of protein inhibitors of the  $\beta$ -lactamases, the deletion of porin proteins in the membrane, the acquisition and activation of efflux exporter proteins, and the modification of the cell wall to minimize  $\beta$ -lactam antibiotic access to their targets. While the alteration in the cell wall biosynthesizing targets and the expression of  $\beta$ -lactamases may be regarded as the primary bacterial defensive measures, none of these defensive measures is unimportant. Depending on the bacterium and the particular circumstance of the  $\beta$ -lactam challenge, the bacterium that devises a successful combination of these responses is the bacterium that survives. This review covers the

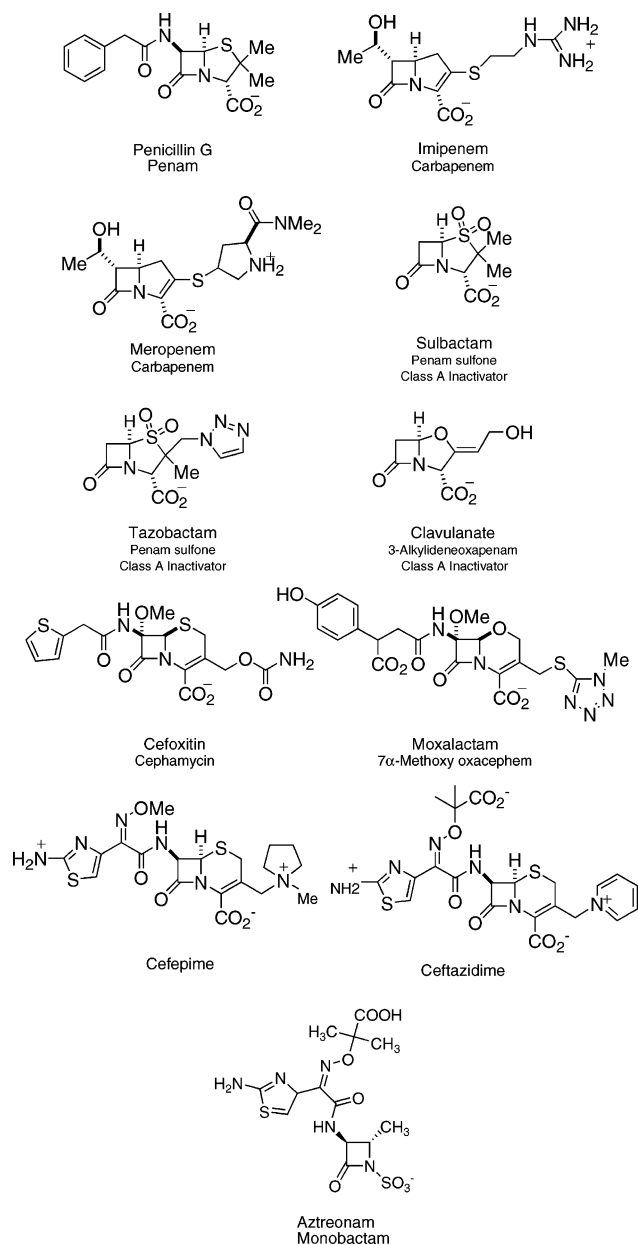


Shahriar Mobashery received his undergraduate (1981) and doctoral (1985) degrees from the University of Southern California and University of Chicago, respectively. After postdoctoral research (1986–1988) at Rockefeller University, he joined the faculty at Wayne State University in 1989. Mobashery and his research group relocated to the University of Notre Dame in 2003, where he holds the position of Navari Family Professor of Life Sciences in the Department of Chemistry and Biochemistry.

primary literature of the past 4 years with citation to the preceding literature via the many outstanding concurrent reviews on this topic.

Notwithstanding the likely familiarity of the reader with the  $\beta$ -lactam antibiotics as mechanism-based enzyme inhibitors of cell wall biosynthesis (inhibiting the  $\beta$ -lactam “binding protein” enzymes) and enzyme substrates (of the hydrolytic antibiotic-destroying enzymes, the  $\beta$ -lactamases),<sup>16</sup> an overview of the chemistry of the  $\beta$ -lactams is essential context. The point of reference is the eponymous  $\beta$ -lactam (that is, a 2-azetidione) four-membered cyclic amide ring of the penicillins and cephalosporins (Scheme 1). The neighboring carboxylate (on the second ring of these bicyclic structures) and the acylamino substituent upon the  $\beta$ -lactam immediately (from our contemporary perspective) define these structures as conformationally constrained tripeptides, having an exposed C-terminus and imbued with the capacity to acylate (with opening of the  $\beta$ -lactam) susceptible nucleophiles. The antibiotic property implies that these peptidomimetics mimic an essential peptide motif possessed by the bacteria and engage this mimicry to the purpose of confounding acylation of a critical enzymatic target. Indeed, this hypothesis coincides to the guiding  $\beta$ -lactam presumption—the Tipper–Strominger hypothesis—that dates from the discovery by Tipper and Strominger (and simultaneously and independently by Wise and Park) that a penicillin-derived entity is irreversibly incorporated into the transpeptidase and carboxypeptidase enzymes of bacterial cell wall biosynthesis.<sup>25,26</sup> Among the cell wall biosynthetic enzymes an obvious candidate for such interference is the peptidoglycan cross-linking transpeptidase wherein the peptidoglycan D-Ala-D-Ala terminus is cleaved in the serine acylation half-reaction (with loss of the terminal D-Ala) and the cross-link is formed in the deacylation half-reaction (by acyl transfer to an amine substituent of the neighboring peptidoglycan strand). Should this enzyme be presented with a substrate mimetic wherein the initial acylation reaction remains enabled, but the capacity for deacylation is abolished,

Scheme 1



then the enzyme will fail to complete its catalytic cycle.<sup>27,28</sup> The loss of these enzymatic activities disrupts the homeostasis of cell wall integrity, leading (through a poorly understood process that ultimately involves activation of cell wall degradative enzymes, termed autolysins) to lethal cell wall defects.<sup>1,29–31</sup> A bacterium unable to maintain the integrity of its cell wall will be unable to reproduce (a bacteriostatic antibiotic) or survive (a bactericidal antibiotic) wherein the impaired cell wall no longer contains the osmotic pressure of the cytoplasm.<sup>32,33</sup>

Within this mechanistic perspective is found the two important strategies for bacterial acquisition of resistance to the  $\beta$ -lactam antibiotics. If a cell wall transpeptidase is deceived by a peptidomimetic in the acylating half-reaction, then resistance can be achieved by alteration of the transpeptidase such that acylation by the peptidomimetic does not happen. Second, if a cell wall transpeptidase is unable to complete deacylation of the peptidomimetic, then resistance

may be achieved by alteration of the transpeptidase so as to enable this reaction. The first answer is that of mutated (resistant)  $\beta$ -lactam (penicillin) binding proteins (PBPs). The second answer is that of the transformation of the PBPs to new, fully capacitated—often operational at the substrate diffusional rate constant—hydrolytic (wherein water is the acyl acceptor) enzymes, the  $\beta$ -lactamases. As these alterations must be accomplished without loss of bacterial fitness, it may be expected that specific circumstance will make one the more effective strategy than the other. This has proven to be so. Both contribute. However, whereas the altered PBP is generally regarded as a relatively recent development in Gram-positive bacteria resistance, the advent of  $\beta$ -lactamases is understood as an ancient evolutionary event in bacteria resistance. With respect to the last 60 years, it is far less the evolutionary development of these resistant enzymes as it is their broadened distribution, which is of immediate concern.

We have posited a relationship between the mechanisms by which  $\beta$ -lactams serve as antibiotics and the primary mechanisms by which bacteria acquire resistance to these mechanisms in terms of enzymatic acylation and deacylation half-reactions. Further development of these concepts requires, however, a clearer description of these events as chemical reactions. Two reactions are pertinent. Under enzymatic control each comprises an acylation event (wherein an enzyme active site serine is involved for both) and a deacylation event (wherein for the transpeptidase the incoming nucleophile is usually a lysine, ornithine, or diaminopimelate amino group and wherein for the  $\beta$ -lactamase the acyl acceptor is water). For the transpeptidase, the net reaction is the cross-linking of the peptidoglycan NAM-pentapeptide, the major constituent of the cell wall. For the  $\beta$ -lactamase, the net reaction is  $\beta$ -lactam hydrolysis to the biologically harmless penicilloate.

An objective of the two following sections—the relationship of the PBPs and  $\beta$ -lactamases to bacterial  $\beta$ -lactam resistance—is to communicate at the simplest possible level the operation of these acylation/deacylation reactions at the protein level. This understanding remains a challenging task. The first aspect of this task is the comparative transition-state energies for ordinary (acyclic) amide (or peptide) cleavage and  $\beta$ -lactam opening. As Page discussed,<sup>34</sup> the historical presumption that  $\beta$ -lactam antibiotic ability correlates to the release of significant strain energy upon  $\beta$ -lactam ring opening is overvalued. Rather, the strain energy is modest and such greater  $\beta$ -lactam reactivity as does exist among the  $\beta$ -lactam family members (for example, the Sweet–Woodward correlation)<sup>35</sup> is unlikely to be expressed in the critical (rate-limiting) enzymatic acylation step.<sup>36</sup> Moreover, the spatial requirements for general acid catalysis of transient tetrahedral species collapse to the acyl-enzyme are rather different for a peptide compared to a  $\beta$ -lactam.<sup>34</sup> The answers to the critical questions as to why  $\beta$ -lactams successfully inhibit the PBPs and have become favorable substrates for the PBP-derived  $\beta$ -lactamases are found in the experiments that address these questions: How is the  $\beta$ -lactam



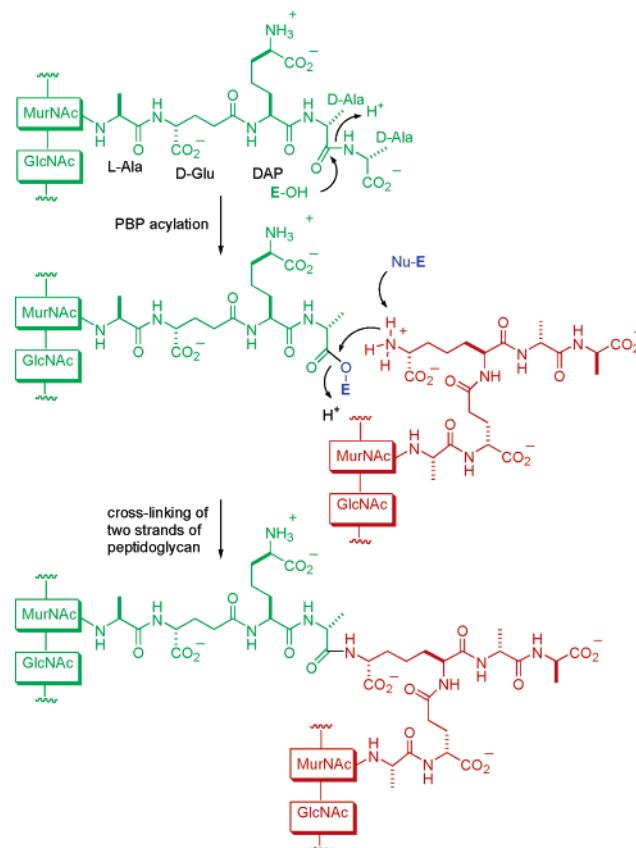
recognized as a peptidomimetic by both enzyme classes? How does the  $\beta$ -lactam exploit for acylation the PBP catalytic machinery, for which it is not intended? How does the resulting PBP acyl-enzyme resist the catalytic machinery for deacylation (or transacylation)? How has the  $\beta$ -lactamase acyl-enzyme become fully competent for acyl transfer to water? The extraction of answers to these questions requires challenging experimental design. This design would be more straightforward if the mechanistic basis for enzyme catalysis was evident from protein structure. It is not!<sup>34,37–39</sup> Enzyme catalysis is the subtle orchestration of a panoply of electrostatic forces, often significantly influenced by distal (non-active site) changes in protein structure. While we can visualize atoms—the structures of several PBPs and of numerous  $\beta$ -lactamases are known—we must intuit forces and conjecture the transition states that they stabilize. The relationship of such conjectures (and transition states) to bacterial  $\beta$ -lactam resistance is the objective of the remainder of this review.

## 2. Penicillin-Binding Proteins

### 2.1. Enzymes of Cell Wall Biosynthesis

To have survived means to have been opportunistic. Among the survivors, across the eons of time, are the single-cell microorganisms of the domain bacteria. The seminal observation that a crystal violet stain is retained by some bacteria, but not by others, is known now to signify two different exoskeleton constructs. The positive staining bacteria have a single multilayered polymeric—that of a cross-linked peptidoglycan—exoskeleton, while the nonstaining bacteria have a thinner (two and in places three layers) of a polymeric (and also a cross-linked peptidoglycan) exoskeleton, further surrounded by a gellike periplasmic layer,<sup>32</sup> itself enclosed by a complex (outer) membrane bilayer. Despite this substantial difference, there are at the functional level and at the molecular level remarkable similarities between the two. The peptidoglycan exoskeleton (termed the murein sacculus) is durable and elastic, strong enough to contain the osmotic turgor of the living bacterium yet permitting nutrient access to the porins and transporter proteins.<sup>40</sup> The cell wall components—synthesized in the cytoplasm and transported across the cytoplasmic membrane for polymerization—of both are remarkably similar.<sup>41</sup> For Gram-negative bacteria (and for many Gram-positive ones), in the final cell wall assembly step the D-Ala terminus of the pentapeptide-functionalized *N*-acetylglucosamine (termed *N*-acetylmuramic acid or NAM, and assembled with *N*-acetylglucosamine or NAG, as a NAG-NAM disaccharide repeat) is removed. The resulting acyl species is then transferred (cross-linked) to an amino group of a neighboring chain, thereby unifying the peptidoglycan sacculus as a single polymeric macromolecule (Scheme 2).<sup>26</sup> Given the sophistication of this process (which is also intimate to the resealing of the sacculus during cell division), cooperative multienzyme catalysis (that includes the “transpeptidase” just described) is implicated. The study of this enzyme ensemble (termed

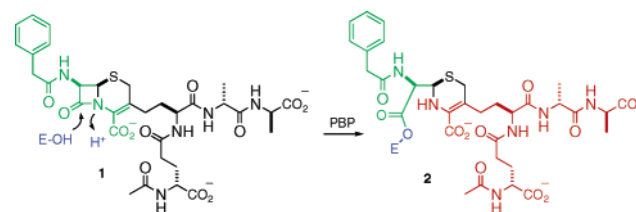
Scheme 2



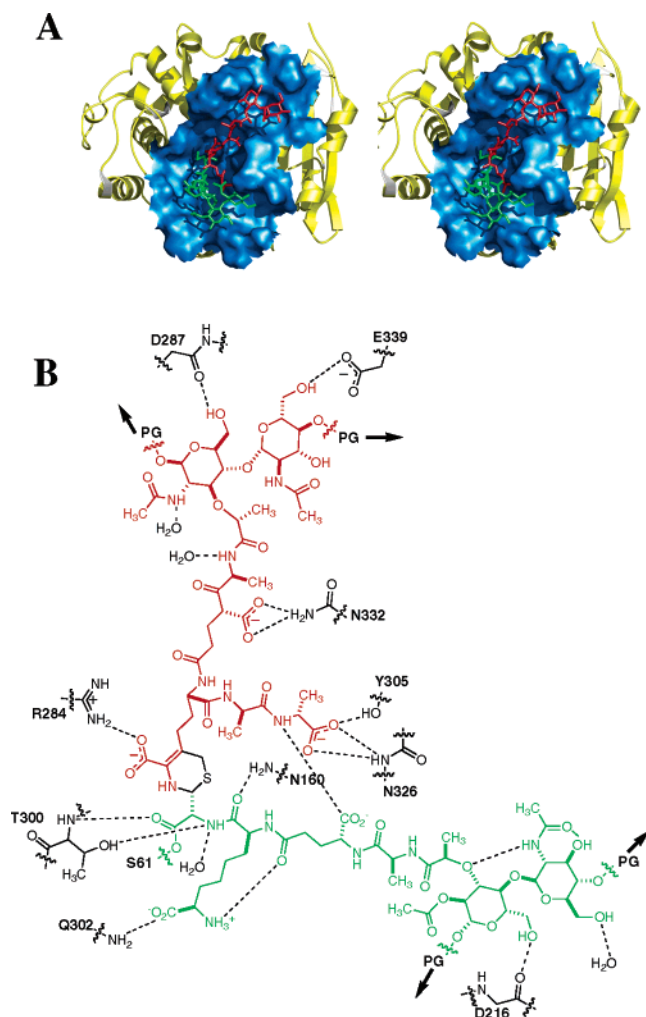
the divisome when as part of cell division) to bacterial shape, integrity, and function is of outstanding scientific importance.<sup>26,32,42–45</sup> With regard to antibiotics (not only the  $\beta$ -lactams, although these are our focus), this ensemble is the story of compelling opportunity and compelling opportunism.

The events that lead to the cross-linking reaction have been elucidated with the 1.2 Å resolution X-ray structure of the acyl-enzyme formed from the D-alanyl-D-alanine carboxypeptidase/transpeptidase from *Streptomyces* sp. strain R61 and a unique cephalosporin.<sup>33</sup> This cephalosporin (compound **1**, Scheme 3) was designed to incorporate components of the cell

Scheme 3



wall into its own structure. As anticipated, compound **1** modified the active site serine, binding between the all  $\alpha$ -helix and  $\alpha/\beta$  domain. The portions of compound **1** that mimic strands 1 and 2 from the peptidoglycan (see the green- and red-colored portions in Figure 1 and Scheme 3, for example) were found to be oriented within the active site. The left portion of compound **1** mimics the acyl-D-Ala-D-Ala portion of the first strand of the peptidoglycan (portion of **1** in green), while the right portion mimics the approach of the nucleophile—the diaminopimelate—from the second



**Figure 1.** (A) Stereoview of the three-dimensional structures of two strands of peptidoglycan bound to the active site of the D,D-transpeptidase/D,D-carboxypeptidase from *Streptomyces* R61 PBP, constructed computationally from the 1.2 Å resolution structure for the acyl-enzyme species with compound 1 (the extension reaches the NAG-NAM units on the peptidoglycan). The protein is shown in yellow ribbon representation, while the bound computational model representing the two strands of the peptidoglycan are shown in green and red capped-sticks representation. The blue van der Waals surface defines the active site. (B) Schematic representing the peptidoglycan from A, showing the various hydrogen-bonding interactions and color-coded according to the three-dimensional model.

strand of the peptidoglycan (red portions in structure 2). The acylation is proposed to occur after activation of the active site Ser62 (located at the N-terminus of an  $\alpha$ -helix, which is expected to modulate the serine  $pK_a$  as proposed by Moews et al.<sup>46</sup>) by the general base Lys65—which is in direct contact with Ser62 at a distance of 3.0 Å. This mechanism is consistent with previously proposed mechanisms of the acylation of PBPs, where the universally conserved lysine acts as the general base, abstracting a proton from serine, followed by the back-donation of the proton (from lysine) to the peptide or  $\beta$ -lactam nitrogen.<sup>47</sup> To provide a more detailed picture of the cross-linking event, a computational model was constructed from the high-resolution structure by extending compound 2 to include the full pentapeptide and a NAG-NAM extension. The resulting model was fully solvated and

energy minimized (shown in Figure 1A). The peptidoglycan strands were found to form a network of electrostatic interactions (shown in Figure 1B). These interactions should play important roles in properly positioning the peptidoglycan strands and for other important events such as deprotonation of diaminiopimelate in the cross-linking event. It is of interest to note that the three-dimensional model differed from a previous model<sup>48</sup> that used only the apo PBP2x structure with respect to the location of the saccharide-binding grooves.

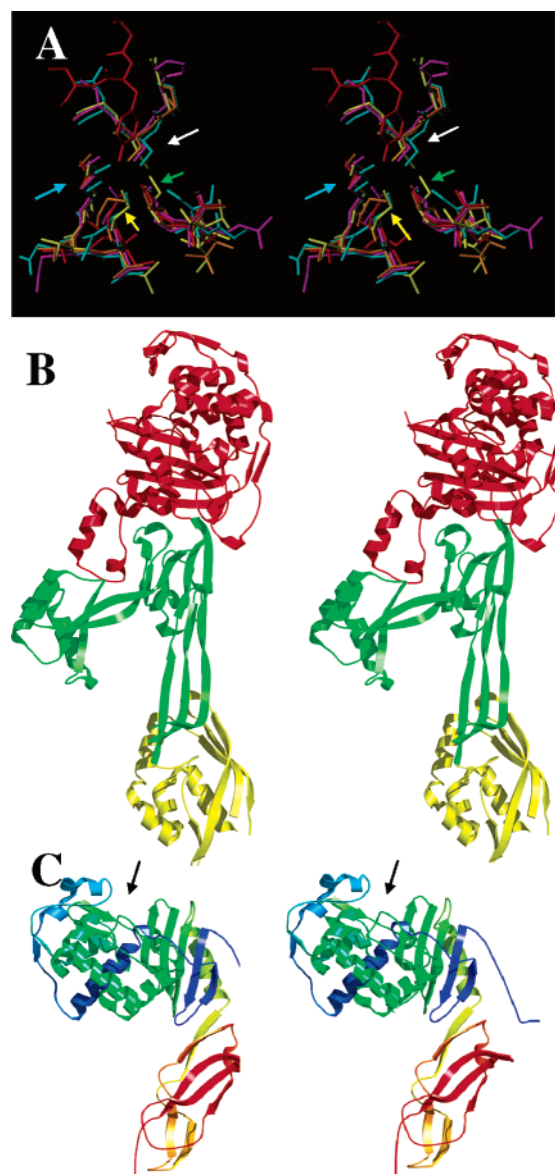
The description of behavior as either moral or immoral is (primarily) a human characteristic. For other organisms this distinction is irrelevant, and the focus of their behavior is survival to the point of reproduction. Among the bacteria (and fungi, for which bacteria are a food source) survival—that is, preservation within an ecological niche—requires exploitation of vulnerability. In addition, the biosynthetic enzymes of bacterial cell wall biosynthesis are vulnerable. The basis of their vulnerability (which is one and the same with that of the bacterium) is the combination of essentiality and exposure. These enzymes are located underneath the very cell wall that they assemble. For the Gram-negative bacteria these enzymes are either within the periplasmic space or—for the most essential of these enzymes—with active sites exposed to the periplasmic space and a transmembrane domain (with small cytoplasmic anchor) within the cytoplasmic membrane. Hence, bimolecular encounter with an inhibitor of these enzymes requires only the successful passage of the inhibitor—intermingling with solute nutrients—through the lipopolysaccharide of the outer membrane into contact with the peptidoglycan surface of the periplasmic space. While this simple requirement cannot be underestimated (especially insofar as antibacterial design and for resistance development) for the penicillin and cephalosporin  $\beta$ -lactams secreted by the biosynthesizing bacteria and fungi within the niche, the passage and encounter with these biosynthetic enzymes is straightforward. Astonishingly, *each* enzyme of the ensemble is capable of inactivation (via the same mechanism of irreversible acylation) by an appropriately substituted  $\beta$ -lactam. The inactivation is facile for susceptible Gram-positive and -negative strains and less so for resistant bacteria. This truly remarkable event is commemorated by historical nomenclature: these enzymes are collectively the penicillin-binding proteins (or PBPs) of the bacteria.

The chemically intriguing aspect of this event is the recognition by each enzyme regardless of the specific cell wall biosynthetic role. The inescapable conclusion is a fundamental of homology of structure and of alignment with the active site. However, despite this homology, all  $\beta$ -lactams do not inhibit all PBPs, likely due to subtle differences in the active site and distal regions in the protein. Regardless of cell morphology (Gram positive or negative) and regardless of individual specific synthetic function, these enzymes must possess such similarity as to implicate a mere handful of, if not a single, ancestral progenitor(s). As the intact  $\beta$ -lactam antibiotic was

recognized by this ancestral enzyme as a mimic of the peptidic terminus of the peptidoglycan, so too there is continued recognition of these antibiotics by the offspring of this enzyme. Once the  $\beta$ -lactam antibiotic is recognized by the PBP, the recognition culminates in the formation of a stable acyl–enzyme species. Hydrolysis of the acyl–enzyme ester bond is slow in PBPs, with half-lives that substantially exceed the doubling time of the organism. To appreciate the degree of inefficiency of this step in PBPs, comparison of the deacylation rate constant with that of  $\beta$ -lactamases—resistance enzymes that are believed to have descended from the PBPs—reveals that the rate of deacylation in PBPs is up to 6 orders of magnitude slower.<sup>49</sup> The irreversibility of the deacylation step in PBPs is at the root of the antimicrobial action of  $\beta$ -lactam antibiotics.

The basis for these differences must clearly derive from the differences in the target–PBP and  $\beta$ -lactamases—structures. The first X-ray diffraction structures of two low molecular weight PBPs were solved nearly 25 years ago.<sup>50–52</sup> Since then only a handful of additional structures have been solved, despite the very large number of PBPs that are now known. Those with solved structures include the low  $M_r$  PBPs from *Streptomyces* R61<sup>53</sup> and *Streptomyces* K15 (PDB code 1SKF),<sup>54,55</sup> a zinc-dependent PBP from *Streptomyces albus* G (PDB code 1LBU),<sup>50</sup> the PBP5 from *Escherichia coli* (PDB code 1NZO),<sup>56</sup> and the high  $M_r$  (and  $\beta$ -lactam resistant) PBP2x (PDB code 1PMD)<sup>48</sup> from *Streptococcus pneumoniae* and PBP2a from *Staphylococcus aureus*.<sup>57</sup> A comparison of these structures reveals similarities but also differences. The fold of the transpeptidase/carboxypeptidase appears to be conserved among PBPs—the exception would appear to be the abundant penicillin-binding protein from *Treponema pallidum*, Tp47, which has a unique multidomain fold.<sup>58</sup> This unit consists of two regions: one is an all  $\alpha$ -helix, and the second is a mixed  $\alpha/\beta$  structure consisting of a  $\beta$ -sheet that is flanked on both sides with  $\alpha$ -helices. An indication of their similarities can be gleaned from a superimposition along the common backbone atoms of the *E. coli* PBP5 and the *Streptomyces* K15 PBP that results in a 1.2 Å rms deviation. Whereas the function of the transpeptidase/carboxypeptidase domain among these PBPs is known, the function of additional domains that are found in the structures of both low- and high- $M_r$  PBPs remain unknown. One example, in PBP5 from *E. coli* the additional unique domain is found in a nearly perpendicular orientation relative to the transpeptidase-like domain (that is shown in Figure 2A).<sup>59</sup> This domain is composed of two- and three-stranded antiparallel  $\beta$ -sheets with noticeable hydrophobic properties.<sup>56,59</sup> Other examples include two high molecular weight class B PBPs: PBP2a from *S. aureus* and PBP2x from *S. pneumoniae*. The three-dimensional structure of PBP2a (Figure 2B) shows a non-penicillin-binding domain and an N-terminal domain whose functions remain unknown.

The similarity in the three-dimensional structure of the carboxypeptidase/transpeptidase domains of PBPs is also matched by a high degree of similarity in the relative position of residues from three highly



**Figure 2.** (A) Stereoview of the active site of the PBP2x from *S. pneumoniae* (cyan), the PBP2a from *S. aureus* (magenta), the PBP from *Streptomyces* K15 (orange), the D,D-transpeptidase/D,D-carboxypeptidase from *Streptomyces* R61 (red), and the PBP5 from *E. coli* (yellow) superimposed along the  $\alpha$ -carbons of the conserved residues (shown in capped-sticks representation). The cyan, yellow, green, and white arrows point to the conserved lysine from the SXXX motif, the serine from the SXXX motif, the lysine/histidine from the KTS/KTG motif, and the serine/tyrosine from the SDN/SGN/TXN motif, respectively. (B) Stereoview of the three-dimensional structure of PBP2a from *S. aureus* shown in ribbon representation. The non-penicillin-binding domain is shown in yellow and green representation; the yellow domain corresponds to the N-terminal domain. The structure that is red corresponds to the D,D-transpeptidase domain. (C) Stereoview of the three-dimensional structure of PBP5 from *E. coli* shown in ribbon representation. The arrow points to the flexible  $\Omega$ -like loop.

conserved motifs (Figure 2C). The first motif is the strictly conserved (both PBPs and  $\beta$ -lactamases) SXXX tetrad. The serine corresponds to the amino acid that is activated to undergo acylation in both peptidase and  $\beta$ -lactamase. It is located at the N-terminus of a helix, which likely modulates its  $pK_a$ ,



thus facilitating its activation as a nucleophile and nucleofugacity as a leaving group.<sup>46</sup> The lysine in this motif is the general base that activates the serine for acylation.<sup>60</sup> The second conserved motif is the (S/Y)-XN tripeptide sequence. This triad is SDN in *Streptomyces* R61, SGN in the *Streptomyces* K15 D,D-transpeptidase, and YSN in the *Streptomyces* R61 D,D-peptidase. The S/Y residue in this motif is thought to be required for back-donation of a proton to the nitrogen atom of the  $\beta$ -lactam ring after formation of the tetrahedral intermediate. From the superimposition of the amino acids within the active sites of these PBPs (as shown in Figure 2C) it appears that the position of the hydroxyl (whether serine or tyrosine) is conserved (comparing the SDN and YXN motifs). The third conserved motif in PBPs is a KTS or KTG motif (except in the case of *Streptomyces* R61 where it is an HT(S/G)). In  $\beta$ -lactamases the role of this lysine (or histidine) is important in modulating the  $pK_a$  of the universally conserved lysine that is two residues downstream of the active site serine.

The overall similarity of the transpeptidase/carboxypeptidase region and the conserved relative positions of highly conserved residues in PBPs do not translate into similar catalytic (or functional) behavior. PBP5 of *E. coli* is a case in point. While most PBPs have low deacylation rate constants, PBP5 has an unusually high deacylation rate constant with a half-life  $t_{1/2} < 10$  min with penicillin G; this is to be contrasted to the more typical deacylation rate constant of  $8.3 \times 10^{-6} \text{ s}^{-1}$  for the acyl-enzyme species of PBP2a with the same substrate. What makes this PBP so unusual? Earlier studies had shown that a G105D mutation reduced the deacylation rate by 30-fold.<sup>61</sup> The X-ray structure of this mutant did not reveal the basis for the reduced deacylation<sup>59</sup> since the mutation does not occur in the active site. However, the X-ray structure of the wild-type enzyme<sup>56</sup> revealed disorder at a loop located near the active site serine. The position of this loop is reminiscent of the  $\Omega$ -loop (vide infra) in class A  $\beta$ -lactamases, based on the superimposition of the TEM-1  $\beta$ -lactamase and PBP5.<sup>59</sup> The different conformation of the loop adopted by the mutant likely contributes to the deacylation rate difference. This understanding takes us to the curious event (and a theme of this review). What are the molecular events that result in failed recognition of  $\beta$ -lactam antibiotics by the PBPs? Alteration of the PBPs is a dominant mechanism of Gram-positive resistance. For this compelling reason the focus of Gram-positive PBP biochemistry has changed from the historical (the enzymes of the nonpathogenic *Bacillus subtilis*)<sup>62</sup> to those of the resistant and pathogenic *S. pneumoniae* and *S. aureus*. How are the PBPs of these resistant pathogens different? Two limiting possibilities exist. The PBPs are the same but exist in increased copy number or the PBPs are altered by selection of mutant variants so as to diminish recognition of the  $\beta$ -lactam without compromise of the peptidoglycan biosynthetic role. Both processes exist, but it is the latter that has proven to be the most versatile—and expanding—mechanism of Gram-positive bacterial resistance.

The expansion of resistant PBPs is a medical problem with microbiological (what is the ecological circumstance where resistance is acquired from one bacterium by another), molecular biological (what are the mutations, and what is the genetic basis for their transfer), biochemical (how do these mutations defeat recognition of the  $\beta$ -lactam as a mechanism-based PBP inhibitor), and chemical (what will be the design of new generation  $\beta$ -lactam antibiotics effective against resistant pathogens) manifestations. A decrease in the spread of antimicrobial drug resistance will require societal change and scientific discovery in response to each of these manifestations. Of these four we will briefly address the microbiological and molecular biological, and focus on the biochemical. The topic of the chemical is reviewed elsewhere.<sup>24</sup>

An excellent point of biochemical entry to the reality of *S. aureus*  $\beta$ -lactam resistance is Pucci and Dougherty's analysis, by saturating penicillin inactivation, of the PBP distribution and stoichiometry in susceptible and resistant *S. aureus*.<sup>63</sup> While it is long known that substituent changes made to the penicillin and cephalosporin periphery influence relative affinity (specificity) among the penicillin-binding proteins, by judicious substituent choice and high concentration it is nonetheless possible to saturate (to titrate to the point of complete inactivation) the entire ensemble and so obtain their relative abundance (copies per bacterium). Susceptible *S. aureus* contains four PBPs, three of which are "high" molecular mass enzymes (70–80 kDa) and one of which is a low molecular mass enzyme (46 kDa). The high  $M_r$  enzymes are PBP1 (approximately 185 enzymes per cell), PBP2 (460 enzymes), and PBP3 (150 enzymes). The low  $M_r$  enzyme is PBP4 (285 enzymes per cell). While this is a smaller number of enzymes than is found in other bacteria species (*B. subtilis* has 12; *E. coli* has 16) the critical biosynthetic enzymes (with respect to  $\beta$ -lactam lethality) for all are the (vide infra) high  $M_r$  enzymes, which are usually bifunctional (one activity is the  $\beta$ -lactam-sensitive transpeptidase activity, and the second is a *non*- $\beta$ -lactam-sensitive transglycosylase activity). For *S. aureus* the bifunctional enzyme target of the  $\beta$ -lactams (wherein the transpeptidase but not the transglycosylase activity is inhibited) is the PBP2 enzyme.

What is the PBP composition of the  $\beta$ -lactam-resistant *S. aureus*? A comparison of the PBP composition of a resistant strain, by saturating penicillin inactivation, shows that the resistant bacterium contains the same four PBPs as the susceptible bacterium (and in the same quantity per bacterium as the susceptible bacterium) and an additional fifth enzyme (termed PBP2a).<sup>63,64</sup> The PBP2a is present in substantial quantity (approximately 800 copies per cell with some variability) and is a "low affinity" enzyme with respect to  $\beta$ -lactam binding and inactivation. When resistant *S. aureus* is challenged by a  $\beta$ -lactam antibiotic, the transpeptidase activity of its PBP2 enzyme is inactivated but the transpeptidase activity of its PBP2a enzyme is unaffected. The PBP2a performs the transpeptidase function of the now inactivated PBP2, and the bacterium survives. Simply stated, the  $\beta$ -lactam concentration attained



during chemotherapy is insufficient to inactivate the transpeptidase activity of this new enzyme. (The PBP2a contains a second domain that is presumed to possess a catalytic function which is not transglycosylase activity. The role of this second PBP2a domain remains unknown.) In the presence of  $\beta$ -lactam antibiotics the functioning transglycosylase domain of the PBP2 (its transpeptidase having been inactivated) works cooperatively with the active transpeptidase of the PBP2a to maintain the cell wall integrity of the resistant *S. aureus*.<sup>65,66</sup> Therefore, the salient issues to the understanding of Gram-positive  $\beta$ -lactam resistance are the circumstance of PBP2a acquisition, the genetic origin of PBP2a, and the molecular alteration(s) within the transpeptidase PBP2a domain that result in a change from high to low susceptibility to  $\beta$ -lactam inactivation.

The appearance of methicillin resistance soon followed (within a year) the introduction of methicillin in the clinic.<sup>67</sup> The mechanism by which the *mecA* gene, carried by mobile genetic elements known as the staphylococcal cassette chromosome *mec* (SCC-mec),<sup>68</sup> was acquired by *S. aureus* remains unknown. It is suggested that this gene was acquired from *Staphylococcus sciuri* (a bacterium found in the gut of animals) which possesses a close *mecA* gene homologue.<sup>69</sup> Upon activation of the *mecA* gene, the PBP2a protein is expressed. It was shown in the mid-1980s that the presence of PBP2a in *S. aureus* conferred resistance to the clinically used  $\beta$ -lactams,<sup>70–72</sup> as evidenced by a 500-fold increase in the MIC (minimal inhibitory concentration) for penicillins. These bacteria came to be known as methicillin-resistant *S. aureus* (or MRSA). Kinetic characterization of the reaction of PBP2a with  $\beta$ -lactams provided valuable mechanistic information and revealed that the resistance to  $\beta$ -lactams was not merely due to a large  $K_d$  value (a commonly held belief) but to the acylation rate constant as well.<sup>73</sup> The dissociation constant of the PBP2a-benzylpenicillin complex was 13 mM,<sup>74</sup> similar to what is found for other (susceptible) PBPs. A much clearer difference is the apparent second-order rate constant for acylation of the active site serine. For the reaction of PBP2a with benzylpenicillin this rate constant is 2–3 orders of magnitude smaller than those found for other high molecular weight PBPs (such as the *S. aureus* PBP2 and *S. pneumoniae* PBP2x).<sup>74</sup> The structural features of PBP2a that are responsible for the poor acylation were elucidated recently with X-ray structures for the apo and acyl-enzyme complex of PBP2a with benzylpenicillin, nitrocefin, and methicillin.<sup>57</sup> Comparison of the apo-PBP and acyl-PBP structures revealed noticeable differences. In the acyl-enzyme structure the C $\alpha$ , C $\beta$ , and O $\gamma$  of Ser403—the acylated serine—are 1.1, 1.4, and 1.8 Å away from the same atoms in the apo-structure, suggesting that the  $\alpha$ -helix that holds Ser403 must undergo a conformational change for acylation to occur.<sup>57</sup>

Structural modifications in PBPs that result in increased resistance are not confined to *S. aureus*. The three-dimensional structures of two additional PBPs have been solved by X-ray crystallography. One is PBP2x from *S. pneumoniae*. PBP2x is a class B

high molecular weight PBP, and its structure was solved nearly a decade ago.<sup>48</sup> The X-ray diffraction structure of a mutant PBP2x provided the first glimpse into the structural bases for resistance by mutation of PBPs.<sup>75,76</sup> Two drug-resistant PBP2x mutants have been characterized. The first X-ray structure describes the effects of mutations Thr338Ala and Met339Phe, which along with other mutations alter the acylation efficiency by 20-fold. Both of these mutations occur close to the active site Ser-337, the residue that is acylated by  $\beta$ -lactam antibiotics. The effect of these mutations is attributed to the disruption of hydrogen-bonding interactions between Thr388 and a conserved water molecule. Also, a conformational change that occurs for the  $\beta$ 3 strand is attributed to the collective effects of the larger Phe339 side chain and smaller Ala338 chain. These conformational changes enable an alternative conformation for Ser337 that might be less prone to activation. The most likely candidate for the base that promotes Ser337 acylation is Lys340, whose amine is in hydrogen-bonding contact (3 Å) with the serine hydroxyl. The structure of another mutant of PBP2x has been recently solved.<sup>75</sup> It reveals similar conformational changes as the PBP2x above except that in this case the effects are attributed to the change in polarity introduced by Gln552Glu and to a narrower active site.

PBP5, a transpeptidase from *Enterococcus faecium*—a bacterium which incidentally does not produce  $\beta$ -lactamases—has also been implicated in resistance to  $\beta$ -lactams through either modification or overexpression of the enzyme.<sup>77,78</sup> The enterococci are less virulent than *S. aureus* and *S. pneumoniae* but have become prominent in the clinic due to their increased levels of resistance to a variety of antibiotics.<sup>79</sup> PBP5fm has low affinity for  $\beta$ -lactam antibiotics. Two reasons for this decreased affinity are suggested from the structure of this protein.<sup>80</sup> A glutamate (Glu622) near the active site may present a steric barrier to  $\beta$ -lactam binding. This interpretation is consistent with the reduced affinity for benzylpenicillin that results when the equivalent site in PBP2x is changed to glutamate.<sup>81</sup> Second, an arginine (Arg464) may interact with its neighbors in the conserved loop spanning residues 461–465, resulting in a more rigid cleft that would lead to the reduced affinity of PBP5fm.<sup>80</sup>

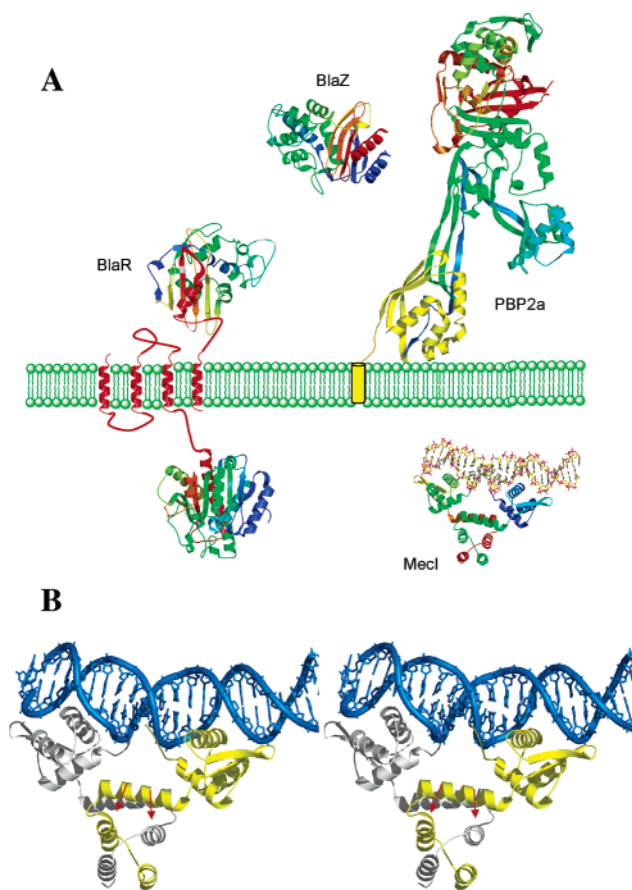
## 2.2. $\beta$ -Lactam-Sensing Proteins

Resistance to antibiotics in Gram-positive and Gram-negative bacteria has manifested itself through various mechanisms, including the production of  $\beta$ -lactamases or PBPs that are insensitive to the action of  $\beta$ -lactams, such as the case of PBP2a from *S. aureus*. In the mid-1980s Lampen and co-workers observed that the synthesis of the  $\beta$ -lactamase from *Bacillus licheniformis* 749/I gradually peaks at 1–1.5 h after exposure to a  $\beta$ -lactam and decreases slowly in the following 1–2 h<sup>82</sup> (induction in *S. aureus* is far more rapid, complete within 11 min<sup>83</sup>). This experiment confirmed that  $\beta$ -lactamase production is inducible and implied the presence of a transduction

mechanism. The identification of a membrane-spanning protein, BlaR, from *B. licheniformis* soon followed. That this enzyme contained a sensor/transducer domain that is highly similar to the class D  $\beta$ -lactamases,<sup>84</sup> a membrane domain consisting of a four-helix bundle<sup>85</sup> and an intracellular domain containing a zinc ion,<sup>84</sup> made it a strong candidate to carry out the signal transmission events in a transduction mechanism. The BlaR protein is the product of the *blaR1* gene, which is a member of a triad of genes from the *bla* divergon; *blaP* and *blaI* are the remaining genes that encode the effector protein ( $\beta$ -lactamase) and repressor protein BlaI.<sup>86</sup> The BlaI protein—a DNA-binding repressor protein that is located immediately upstream of the genes *blaP* and *blaR*—blocks expression of both structural and regulatory genes, including itself.<sup>87</sup>

The regulation of the production of resistance enzymes has also been recently studied in *S. aureus*, an organism that, in addition to  $\beta$ -lactamases, produces a low-affinity PBP, namely, PBP2a, which is regulated by a similar mechanism that involves a sensor–transducer (*mecR*), a DNA-binding repressor (*mecI*), and a structural gene (*mecA*, Figure 3A). It is worth noting that the mere presence of the *mecA* gene is insufficient for expression of resistance in *S. aureus* as yet other (and yet unknown) genetic changes are also necessary.<sup>88,89</sup> The *bla* or *mec* regulatory genes regulate production of PBP2a and  $\beta$ -lactamase due to a high degree of homology between the two systems.<sup>90</sup> However, the inability of  $\beta$ -lactams to induce PBP2a in *S. aureus* and the fact that the *blaI/blaR* system interacts with the *mecA* promoter indicate that this system could also be responsible for the induction of *mecA* transcription.<sup>91</sup> The sequence of events that lead to expression of the *blaZ* gene (for  $\beta$ -lactamase) in *S. aureus* is similar to that of *B. licheniformis*: following the binding of a  $\beta$ -lactam to the sensor domain of BlaR, a signal is transmitted across the membrane and leads to activation and autocatalytic cleavage of the intracellular zinc-ion-dependent domain of blaR; the activated metalloprotease either directly or with the aid of cofactors cleaves the DNA-bound repressor protein BlaI,<sup>92</sup> which is left unable to dimerize and efficiently bind to its operator for blockage of expression of the structural genes.<sup>90</sup> Autocleavage of BlaR leads to incapacitation of the protein, which has to be regenerated continuously. The protein presumably expressed by the *blaR2* gene is proposed to play a role in the induction mechanism, but its exact role remains unelucidated.

Transcription of the genes encoding  $\beta$ -lactamases is set in motion by the binding of a  $\beta$ -lactam antibiotic to the extracellular sensor domain of BlaR. The kinetics of this process have been characterized for *B. licheniformis* and *S. aureus*. Kinetics of BlaR from *S. aureus* with various  $\beta$ -lactams shows that a single acylation event occurs over the lifetime of the organism, making BlaR like a PBP.<sup>93</sup> The acylation step is efficient (second-order rate constant  $k_2/K_s$  of  $10^4$ – $10^6$  M<sup>-1</sup> s<sup>-1</sup>, while the first-order deacylation rate constant has an exceedingly slow value of  $10^{-5}$  s<sup>-1</sup>.<sup>93</sup> The three-dimensional structure of BlaR from *B.*



**Figure 3.** (A) Schematic of the various proteins that are involved in regulation of  $\beta$ -lactamase and PBP2a. All proteins are color-coded based on their secondary structures and shown in ribbon representation. The lipid bilayer of the inner membrane is shown with green spheres representing the phospholipid head, and the lines represent the lipid tail. The sensor/transducer BlaR is anchored to the surface through a helix bundle, shown in red ribbon representation, which culminates in the C-terminal metalloprotease domain, located in the cytoplasm. PBP2a is shown anchored to the membrane, and a class A  $\beta$ -lactamase (BlaZ) is shown unanchored in the periplasm. Shown in the cytoplasm is the MecI dimer (ribbon) in complex with its operator DNA (capped-sticks). (B) Close-up depiction of the complex of MecI dimer with its operator DNA. The MecI dimer is shown in ribbon representation with the monomers colored in gray and yellow, while the DNA oligonucleotide is shown in blue capped-sticks representation with a ribbon along the duplex backbone. The red arrows point to the cleavage sites in the two monomers.

*licheniformis* has been recently solved by X-ray crystallography<sup>94</sup> and confirms the postulated high degree of similarity between the C-terminal domain of BlaR and the class D  $\beta$ -lactamases.<sup>84</sup> While the three-dimensional structure of BlaR is nearly identical to that of the class D  $\beta$ -lactamases, the X-ray structure of this enzyme does not reveal *N*-carboxylation at the active site lysine,<sup>94</sup> an event that is widely believed to occur in the class D  $\beta$ -lactamases.<sup>95–97</sup> Previous kinetic studies of the BlaR protein from *S. aureus* had found that BlaR from *S. aureus* contains an active site lysine (Lys392) that reacts with CO<sub>2</sub> to form a carbamate.<sup>93</sup> The authors of the X-ray structure suggest that the presence of a threonine residue—two residues downstream of the



active serine residue where a valine residue would be usually located in class D  $\beta$ -lactamase—is likely the reason behind the lack of carboxylation of that lysine residue.<sup>94</sup> However, the signature  $^{13}\text{C}$  NMR signal for *N*-carboxylated lysine in BlaR of *S. aureus* has been documented in solution.<sup>93</sup>

More recently, the structures of the acyl–enzyme complex of BlaR from *S. aureus* with benzylpenicillin and ceftazidime have been independently solved by X-ray crystallography.<sup>98,99</sup> These confirm the similarities in the structures of BlaR from *S. aureus* to BlaR of *B. licheniformis* and the class D  $\beta$ -lactamases. Interestingly, both of these studies found that BlaR was not carboxylated in the acyl–enzyme complex, in contrast to the  $^{13}\text{C}$  NMR, fluorescence, and mutagenesis studies identifying the carboxylated lysine in the resting BlaR protein.<sup>93</sup> QM/MM calculations carried out by Birck et al.<sup>99</sup> reveal that upon protonation of the carbamate nitrogen in the class D  $\beta$ -lactamases a barrierless decarboxylation occurs. The authors postulate that the same *N*-decarboxylation event occurs in BlaR, but unlike the class D  $\beta$ -lactamases decarboxylation results in an inactive enzyme unable to recarboxylate due to a hydrogen bond between Lys392 and Asn439. This conclusion is consistent with the fact that BlaR undergoes a single acylation event over the lifetime of the organism.

The nature of the signaling event across the membrane that transpires as a result of  $\beta$ -lactam binding to the sensor domain of BlaR is not well understood. Golemi-Kotra and co-workers have shown that binding of the antibiotic to BlaR of *S. aureus* is accompanied by significant conformational changes that likely have a role in the signal transduction mechanism.<sup>93</sup> A recent study of the transduction mechanism in *B. licheniformis* did not identify a conformational change in the C-terminal domain.<sup>100</sup> An alternative mechanism was noted based on an interdomain conformational change in the membrane protein consisting of the loss of interaction between the C-terminal domain and an L2 loop of BlaR that connects two  $\alpha$ -helices of the four-helix bundle. It was shown that in the absence of the antibiotic the sensor domain and the L2 loop form an interaction. Recent mutations of highly conserved residues in the L2 loop appear to be lending credence to this mechanism, as the organisms exhibited no  $\beta$ -lactamase activity, since the level of antibiotic remained at similar levels to that of a  $\beta$ -lactamase-negative control.<sup>98</sup> Whereas the mechanism that leads to the transduction of the signal appears to be contentious, the event that follows is accepted to consist of autocleavage of the zinc-containing intracellular domain,<sup>87</sup> which in the case of *S. aureus* is followed by inactivation of the repressor proteins MecI/BlaI, while in *B. licheniformis* this process is thought to occur through an intermediary coactivator.<sup>101</sup>

The structures of BlaI<sup>102</sup> and MecI<sup>103,104</sup> have been recently solved by NMR and X-ray crystallography, respectively. The BlaI and MecI proteins from *S. aureus* share 60% identity and 31 to 41% identity to BlaI from *B. licheniformis*.<sup>102</sup> The structure of MecI consists of a dimer in the shape of a triangle (shown

in Figure 3B).<sup>104</sup> Dimerization occurs at the C-terminal domain, while the DNA-binding domain is located at the N-terminus (see Figure 3B for structure of MecI–DNA complex). The topology of MecI follows a “winged-helix” architecture<sup>103,104</sup> with a helix–turn–helix DNA-binding motif; the second helix of this motif binds to the major groove of DNA with up to 16 hydrogen bonds and salt bridges (see Figure 3B).<sup>104</sup> The high level of conservation of the residues that form contact between the repressor protein and the operator DNA in *S. aureus* and *B. licheniformis* suggests that this complexation is likely similar in BlaI and MecI.<sup>104</sup>

### 3. $\beta$ -Lactamases

#### 3.1. Overview and Classification

The  $\beta$ -lactamases predate the antibiotic era. The evolution of these enzymes is presumed to have taken place in parallel to the biosynthetic steps leading to  $\beta$ -lactam antibiotics.<sup>105</sup> Indeed, the first  $\beta$ -lactamase was identified in the early 1940s prior to the first large-scale use of penicillins in Boston 2 years subsequent.<sup>16</sup> However, extensive clinical use of these antibiotics— $\beta$ -lactams comprise approximately 55% of all antibiotics used currently—has accelerated the selection process for the emergence of once rare genes for these antibiotic-resistant enzymes. The rare bacterium that harbored the gene for a  $\beta$ -lactamase would have had the opportunity to grow unencumbered once the susceptible organisms in a heterogeneous population of bacteria were eliminated in the course of an antibiotic treatment regimen. In essence, the less fit bacterium is eliminated by the  $\beta$ -lactam challenge and the resistant organism experiences unlimited nutritional resources to propagate. The once rare gene for the  $\beta$ -lactamase is amplified.

Sharing of genetic materials among microbial populations is relatively facile. Genes, often residing on inherently mobile genetic elements such as plasmids and transposons, are shared not merely members of a given species of bacteria but also among unrelated genera. The facility of genetic sharing is underscored by the observation that some organisms, such as the *Streptococci*, are able to acquire free-standing stretches of nucleic acids (containing entire genes) directly from the environment. All these processes have contributed by clinical selection to the amplification of once rare antibiotic-resistant genes and their liberal sharing among various bacterial populations.

The account given above outlines the plausible events that have given rise to the emergence of the “parental” genes for  $\beta$ -lactamases. As a consequence of the inappropriate use of  $\beta$ -lactam antibiotics for the past half a century, especially in the community, many variants of the parental enzymes have emerged. This accelerated evolution of the antibiotic-resistant genes has been abetted by the creative molecular tinkering of medicinal chemists in the past decades, the fruits of which are an ensemble of  $\beta$ -lactam antibiotic structures. The dynamics between the discovery, the creation of new  $\beta$ -lactam antibiotics, and the clinical responses by microbial population to



these developments have been outlined by Bush and Mobashery.<sup>106</sup> In light of the different properties of these various types of  $\beta$ -lactam antibiotics—for example, antibiotics that target different sets of PBPs or those that have been imparted with resistance to the action of  $\beta$ -lactamases—the clinical response by bacteria has been the selection of mutant variants of  $\beta$ -lactamases that often have broadened the catalytic ability of the enzymes. For example, the TEM-1  $\beta$ -lactamase, a plasmid-borne enzyme described first in 1963, has given rise to 133 variants (as of February 2004).<sup>15</sup> While this variety does reflect the successful therapeutic use of  $\beta$ -lactams, it also may be taken as presaging the obsolescence of these versatile antibiotics sometime in the future.

Prompted by a critical biochemical requirement, nature deftly develops catalysts to meet the need. Despite the outstanding stability of the peptide bond (half-life of approximately 500 years when uncatalyzed for hydrolysis),<sup>107,108</sup> multiple classes of proteases have evolved to hydrolyze this bond, in light of the central importance of proteolysis to many biological processes. The same has been true for  $\beta$ -lactamases in the face of the life or death options to the organisms presented by the antibiotics. There are four known classes of bona fide  $\beta$ -lactamases, each of which operates by a distinct reaction mechanism.<sup>15,105,109,110</sup>

While a handful of  $\beta$ -lactamases were known in the early 1970s, the number at the present exceeds 470 (communication by Dr. Karen Bush). Two general types of  $\beta$ -lactamases are known: those that require the zinc ion for their function, and those that pursue a transient serine acylation/deacylation strategy (if the unique  $\beta$ -lactamase activity of the *T. pallidum* PBP is also found in other organisms, this will yet be another type of  $\beta$ -lactamase as it does not require a zinc ion nor does it pursue the covalent catalytic strategy of the serine enzymes).<sup>58,111</sup> The widely accepted molecular classification places  $\beta$ -lactamases into four classes: three serine-dependent enzyme classes (classes A, C, and D) and one metal-dependent (class B). This classification is not to be confused with that of Ghuysen for the PBPs in which the two groups of low molecular weight and high molecular weight PBPs are divided among classes A, B, and C (for a total of six PBP classes).

Both PBPs and  $\beta$ -lactamases are present in the periplasmic space of Gram-negative bacteria. In Gram-positive organisms (which lack the outer membrane) the PBPs are located on the outer surface of the cytoplasmic membrane and the  $\beta$ -lactamases are either excreted or bound to the cytoplasmic membrane.<sup>112</sup> All  $\beta$ -lactamases are expected to have divested completely their ability to bind the peptidoglycan substrate of their ancestral PBPs.<sup>14,113</sup> If not, the opportunity to function as a vanguard against the incoming antibiotics would be lost (the structural means to this end was revealed recently for the class C  $\beta$ -lactamases).<sup>113</sup> Moreover, this same conclusion may be intuited from the ability of many of the class A and C  $\beta$ -lactamases to act at the diffusion-limited rate for their preferred substrates.<sup>114,115</sup>

A summary of the mechanistic expectations for  $\beta$ -lactamase catalysis is a useful prelude to the discussion of the relationship between  $\beta$ -lactamase structure and the evolution of function (and mechanism) to confer  $\beta$ -lactam resistance. Entry of the  $\beta$ -lactam substrate is guided by relatively long-range electrostatic attractions between the cationic side chain of an active site amino acid and the carboxylate of the  $\beta$ -lactam. Positioning of the  $\beta$ -lactam then occurs by shorter range attractive electrostatic interaction involving hydrogen bonding from the protein to the  $\beta$ -lactam carbonyl (the oxyanion hole, formed in the class A  $\beta$ -lactamases by the hydrogen bonding to the  $\beta$ -lactam carbonyl by the backbone amide nitrogens of Ser-70 and Ala-237).<sup>116</sup> Appropriately functionalized  $\beta$ -lactams (here with special reference to the bicyclic structure of nearly all of the antibacterial  $\beta$ -lactams) sequester in the active site. This complex formation results in *localized* (ground state) destabilizing electrostatic interactions at the locus that enable catalysis, compensated by stabilizing hydrophobic interactions elsewhere in the enzyme–substrate complex. The higher carbonyl IR stretching frequency of the  $\beta$ -lactam when it is in the oxyanion hole, indicative of enhanced reactivity toward nucleophile addition, exemplifies localized ground-state destabilization.<sup>117</sup> Moreover, within the complex the total ensemble of the remaining amino acids are now predisposed to initiate turnover. The outcome of this positioning is a decrease in the transition-state energy by the *simultaneous* operation of Lewis (clearly exemplified by the zinc atom of the metalloproteases but other through-space electrostatic interactions as well) and Brønsted catalysis. The rate-limiting step of nonenzymatic  $\beta$ -lactam hydrolysis is oxyanion addition to the  $\beta$ -lactam carbonyl, and there is little doubt that this event is also rate limiting for hydrolysis by class A  $\beta$ -lactamases.<sup>34,116</sup>

For the serine (classes A, C, and D)  $\beta$ -lactamases (the metallo- $\beta$ -lactamases are discussed subsequently) the critical result of Henri–Michaelis complex formation is to attain such stabilization of the incipient tetrahedral species so as to enable general base catalysis for serine addition to the  $\beta$ -lactam. Upon serine oxygen addition considerable basicity develops on the nitrogen. At the point in the transition state of (substantial) negative charge transfer (from the general base, through the serine) to the tetrahedral species, a concomitant increase in the  $\beta$ -lactam nitrogen basicity is attained as to accept proton donation to this nitrogen. The source of this proton is debated. Nonetheless, nitrogen protonation is an obligatory event for productive tetrahedral collapse. As noted by Page and Laws,<sup>34</sup> oxyanion addition to the less hindered *re* face of the  $\beta$ -lactam and the Brønsted general acid N-protonation both must occur on this same face. This imposes a spatial (stereoelectronic) constraint on the positioning of these two catalytic groups within the active site (if they are allowed contact with each other they will simply self-annihilate by proton transfer). An obvious possibility is the use of the protonated amino acid that was used as the general base for oxyanion addition (as it is

already on this face and makes the self-annihilation issue moot). Hydrolysis of the serine acyl–enzyme, the deacylation step, is energetically less demanding (a more reactive carbonyl and thus having a diminished need for general acid catalysis in tetrahedral collapse).

Within the active sites of the serine  $\beta$ -lactamases are, therefore, two ensembles of amino acids. The first is the catalytic ensemble comprised of a minimum of five amino acids: the serine, the general base for the serine, the oxyanion hole (two amino acids), and the cationic recognition site for the carboxylate. These amino acids are expected to be invariant (or very highly conserved). The second is the recognition ensemble (in one form or another, all of the remaining amino acids) that complements the hydrophobic and hydrophilic segments presented by the remaining structure of the  $\beta$ -lactam. These recognition amino acids will be variable and correspond to the amino acids that will mutate under selection pressure. For a given  $\beta$ -lactamase and given substrate the complementarity to the rate-limiting transition state and to overall recognition of the  $\beta$ -lactam will vary. This variability is, of course, quantified as the unique  $k_{\text{cat}}/K_{\text{m}}$  for the substrate. While this may seem obvious, there is a less appreciated corollary. The relative individual role played by any member of these ensembles during catalysis (measured, say, in terms of an amino acid  $\text{p}K_{\text{a}}$ ) is therefore substrate dependent. Deletion of an essential amino acid—while an essential tool of mechanistic enzymology—alters the energetic landscape of the active site in such fashion as to make all subsequent interpretation cautionary. The criteria that determine which amino acids of the recognition ensemble transform under selection pressure are straightforward. The integrity of the catalytic ensemble must be preserved, and the integrity of the protein as the whole (for example, as measured in terms of thermal stability or expression/folding capability) can only be lightly varied.<sup>118–121</sup> Likewise, mutations that require only a single nucleotide change and that preserve common codon usage are more probable than those that do not.<sup>120</sup> This implies that certain  $\beta$ -lactamase families are anticipated to be more “plastic” than others, and indeed, the serine  $\beta$ -lactamases provide such a contrast with the great phenotypic diversity of the serine class A TEM enzymes as compared to the class A SHV enzymes, which have diversified but without substantial phenotypic evolution.<sup>15</sup> Given these boundary conditions and superlative kinetic and structural data for these enzymes, one might presume that the assignment of function within the catalytic ensemble and within the recognition ensemble as these develop under selection pressure would be straightforward. One would be wrong.

### 3.2. Class A $\beta$ -Lactamases

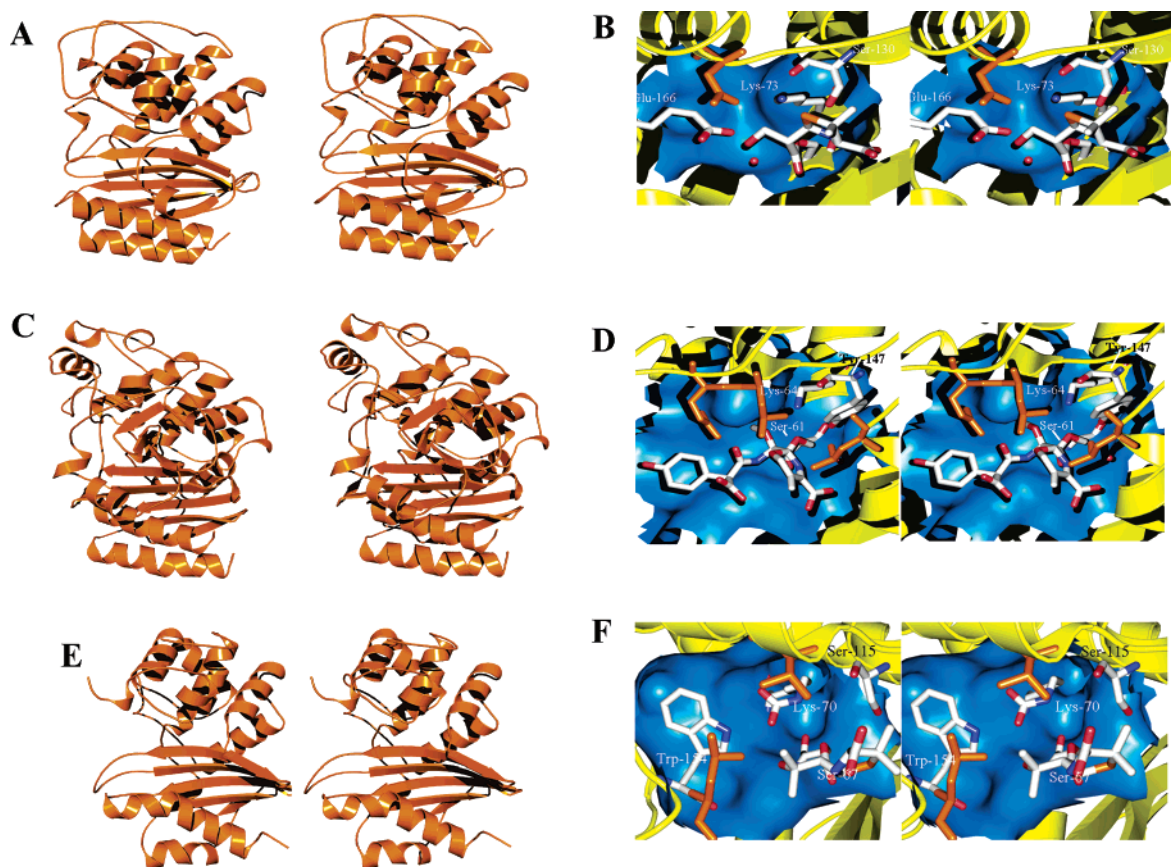
This is the largest and best mechanistically characterized serine  $\beta$ -lactamase class. Historically, these  $\beta$ -lactamases were described as “penicillinases” as their ability to catalyze penicillin hydrolysis was greater than that for cephalosporins. They have become so efficient at their function that they are

diffusion controlled, where the apparent second-order rate constant  $k_{\text{cat}}/K_{\text{m}}$  has reached the (upper) diffusion limit estimated from collision theory. As a result, class A  $\beta$ -lactamases may be described as having reached catalytic perfection for their preferred substrates.<sup>114</sup> Variants with much broader substrate preferences are now known, including enzymes imparting clinical resistance to late generation  $\beta$ -lactams.<sup>15,109,122–124</sup> The class A  $\beta$ -lactamases are closely related in sequence to low molecular weight class C PBPs such as PBP4 of *E. coli*, *H. influenza*, and *M. tuberculosis*.<sup>60</sup> As judged by the comparison of crystal structures, the catalytic domain of the larger *E. coli* PBP5 (low  $M_{\text{r}}$  PBP class A) shows high similarity as well.<sup>56,125</sup> In terms of bacterial resistance, three class A  $\beta$ -lactamases subclasses dominate: the (historically Gram-negative plasmid penicillinase) TEM/SHV, the *P. aeruginosa* PER/OXA/TOHO cephalosporinases, and the CTX-M (NMC-A) carbapenemase subclasses.<sup>126</sup> As of October 2004, 135 TEM and 57 SHV  $\beta$ -lactamase variants are known (<http://www.lahey.org/studies/webt.htm>). While the sequence homology among the three is easily recognizable and the fundamental catalytic mechanism for each is the same, the differences render broad structural and mechanistic generalizations—especially as they relate to resistance development—unwarranted.

Several class A variants resist inactivation by the mechanism-based inhibitors (clavulanate and sulbactam) used in clinical formulations with otherwise  $\beta$ -lactamase-susceptible penicillins. Until very recently the occurrence of these inactivation-resistant class A  $\beta$ -lactamases was limited to the aforementioned TEM subclass, and hence, the term “inhibitor-resistant TEM” (IRT) was coined. However, in light of the discovery of inhibitor resistance in the SHV-type enzymes, this group is better referred to as “inhibitor-resistant  $\beta$ -lactamases”. Furthermore, new class A  $\beta$ -lactamases that are active against the more recent cephalosporins (ceftazidime and cefotaxime and the monobactam aztreonam) and others that are active against the carbapenems are known collectively (also with other class C and D enzymes) as “expanded-spectrum  $\beta$ -lactamases” (ESBL).<sup>124</sup>

The crystal structure of several Gram-positive (including enzymes from *B. licheniformis* and *S. aureus*) and Gram-negative (from *E. coli*) class A  $\beta$ -lactamases were solved in the late 1980s and early 1990s.<sup>127–131</sup> The TEM-1  $\beta$ -lactamase structure has two domains: an  $\alpha/\beta$  domain consisting of five-stranded  $\beta$ -sheet and three  $\alpha$ -helices and an  $\alpha$  domain consisting of eight  $\alpha$ -helices.<sup>128,131</sup> Together, these two domains sandwich the core of the active site. The class A  $\beta$ -lactamases reveal striking similarities to the PBP structures<sup>132</sup> (compare Figure 4A and B, D, and F). Further similarities can be found in the relative three-dimensional position of several highly conserved residues, including Ser70, Lys73, Lys234, and Ser130. While some of these residues are not invariant, their substitutes contain similar functional groups capable of carrying out the same functions (for example, Ser130 is typically replaced by tyrosine in the class C  $\beta$ -lactamases, and Lys234 is sometimes replaced by a histidine as in the





**Figure 4.** Stereoviews of the three-dimensional structures of (A) a class A  $\beta$ -lactamase (TEM-1; PDB code 1TEM), (C) a class C  $\beta$ -lactamase (AmpC; PDB code 1FCO), (E) and a class D  $\beta$ -lactamase (OXA-10; PDB code 1K57). Close-up stereoviews of the active sites of the acyl–enzyme complex are shown as (B) TEM-1 with 6 $\alpha$ -hydroxymethylpenicillate, (D) AmpC with moxalactam, and (F) OXA-10 with 6 $\beta$ -(1-hydroxy-1-methylethyl)penicillanic acid. The enzymes are in yellow ribbon representation with a van der Waals surface in blue for the active site. The important active site residues are depicted in capped-sticks representation (color-coded according to atom type: S, yellow; O, red; N, blue; C, white). The hydrolytic water molecule is shown as the red sphere. Hydrophobic residues in the active site and other residues that are close to important residues but are not directly involved in the catalytic process are shown in orange capped-sticks representation.

structure of the *Streptomyces* R61 D,D-peptidase/transpeptidase).

The remaining invariant amino acid is Glu166. This glutamate is located on a loop (termed the  $\Omega$ -loop) that sequesters, by hydrogen bonding, a single water molecule as a bridge to the Ser70 adjacent to the *re* face of the  $\beta$ -lactam carbonyl. This glutamate has been proposed by some to have a role in the rate-limiting acylation step.<sup>133–137</sup> This mechanism envisions that this glutamate, acting through the bridging water as a proton shuttle, activates Ser70 for nucleophilic addition to the  $\beta$ -lactam.<sup>136,138</sup>

The alternative possibility for the general base, the invariant Lys73,<sup>129,131</sup> must then be electrostatic stabilization (such as to increase the Ser70 acidity and hence nucleophilicity). The evidence in favor of Glu166 as the serine-activating general base is summarized. A compelling argument in favor of a Lewis acid, rather than a Brønsted base, role for Lys73 was the <sup>15</sup>N NMR determination of its  $pK_a$  as greater than 10.<sup>139</sup> This determination was followed by Poisson–Boltzmann electrostatic calculations by Lamotte-Brasseur et al. supporting the  $pK_a > 10$  assignment,<sup>140,141</sup> although others (using the same calculation method) estimated a value of 8.<sup>142</sup> More recently each of three methods—enzyme kinetics, <sup>15</sup>N NMR, and free-energy calculations using the ther-

modynamic integration—supports a  $pK_a$  value for Lys73 of 8.0–8.5.<sup>143</sup> Ultrahigh-resolution crystal structures of the TEM-1<sup>133</sup> and SHV-2<sup>136</sup> enzymes show a spatial arrangement of Glu166 and the invariant water, in the presence of a bound transition state mimic, to be consistent with serine activation via the proton shuttle.<sup>133</sup> The TEM-1 structure resolves a hydrogen atom on Glu166, while the SHV-2 structure shows the hydrogen of the Ser70 hydroxyl pointing to the conserved water molecule. Following serine addition the Ser130 hydroxyl is positioned ideally to shuttle the proton on Lys73 to the  $\beta$ -lactam nitrogen of the tetrahedral species (Figure 4B), driving ring opening to the acyl–enzyme species. It is widely accepted that Glu166 is the general base for hydrolysis of the acyl–enzyme ester. In fact, site-directed mutagenesis of Glu166 (E166A) abolishes deacylation while impairing (but not abolishing) the acylation process by a factor of  $10^3$ .<sup>127,137</sup>

Over the past three decades several strategies have emerged, in the guise of new  $\beta$ -lactams, to incapacitate the class A  $\beta$ -lactamases. The first strategy is exemplified by clavulanate and the penam sulfones (sulbactam and tazobactam), which are poor PBP inactivators but excellent  $\beta$ -lactamase inactivators.<sup>144</sup> The key mechanistic event for both is a quite similar fragmentation reaction of the respective serine acyl–



enzyme intermediates that is competitive with hydrolytic deacylation and gives a new acyl-enzyme intermediate improperly positioned for catalytic deacylation.<sup>145–150</sup> As noted previously, these  $\beta$ -lactams are formulated with other  $\beta$ -lactam PBP inactivators to target  $\beta$ -lactamase-expressing pathogens. The second strategy is exemplified by the carbapenems (such as imipenem) and the cephamycins (exemplified by cefoxitin), which resist  $\beta$ -lactamase hydrolysis by diminished ability at acylation and/or (especially) deacylation events of  $\beta$ -lactamase catalysis. Both of these  $\beta$ -lactams have unusual  $\beta$ -lactam substituents that are believed to interfere with the proper positioning of their  $\beta$ -lactam segments when in complex with the  $\beta$ -lactamase. As these structural features do not interfere with PBP affinity, these are used therapeutically as single agents. The third strategy is that of the third- (and fourth) generation cephalosporins (exemplified by cefotaxime, ceftazidime, and cefepime), which are highly functionalized cephalosporins that are poorly recognized by the class A  $\beta$ -lactamases. These too are used as single-agent therapies, although this may change. With respect to  $\beta$ -lactam antibacterial design, the structural and mechanistic basis for the evolution of  $\beta$ -lactamases that have overcome these barriers and now recognize these  $\beta$ -lactams as substrates is a topic of more than idle curiosity.

In the event the acquisition of clavulanate and penam sulfone inhibitor-resistant TEM  $\beta$ -lactamases is accomplished by single-point mutations at one of several key amino acids,<sup>146–148,151–156</sup> a compensatory second point mutation, unrelated to resistance development but rather to restore enzyme stability,<sup>119,154</sup> is also seen in some clinical isolates. The relative ease of this transformation may be understood in terms of the required clavulanate (or penam sulfone) acyl-enzyme fragmentation as an “off-pathway” event, unrelated to normal catalysis, and hence easily disposed.<sup>155</sup> Moreover, it is evident from the kinetic properties of the enzymes that incremental adjustment of the kinetic parameters suffice to impart resistance to these inactivators. For example, N276D mutation of TEM-1 is representative of common clavulanate-resistant IRT variants wherein the clavulanate  $K_i$  increases from 0.4 (TEM-1) to 17  $\mu$ M (N276D TEM-1) and  $k_{\text{cat}}$  increases from 0.02 to 0.16  $\text{s}^{-1}$ .<sup>151</sup> The other kinetic parameters ( $k_{\text{inact}}$ ,  $k_{\text{rec}}$ ) are less altered. The critical fragmentation event in clavulanate inactivation of the TEM  $\beta$ -lactamase is known to require a protonation event wherein the proton is provided by a conserved structural water.<sup>146</sup> Replacement of the neutral Asn276 with the charged Asp276 results in substantial movement of the Asp side chain so as to engage the Arg244 guanidinium that is ordinarily involved in substrate carboxylate recognition. The resulting electrostatic modulation manifests in dissociation (and thus loss) of this conserved water. Very similar kinetic changes are seen with respect to clavulanate inactivation of the M69L TEM-33 variant.<sup>152</sup> This methionine, while clearly not a conserved TEM residue, is nonetheless located in a region of strong structural constraint (at the beginning of the TEM H2  $\alpha$ -helix and in contact

with the B3 and B4  $\beta$ -strands and thus while removed from the active site influences the active site geometry).<sup>152,154</sup> Replacement of the methionine with leucine gives a  $\beta$ -lactamase (the TEM-33 enzyme) having an identical ability to hydrolyze penicillin G. Clavulanate, however, binds more poorly (by  $1.9 \pm 0.2 \text{ kcal mol}^{-1}$  for the pre-acylation complex).<sup>152</sup> An explanation for this difference is not evident from the protein structure but is suggested by computational analyses that indicate less favorable van der Waals and electrostatic energies for clavulanate binding to the M69L mutant. Conversely, improved clavulanate inactivation is seen for the (clinically not observed) M69G TEM-1 mutant.<sup>154</sup> Yet another mechanism by which the TEM enzymes evade clavulanate has been suggested to involve disruption of the active site interactions of the Ser130 and whose oxygen engages customarily in postfragmentation cross-linking to the clavulanate (and penam sulfone) remnant acyl-enzyme.<sup>154</sup> For both the TEM-32 (M69I/M182T) and TEM-34 (M69V) variants the local environment of this serine is perturbed such that the cross-linking, leading to a long-lived acyl-enzyme, does not occur.<sup>154</sup> An evaluation of Ser130 SHV mutations identified only S130G as conferring clavulanate resistance, again resulting from destabilization of the clavulanate pre-acylation complex.<sup>149</sup> It is evident that the basis for the evasion is not loss of the ability to cross-link<sup>157</sup> but rather the simple result of an overall diminished affinity for these variants to bind these inactivators (even when this is accomplished at the cost of loss of catalytic function, as measured by  $k_{\text{cat}}/K_m$ , toward  $\beta$ -lactam substrates).<sup>121,158</sup> Last, the ESBL  $\beta$ -lactamases generally retain susceptibility toward clavulanate and penam sulfone inactivation (in contrast to the IRT enzymes), and thus, the recent generation cephalosporins may eventually be combined with these inactivators in clinical practice.<sup>159</sup> This likelihood is also reflected in the continuing interest in other  $\beta$ -lactam templates, such as the 6-methylidene penam sulfones and penems,<sup>160,161</sup> that have a broad-based ability to inactivate  $\beta$ -lactamases by acyl-enzyme fragmentations and nucleophile additions.

Among the most common of the IRT TEM variants are those with replacement of arginine-244 (alone and in cooperation with other mutations).<sup>158</sup> Arg244 is a conserved residue of the  $\beta 4$  strand of the parent TEM  $\beta$ -lactamase. Its replacement by serine gives the TEM-30 (= TEM-41)/IRT-2 enzyme, by cysteine the TEM-31/IRT-1 enzyme, and by histidine the TEM-51/IRT-15 enzyme. As this arginine is an active site residue and as its replacement dramatically alters the substrate specificity of the enzyme (exemplified by a greater than 10-fold decrease in  $k_{\text{cat}}/K_m$  for penicillin substrates), considerable effort has been made to identify its role in catalysis. This effort is further driven by the unique properties acquired upon replacement of this arginine by these three amino acids (the TEM-30, -31, and -51  $\beta$ -lactamases are virtually identical).<sup>162</sup> The most important of these properties is resistance to the TEM inactivators clavulanate, sulbactam, and tazobactam, accomplished by perturbation of the partitioning of the

acyl-enzyme between hydrolysis (where there is little change in normal turnover) and the slower fragmentation and cross-linking (where this inactivation event is even further suppressed).<sup>145,154,163</sup> Two possible roles for Arg244 in normal substrate turnover have been proposed; both are consistent with the alteration in the steady-state kinetics (decreased  $k_{\text{cat}}/K_m$ ). The first possibility is that the arginine side chain participates, with a highly ordered proximal water, in  $\beta$ -lactam substrate recognition and active site orientation via electrostatic pairing with the carboxylate substituent, which is found in all bicyclic  $\beta$ -lactam antibiotics.<sup>155,164</sup> The likelihood of such an interaction is well substantiated both by active site simulation and by crystallography.<sup>118,119,154</sup> The second proposal is that Arg244 facilitates turnover by assisting, now via electrostatic repulsion, in product dissociation.<sup>165</sup> These two possibilities are not mutually exclusive. With respect to resistance, the paramount question is clearly that of the structural consequence of arginine replacement and the correlation of that consequence to the mechanism of clavulanate and penam sulfone TEM  $\beta$ -lactamase inactivation. The answer, it appears, is the pivotal role of the proximal (to the arginine guanidinium) water that is *lost* to the active site when this arginine is replaced.<sup>146,154</sup> This water molecule provides the critical proton catalyst necessary to the fragmentation event of the clavulanate acyl-enzyme. Upon arginine replacement this water molecule is lost and these inactivators of the parent TEM become ordinary substrates for these IRT TEM variants. Other IRT variants (such as occur at Met69) that retain this arginine are nonetheless also able to resist these inactivators by perturbing the second residue, Ser130, critical to this fragmentation (and its sequelae).

With respect to resistance development, a particular objective is the understanding of the relationship between the mutations securing the IRT class A variants and the mutations (such as the R164S) that secure to the class A  $\beta$ -lactamases the ability to hydrolyze late generation cephalosporins (the class A ESBL).<sup>166–170</sup> The consensus—for the moment—is that these two phenotypes are mutually exclusive. For example, the TEM  $\beta$ -lactamase double mutant (R164S, R244S) retains clavulanate resistance but is no longer capable of ceftazidime hydrolysis.<sup>166,168</sup> The obvious possibility identified by this observation that inactivator/late generation cephalosporin combination therapy might prove clinically advantageous is now in the process of preliminary evaluation.<sup>159</sup>

The outstanding features of the carbapenem (e.g., imipenem) and cephamycin (e.g., cefoxitin) classes of  $\beta$ -lactamase inhibitors are the respective 6 $\alpha$ -hydroxyethyl and 7 $\alpha$ -methoxy substitution of these  $\beta$ -lactams. The potential for these substituents to interfere with nucleophile approach to the  $\beta$ -lactam carbonyl is immediately evident, and this hypothesis is proven especially with respect to catalytic deacylation.<sup>171</sup> Of the two, cefoxitin is the more straightforward. It has a standard cephalosporin scaffold but with an unusual 7 $\alpha$ -methoxy substituent in a position occupied customarily by a hydrogen in the cephalosporins. Thus, cefoxitin engages many of the standard recog-

nition features (albeit abnormally) for class A  $\beta$ -lactamase substrates. The critical mechanistic event occurs upon serine acylation. In the cefoxitin acyl-enzyme intermediate the 7 $\alpha$ -methoxy not only displaces the catalytic water<sup>172,173</sup> but also interferes with the Asn132 side chain. This side chain is compelled to move from its customary location (where with normal substrates it is engaged in a hydrogen bond with the substrate 7 $\beta$ -amide).<sup>173</sup> This movement alters the cefoxitin 7 $\beta$ -side chain in such a way as to induce further active site distortion, especially of the  $\Omega$ -loop. The cumulative effect is a remarkably stable acyl-enzyme. Subtler (but no less complex nor less intriguing) mechanisms operate for the class A carbapenemases. As the dominant mechanism for carbapenem resistance in pathogens involves the acquisition of class B or metallo- $\beta$ -lactamases, however, the enzymatic basis for resistance due to expression of a modified class A  $\beta$ -lactamase has been less well studied. Yet there is no doubt that the new mechanism coincides, in part, with a stunning structural transformation of the TEM peptide, the introduction of a second disulfide bond (Cys69–Cys238). The presence of this cystine is intimately related to the acquisition of the carbapenemase activity.<sup>174–176</sup> The role(s) that this insertion has with respect to the mechanism has only begun to be understood. To begin with, for the *E. cloacae* NMC-A enzyme a nearly 100-fold diminution in the ability to hydrolyze penicillins but a 100-fold improvement in the ability to hydrolyze imipenem is seen.<sup>177</sup> Crystallographic inspection of a stable acyl-enzyme species shows that the positioning of the acyl-enzyme is very similar to that seen for TEM acyl-enzymes (notably normal oxyanion hole hydrogen bonding) but importantly a repositioning of Asn132 so as to open the active site for efficient catalytic delivery of the deacylation water. That even further structural and mechanistic accommodation may be anticipated is suggested by the structure of the related (70% sequence identity to NMC-A) class A SME-1 carbapenemase (and which also shows impaired penicillin hydrolysis). In the resting enzyme the presumptive acylation/deacylation general base Glu166 (on the  $\Omega$ -loop) hydrogen bonds *directly* to Ser70 (without a water bridge).<sup>175</sup> This suggests that the role of the second cystine is to enable an alternative approach (evading the steric barrier of the 6 $\alpha$ -hydroxyethyl substituent) of the serine in the acylation half-reaction.

The remaining aspect of class A  $\beta$ -lactamase evolution as it relates to the acquisition of  $\beta$ -lactam resistance by bacterial pathogens is the ESBL enzymes.<sup>15</sup> Following the clinical appearance of the third-generation oxyimino-cephalosporins (ceftriaxone, cefotaxime) some two decades ago, resistant bacteria appeared. The basis for the resistance was the acquisition and dissemination of class A, C, and D  $\beta$ -lactamases capable (often with a narrow substrate spectrum) of hydrolysis of these oxyimino-cephalosporins. Three related class A groups are pertinent: the TEM and SHV ESBL variants initially among the *Enterobacteriaceae* but increasingly among the *Pseudomonas*,<sup>122</sup> the VEB and PER variants

among the *Pseudomonas*; and the CTX-M variants.<sup>123</sup> Of these three the CTX-M are of greatest concern. These enzymes, formerly most commonly found in nosocomial pathogens, are now found in community strains (*Vibrio*, nontyphoid *Salmonella*, and *Shigella*). Moreover, the recent D240G CTX-M variants show improved ability to hydrolyze ceftazidime.<sup>123</sup> For the moment these enzymes remain capable of inactivation by clavulanate, penam sulfones, cefoxitin, and imipenem, but there is no reason to believe that this will not change. For this reason evaluation of the structural basis for the acquisition of the oxyiminocephalosporins as substrates by all of these enzymes is a major current focus of  $\beta$ -lactamase enzymology.

The efforts concerning the simplest class A transformation into an ESBL—that of the SHV (and TEM) single G238S (SHV-2) point mutation—are instructive as to the difficulties inherent to such an understanding. This single change results in an MIC increase (*E. coli*) from 2 to 8  $\mu\text{g mL}^{-1}$  for ceftazidime and from 0.125 to 16  $\mu\text{g mL}^{-1}$  for cefotaxime (comparing to *E. coli* with the parent SHV-1  $\beta$ -lactamase, for which neither oxyiminocephalosporin is a substrate).<sup>178</sup> The G238S change gives a 4-fold improvement in  $k_{\text{cat}}/K_{\text{m}}$  for a typical cephalosporin (cephaloridine). Moreover, this mutation is exceptionally well expressed, suggesting a basis (also with the optimal kinetics) for the clinical selection of Ser238 as acquisition of (albeit weaker) ESBL activity occurs for other G238 mutants (including G238A, G238N, G238M, G238C, and G238I).<sup>120</sup> An analogous transformation (appearance of cefotaxime and ceftazidime as substrates) is seen for the G238S TEM mutant.<sup>179</sup> The structural basis for this transformation (after speculation, as summarized by Hujer et al.<sup>120</sup>) was resolved by crystallography.<sup>136,180</sup> Glycine 238 is located on the b3  $\beta$ -strand, in close proximity to the Asn170, Met69, and Ala237 residues of the active site. Replacement by serine results in a significant conformational alteration spanning the 238–242 positions but with overall preservation of  $\alpha$ -carbon positions elsewhere in the enzyme (especially those of the  $\Omega$ -loop). The deformation that results from the conformational alteration is borne fully by the b3  $\beta$ -strand, opening the distance between the lower (active site) portion of this strand and the  $\Omega$ -loop by nearly 3 Å.<sup>136,180</sup> A hydrogen bond from the Ser238 hydroxyl to the main-chain carbonyl of the  $\Omega$ -loop Asn170 is seen, which may be presumed (from its orientation and distance and relatively poor solvent accessibility) to be of sufficient stability as to be unlikely to engage bound substrate. The most consistent explanation for the acquisition by this enzyme of the oxyiminocephalosporins as substrates is that the serine opens the active site just enough to accommodate the larger mass of the oxyimino side chain while preserving the orientations and roles of all of the catalytic residues.

It is probable that a similar process (shape-selective expansion of the active site) is operative for the other class A ESBL enzymes, although the process (in terms of protein adjustment) differs for each. The *P. aeruginosa* PER-1 enzyme, for example, is char-

acterized inter alia by an altered  $\Omega$ -loop,<sup>126</sup> and the *P. vulgaris* K1 enzyme lacks the Arg244 customarily thought to be involved in substrate recognition and has atypical residue replacements (notably a Ser237) that may have specific roles in substrate recognition or protein adjustment.<sup>181</sup> Extensive evaluation of the CTX-M Toho-1 structure by Shimamura et al.<sup>182</sup> (acyl-enzymes with the E166A defective mutant) and Ibuka et al.<sup>183</sup> (apo-enzyme) demonstrates that similar accommodations operate to embrace the oxyimino cephalosporins as substrates in this class A enzyme.

These observations emphasize the remarkable ability of bacteria to alter protein structure, under selection pressure, to acquire new function. While the “perfect”  $\beta$ -lactamase—one that hydrolyzes all  $\beta$ -lactam structures regardless of their substitution—has yet to be encountered, it is not necessary for such an enzyme to be created in order to attain this high level of resistance. As will become evident from the following sections, it is only necessary for the bacterium to acquire a *selection* of complementary resistance mechanisms. The CTX-M Toho-1 enzyme, for example, is quite susceptible to inactivation by cefoxitin. The bacterium does not need for this enzyme to evolve to this function as the alternative—acquiring a second  $\beta$ -lactamase with this function—is operationally more facile. Hence, the value of these exhaustive efforts to understand the relationship between structure and function of these  $\beta$ -lactamases is strategic: as the characteristics of the enzyme and the design limits for its evolutionary adaptation are better understood, the design of improved drug structure (or the use of a new drug regimen) is made possible.

A new and encouraging event (where there are not many), with respect to our understanding of this structure–function relationship, is the ability to quickly assess the capability of  $\beta$ -lactamases (not just class A) to acquire new  $\beta$ -lactams as substrates. In the years following the classic experiment of Hall and Knowles<sup>184</sup> with the TEM  $\beta$ -lactamases, which proved that such a capability existed, new methods have been developed to critically assess the structural outcome on the  $\beta$ -lactamase from the selection pressure exerted by a particular  $\beta$ -lactam. Using DNA shuffling (and *E. coli* hypermutator expression) Stemmer et al. isolated the triple-point mutation (E104K/M182T/G238S) TEM-52 variant with ESBL cefotaxime activity.<sup>180,185</sup> By the use of a highly error-prone DNA polymerase, Camps et al.<sup>186</sup> isolated three TEM mutants (E104K/R164S, E104K/R164H/G267R, and E104K/R164S/G267R) conferring a >50-fold resistance phenotype to aztreonam, a monobactam that is a very poor substrate of the parent TEM-1  $\beta$ -lactamase. Two of these point mutations are known, but the third (G267R) is new. Barlow and Hall developed an in-vitro error-prone PCR method (see also Vakulenko et al.<sup>187</sup> for the application of PCR to evaluate the  $\beta$ -lactamase response to clavulanate/sulbactam with ampicillin) that is argued as predictive of  $\beta$ -lactamase evolution under clinical  $\beta$ -lactam pressure. Using this method TEM  $\beta$ -lactamase variants resistant to the entire ensemble of third-generation



cephalosporins (cefotaxime, cefuroxime, ceftazidime, and cefepime) as well as aztreonam were identified.<sup>188–190</sup> Notwithstanding these examples, this method also has identified examples where resistance phenotypes have not emerged (as exemplified by metallo- $\beta$ -lactamases with imipenem, as discussed later), strongly arguing against the presumption that these enzymes have an unconstrained ability to adapt to all variations in antibiotic structure.<sup>190</sup> The value of these methods to drug design and to the design of clinical drug regimens is self-evident.<sup>189,190</sup>

### 3.3. Class C $\beta$ -Lactamases

Class C  $\beta$ -lactamases share with the class A  $\beta$ -lactamases a similar mechanism—active site acylation and hydrolytic deacylation—for  $\beta$ -lactam hydrolysis. This ability was inherited from their respective ancestral PBPs. Nonetheless, at the catalytic level there is a significant difference for deacylation. As first documented by Knox and colleagues,<sup>191</sup> the two classes use opposite faces of the acyl–enzyme species for the approach of the hydrolytic water. In the class C enzymes this water approaches from the  $\beta$ -direction. This distinction refutes any possibility of a direct evolutionary link between the two classes. Indeed, class C  $\beta$ -lactamases are evolutionarily closer to low  $M_r$  class B PBPs.<sup>15,60</sup> Furthermore, the residue responsible for activation of the hydrolytic water in the deacylation has been proposed to be Tyr150,<sup>191–193</sup> a process that has been suggested to be assisted by the amine of the acyl–enzyme species that was previously the  $\beta$ -lactam nitrogen of the antibiotic.<sup>194,195</sup> Therefore, the deacylation mechanism of the class C enzymes is entirely distinct from that of class A  $\beta$ -lactamases.

The class C  $\beta$ -lactamases originally were termed cephalosporinases due to a substrate preference for cephalosporins. They are found, with few exceptions, in most Gram-negative bacteria and are chromosomally encoded in several organisms (including *Citrobacter freundii*, *Enterobacter aerogenes*, and *Enterobacter cloacae*).<sup>196</sup> An increased incidence of plasmid-encoded class C  $\beta$ -lactamases<sup>15,196</sup> was observed 15 years after their first discovery.<sup>197</sup> Plasmid-encoded class C enzymes have been found in *E. coli*, *K. pneumoniae*, *Salmonella* spp., *C. freundii*, *E. aerogenes*, and *Proteus mirabilis*.<sup>198–200</sup> Most worrisome is that the rate of incidences of these enzymes is highest in *K. pneumoniae* and *E. coli*, organisms common to the hospital and community settings.<sup>196</sup>

Class C  $\beta$ -lactamases have reached “catalytic perfection” for their preferred substrates, the cephalosporin-based  $\beta$ -lactams.<sup>115</sup> However, the efficiency of turnover of the penicillins by the class C  $\beta$ -lactamases also remains high. This is attributed to low  $K_m$  values as a result of deacylation as the rate-limiting step for the class C  $\beta$ -lactamases (unlike class A  $\beta$ -lactamases), resulting in high  $k_{cat}/K_m$  values ( $10^5$ – $10^8$  M<sup>-1</sup> s<sup>-1</sup>).<sup>201</sup> The structure of these enzymes was first revealed for the class C  $\beta$ -lactamase from *C. freundii* (and *E. cloacae* strain P99).<sup>191,192</sup> The *C. freundii* enzyme has also been determined with aztreonam bound, at 2.5 Å resolution.<sup>192</sup> The structures reveal an overall similarity to the class A

$\beta$ -lactamases (see Figure 4A and C). Superimposition of the class C  $\beta$ -lactamase (from *E. cloacae*) and a representative class A  $\beta$ -lactamase reveals a handful of active site residues that occupy similar positions. In the class C  $\beta$ -lactamase these are Ser64, Lys67, Lys315, and Tyr150 that correspond, respectively, to the class A residues Ser70, Lys73, Lys315, and Ser130.<sup>191</sup> On the basis of the structures released to date, this correspondence is common among the classes A, C, and D  $\beta$ -lactamases and the PBPs.

The first crystal structure for a class C  $\beta$ -lactamases prompted the proposal that the role of general base, assisting Ser64 acylation, is carried out by the conserved residue Tyr150.<sup>192</sup> It is important to note that the side chain functions of both Lys67 and Tyr150 are in hydrogen-bonding contact with the serine hydroxyl. After accepting a proton from the serine in the formation of the tetrahedral intermediate, this tyrosine then donates the proton back to the  $\beta$ -lactam nitrogen to drive forward the collapse of the tetrahedral intermediate.<sup>192</sup> The same Tyr150 then promotes a water molecule to achieve deacylation of the acyl–enzyme species to complete the catalytic cycle. This hypothesis is based in part on the structural superposition of the active sites of the class C  $\beta$ -lactamase with that of chymotrypsin (a serine protease). In this superimposition the  $\beta$ -lactamase Tyr150 occupies a similar position to the histidine general base in chymotrypsin.<sup>192</sup> The viability of this proposal is diminished by recent NMR and site-directed mutagenesis studies. <sup>13</sup>C NMR evaluation shows that the chemical shifts of Tyr150 are invariant up to pH 11,<sup>202</sup> implying a neutral Tyr150 in the substrate-free enzyme. This result challenges earlier calculations (using Poisson–Boltzmann methodology) that predicted an unusually low  $pK_a$  value (of 8.3) for the Tyr150 phenol.<sup>203</sup> The second line of evidence arguing against Tyr150 as the general base is the site-directed mutagenesis study by Dubus et al.<sup>204</sup> The steady-state kinetics were not substantially altered by replacement of the Tyr150 with a phenylalanine. This result is inconsistent with a role for Tyr150 as a direct participant in the turnover events. Tyr150 is suggested to contribute to the acylation process indirectly, perhaps as a proton shuttle to the  $\beta$ -lactam ring nitrogen (with water serving this role in the Phe mutant). It is of interest to note that the effects on the kinetic parameters were akin to those obtained with the D,D-transpeptidase/D,D-carboxypeptidase from *Streptomyces* species R61 at Tyr159, a residue equivalent to Tyr150.

In light of the recent data by Kato-Toma et al. indicating a normal  $pK_a$  value for Tyr150,<sup>202</sup> the proposed mechanism for the second half of the reaction of the class C  $\beta$ -lactamases has to be reevaluated as well. Oefner et al. indicated that the deprotonated Tyr150 serves as the general base for the second step of the reaction in activation of a water molecule for the deacylation step, a proposal that has been widely accepted.<sup>192</sup> A protonated Tyr150 cannot perform this function.

These observations invoke the involvement of Lys67 (as a free-base) in activation of the active site serine for the acylation event in class C enzymes or

that it may abstract a proton from Tyr150, which in turn activates the serine. Furthermore, for the deacylation step, in the absence of a residue equivalent to Glu166 in class A  $\beta$ -lactamases, crystal structures from *E. cloacae* P99<sup>191</sup> and *C. freundii*<sup>192</sup> argue for the approach of the hydrolytic water from the  $\beta$ -face of the  $\beta$ -lactam antibiotic. Bulychew et al. propose that the nitrogen of the thiazoline (from  $\beta$ -lactam opening) is ideally positioned to promote hydrolytic water addition to the acyl-enzyme to accomplish deacylation.<sup>193</sup> This would be an example of a substrate-assisted catalysis. Two non- $\beta$ -lactam synthetic molecules were used to test this concept. These compounds were chemically predisposed to acylate the active site serine in the *E. cloacae* class C  $\beta$ -lactamase, resulting in acyl-enzyme species. One compound lacked the amine of the acyl-enzyme species, whereas the other possessed it. The one with the amine experienced turnover, whereas the one without it served merely as an irreversible inhibitor of the enzyme.<sup>194</sup> A recent X-ray crystallographic structure of an acyl-enzyme species of AmpC  $\beta$ -lactamase and moxalactam lends further evidence to this proposal. The authors note that a water molecule is organized to take advantage of the ring nitrogen in the hydrolytic step.<sup>195</sup>

Third-generation cephalosporins have been effectively used as a strategy against class C  $\beta$ -lactamases for over a decade. However, class C  $\beta$ -lactamases capable of hydrolyzing most third-generation cephalosporins were first isolated in the 1980s<sup>205</sup> and yet more recently from a virulent strain of *E. cloacae*.<sup>206</sup> These enzymes, termed extended-spectrum  $\beta$ -lactamases (ESBLs),<sup>124</sup> mediate resistance to (extended-spectrum) third-generation cephalosporins (exemplified by ceftazidime, cefotaxime, cefepime, and ceftriaxone) and monobactams (aztreonam) but do not affect cephamycins (cefoxitin and cefotetan) or carbapenems (Meropenem or imipenem). The structural basis for resistance mediated by these ESBLs was revealed by the structure of the *E. cloacae* GC1 enzyme, solved by Crichlow et al.<sup>207</sup> These authors suggest that conformational flexibility of the expanded  $\Omega$ -loop facilitates hydrolysis of third-generation cephalosporins by enabling greater mobility of the acyl moiety. Further evidence implicating the  $\Omega$ -loop modification with resistance to these third-generation cephalosporins came from the structures of the AmpC  $\beta$ -lactamase in complex with various third-generation cephalosporins.<sup>208</sup> A more recent structure of a phosphonate transition-state mimetic bound to the *E. cloacae* GC1 and the wild-type *C. freundii* GN346 class C enzymes afforded further insight to the basis for resistance. It was found that the designer molecule adopted different conformations in both enzymes, with the mutated  $\Omega$ -loop of the GC1 enzyme able to accommodate the cefotaxime side chain in a different conformation, enabling it to allow the approach of the hydrolytic water to the acyl-enzyme species.<sup>209</sup>

### 3.4. Class D $\beta$ -Lactamases

The class D  $\beta$ -lactamases are increasingly encountered among the defensive  $\beta$ -lactamase ensemble of

certain Gram-negative pathogens.<sup>15,109,124,210</sup> These  $\beta$ -lactamases were first termed as oxacillinases (and for this reason are still described as OXA  $\beta$ -lactamase variants) for their ability to hydrolyze the 5-methyl-3-phenylisoxazole-4-carboxy side chain penicillin class, exemplified by oxacillin and cloxacillin. Over 50 class D OXA variants are now known,<sup>211</sup> including variants that have dispensed with their oxacillinase activity in the process of acquiring ESBL activity against carbapenem and third-generation cephalosporins.<sup>212</sup> In the course of these transformations the class D enzymes have expanded from their historical *P. aeruginosa* niche<sup>213,214</sup> into other Gram-negative pathogens including *E. coli*,<sup>215,216</sup> *P. mirabilis*,<sup>217</sup> *Salmonella* sp.,<sup>218</sup> *K. pneumoniae*,<sup>219,220</sup> and especially *Acinetobacter baumannii*.<sup>221–228</sup> While at present the clinical impact of the OXA  $\beta$ -lactamases is associated with infections by *P. aeruginosa* and *A. baumannii*, the widening Gram-negative distribution provides powerful support for the concern that the clinical value of carbapenems (and third-generation cephalosporins) may quickly diminish.<sup>211,220,222,225,229</sup>

These enzymes only recently have become the subject of detailed structural and mechanistic studies. This is due in some measure to their very recent appearance in clinically relevant pathogens (as distinct from the other three  $\beta$ -lactamase classes) but in greater measure to their heterogeneous properties (notwithstanding their interrelatedness) and the historical difficulty of their *in vitro* assay. The literature is replete with descriptions of poorly reproducible biphasic (burst-type) progress curves for some variants, corresponding (in the apparent steady-state) to unusually low  $k_{\text{cat}}/K_m$  values (as exemplified by the data of Danel et al.,<sup>230</sup> Franceschini et al.,<sup>231</sup> Pernot et al.,<sup>232</sup> and Heritier et al.<sup>226</sup>). Among the enzyme properties explored as possible explanations for these difficulties were a monomer-dimer equilibrium with possible additional divalent metal dependency.<sup>230,231,233,234</sup> No credible explanation was found. For example, some OXA variants are monomeric,<sup>97</sup> and the dimer  $K_d$  in any case (typically micromolar) is relevant only to the *in-vivo* and not *in-vitro* kinetics, while other variants show little capacity for divalent metal binding.<sup>224</sup> As a consequence of this dilemma, the possibility that other mechanisms (in addition to the OXA  $\beta$ -lactamase) contribute to the  $\beta$ -lactam resistance phenotype has been discussed.<sup>221</sup> Also, while the importance of porin deletion<sup>211,216,219</sup> and peptidoglycan remodeling (in addition to the assembly of mixed class  $\beta$ -lactamase ensembles or single  $\beta$ -lactamase hyperexpression) to Gram-negative resistance cannot be underestimated, the identity of the likely critical and confounding variable contributing to the *in vitro* assay variability—CO<sub>2</sub>—was identified by Golemi et al.<sup>95,96</sup>

The structural basis by which CO<sub>2</sub> activates the class D enzymes for  $\beta$ -lactam hydrolysis was elucidated concurrent with several independent crystallographic studies of the class D  $\beta$ -lactamases. The first studies on a class D enzymes (the *P. aeruginosa* OXA-10  $\beta$ -lactamase) showed that the class D domain folding was similar to the other two serine  $\beta$ -lacta-



mase classes.<sup>235,236</sup> However, subsequent crystallographic analysis revealed that the active site lysine was *N*-carboxylated as a result of addition of the lysine side chain amine (as the free-base amine) to CO<sub>2</sub>.<sup>96,237</sup> The resulting carbamic acid ionizes to give a carbamate functional group in hydrogen-bonding contact with the active site serine. As the formation of this carbamate is reversible, the earlier reports of the absence of lysine carboxylation are explained. In light of the high physiological concentration of CO<sub>2</sub> (low millimolar) this lysine is expected to be fully carboxylated in vivo.<sup>95</sup> Kinetic analysis of a mutant enzyme where this lysine is replaced shows the total loss of catalytic activity, indicating a direct involvement in catalysis.<sup>95</sup> The role for this unusual lysine-derived carbamate is general base activation for both acylation (activation of the serine) and deacylation (activation of water) steps of catalysis.<sup>95,237</sup> Moreover, the assignment to this CO<sub>2</sub>-derived lysine carbamate of this role as general base catalyst is consistent with the absence of alternative possibilities. Not only does the class D  $\Omega$ -loop not contain a counterpart for the class A Glu166, but the tyrosine of the conserved parental class D Y144-G145-N146 motif on this loop is replaced by phenylalanine in ESBL (both carbapenem and cephalosporin) class D variants, obviating direct participation of this tyrosine in catalysis (as is more fully discussed elsewhere).<sup>97,222,226,238</sup> The OXA-1 crystal also shows the lysine carbamate<sup>97</sup> whereas the OXA-13 enzyme<sup>232</sup> does not (almost certainly an artifact of crystallization). The relationship of the CO<sub>2</sub> to the complicated kinetics extends beyond the relative portion of the lysines that are activated for catalysis. During catalysis the lysine carbamate is prone to spontaneous decarboxylation in the middle of the turnover process, thus arresting catalysis at the acyl-enzyme stage. This must, however, be regarded as an artifact of in vitro assay since supplementation of the medium with bicarbonate (as a CO<sub>2</sub> source) restores the kinetic profile.<sup>95</sup> It has been argued that the more complicated biphasic turnover profile for these enzymes with some substrates is due to a branching mechanism. As the enzyme experiences decarboxylation midcatalysis, it becomes inactivated (the branching species), pending the availability of a CO<sub>2</sub> molecule to restore the lysine to its active carboxylated form. The enzyme is then able to complete the second step of catalysis.<sup>95,236</sup> With a few substrates, however, it was shown that supplementation of the medium with bicarbonate does not simplify the turnover process. For these few cases a branching mechanism (as might occur by a conformational change at the acyl-enzyme state) has been invoked.

[Dr. Roger Labia, a pioneer of studies of  $\beta$ -lactamases, kindly communicated that his early investigations of the class D  $\beta$ -lactamases in 1970s were frustrating because of the complicated and erratic kinetic behavior of the enzymes. He opted to abandon studies of the class D enzymes. He now attributes the erratic behavior of the enzymes to the seasonal variations in the quality of the water, which has high carbonation in the summers and low carbonation in the winters.]

While there are numerous reports evaluating the  $\beta$ -lactam substrate profile for the class D OXA enzymes, essentially all of these predate the discovery of requirement for in-vitro CO<sub>2</sub> (but most unlikely in vivo) activation. These earlier data may not be reliable. Nonetheless, all these data suggest that the substrate profile for these enzymes, individually and as a class, is not broad.<sup>211</sup> The value of the class D enzymes to bacteria is the ability of this enzyme to adapt, under selection pressure, to the specific  $\beta$ -lactam. For example, while most of the ESBL OXA variants hydrolyze ceftazidime better than cefepime, the reverse is true for the OXA-30 variant (derived from OXA-1).<sup>215</sup> Full discussions of the orientation (and contacts) within the class D active site for (inhibitor) acyl-enzymes<sup>232,237</sup> and possible orientations for substrates<sup>97</sup> are presented elsewhere.

A final issue is the origin of the class D enzymes in comparison to the two other serine  $\beta$ -lactamase classes. All three classes are now encountered as both chromosomal- and plasmid-borne genes. However, the evolutionary history of each class is distinct.<sup>15</sup> In contrast to the class C AmpC family, where the mobilization to plasmids is a modern (antibiotic era) event, plasmid mobilization of the class A TEM and class D OXA are ancient events.<sup>239</sup> The primary distinction between the class A and C  $\beta$ -lactamases is the much more rapid and extensive diversification—at this point in time—of the former within the Gram-negative bacteria. Also of particular interest is the complete absence of this diversification within the Gram-positive bacteria given the estimate by Hall and Barlow that the horizontal transfer of the class C gene from the Gram-negative to the *Bacillus* Gram-positive bacteria is an ancient event of some 320–575 Myr (but is after the divergence of *B. subtilis* from *S. aureus*).<sup>15</sup> A homology between the hydrophilic carboxy domain of the BlaR/MecR  $\beta$ -lactam-signaling receptors and the class D  $\beta$ -lactamases was noted previously.<sup>84,240</sup> The recent discovery within *B. subtilis* of a gene encoding an enzyme with weak class D  $\beta$ -lactamase activity, yet also resembling a penicillin-binding protein,<sup>241</sup> suggests that study of the class D gene within those Gram-positive bacteria that possess it may further refine our understanding of the structural and functional relationships between the PBP and  $\beta$ -lactamase enzymes.

### 3.5. Class B Metallo- $\beta$ -lactamases

The second—and in many respects no less foreboding—vision of the  $\beta$ -lactamase future is that of the disseminated metallo- $\beta$ -lactamases (MBLs).<sup>242</sup> First observed in 1967 by Kawabata and Abraham as chromosomal enzymes of the innocuous Gram-positive *B. cereus*, these enzymes occupy a position of concern (in terms of breadth of distribution and breadth of  $\beta$ -lactam catalytic activity) with respect to the inexorable expansion of  $\beta$ -lactam resistance.<sup>109,229,243,244</sup> These metal-dependent (almost always divalent zinc)  $\beta$ -lactamases have a broad  $\beta$ -lactam substrate tolerance that encompasses many of the newer generation cephalosporins, carbapenems, and other  $\beta$ -lactamase inhibitory (clavulanate and penam sulfones)  $\beta$ -lactams important to the treat-



ment of Gram-negative infection.<sup>210,229</sup> As a different chemical mechanism (compared to the serine  $\beta$ -lactamases) is used by the metallo- $\beta$ -lactamases for  $\beta$ -lactam hydrolysis—notably, a mechanism without a covalent enzyme intermediate—an entirely different strategy for their inhibition (or inactivation) will be required should (or is it when) these enzymes expand beyond their present niche (that of minor, and opportunistic, Gram-negative pathogens). While several inhibition strategies have been identified, none has yielded anything resembling that of a clinically effective inhibitor. Also, although there exist as yet chemical reasons (in terms of  $k_{\text{cat}}/K_{\text{m}}$  these are not optimized enzymes) and biochemical reasons (zinc as a limiting nutrient) that ultimately may limit the role the metallo- $\beta$ -lactamases will have as a resistance mechanism (*vide infra*), the proposal discussed by Fast et al.,<sup>245</sup> Fabiane et al.,<sup>246</sup> and Wommer et al.<sup>247</sup> that these are young enzymes, only now in the process of maturation under evolutionary pressure, is both credible and worrisome.

The metallo- $\beta$ -lactamases (also termed Ambler class B and Bush–Jacoby–Medeiros Group 3  $\beta$ -lactamases)<sup>105,243</sup> are small enzymes sharing a common four-layer  $\alpha\beta\beta\alpha$  motif with a central  $\beta$ -sandwich and two  $\alpha$ -helices on either side.<sup>246,248</sup> This motif, arising possibly by a gene duplication event,<sup>60,245</sup> is found also in other proteins (glyoxalase and certain flavoenzymes). The motif has an intrinsic metal-binding site located at an edge of the  $\beta$ -sandwich.<sup>249,250</sup> For the metallo- $\beta$ -lactamases this site is occupied by a divalent zinc ion having a tetrahedral array of three histidines and water. The importance of the zinc ion to  $\beta$ -lactam substrate binding is unquestionable.<sup>251,252</sup> The role of the zinc in the hydrolytic mechanism, beyond that of Lewis acid catalysis, is less certain. Nonetheless, there is a consensus that the water ligand of the zinc ion is the  $\beta$ -lactam ring-opening nucleophile (via a mechanism likely with parallel to that of the zinc metalloproteases), but there are no direct data establishing this role.

Beyond these structural commonalities the metallo- $\beta$ -lactamases possess a surprising breadth of primary structure that most notably includes the creation (in some enzymes) of a second zinc binding site.<sup>243</sup> In these binuclear enzymes the two zincs are proximal (approximately a 3.5 Å separation) with both participating in the water coordination. The ligand environment of the second zinc ion is very different from that for the first both in array (trigonal bipyramid) and amino acid ligands (variable among the binuclear class). The purpose of the second zinc ion is regarded as catalytic augmentation—that is, accomplishing an incremental increase in the  $\beta$ -lactam substrate  $k_{\text{cat}}/K_{\text{m}}$ —and not a role of catalytic necessity.<sup>245,247,253–255</sup> Nonetheless, the specific environment of the zinc ensemble determines the individual enzyme catalytic behavior toward substrates and the detail of the rate-limiting (highest energy) catalytic step.<sup>245,255–259</sup> The relative affinity of the enzyme for the two zinc ions is unequal, and in-vitro enzyme kinetic analysis requires added  $\text{Zn}^{2+}$ .<sup>247,255,260</sup> Wommer et al.<sup>247</sup> discussed in detail the role of the  $\beta$ -lactam substrate in the recruitment of zinc to, and

hence activation of, the  $\beta$ -lactamase. This process is argued as one of physiological necessity wherein the  $\beta$ -lactamase exists as an apoenzyme and the available zinc is reserved to other enzymes until the  $\beta$ -lactam antibiotic is encountered. The likelihood of a fully zinc complemented metallo- $\beta$ -lactamase (binuclear site fully occupied) in vivo is regarded as small. It is therefore understandable why the bacterium possessing a metallo- $\beta$ -lactamase is often found with a serine  $\beta$ -lactamase as well.<sup>261,262</sup> The demonstrable fact that these enzymes are disseminating is proof of evolutionary pressure for resistance development within human clinical practice.

While the details<sup>245,255,263</sup> of the hydrolytic mechanism used by these enzymes is beyond the scope of this review, the mechanistic fundamental is assuredly not. This fundamental is delivery of the zinc-coordinated water, possibly as a hydroxide ion ( $\text{pK}_{\text{a}} = 4.9$  to 5.6),<sup>245,260,264,265</sup> to the  $\beta$ -lactam carbonyl. (The metalloprotease carboxypeptidase A has a similar catalytic  $\text{pK}_{\text{a}}$  that is also assigned to a zinc hydroxide. However, the recent  $^{67}\text{Zn}$  NMR study by Lipton et al. forcefully argues against this assumption.<sup>266</sup> Accordingly, a wholesale mechanistic revision for metallo- $\beta$ -lactamase catalysis may be necessary.) The presumed involvement of a zinc hydroxide intermediate for the metallo- $\beta$ -lactamases has stimulated exquisite studies on the in-vitro mechanism of metal-catalyzed  $\beta$ -lactam solvolysis.<sup>34,267–269</sup> These studies suggest the rate-limiting step to be either metal-coordinated hydroxide addition or (following the hydroxide addition) the collapse of the tetrahedral species. The mechanism is dependent on  $\beta$ -lactam structure in such fashion as to strongly implicate zinc coordination of the  $\beta$ -lactam in the transition state.<sup>34,252,253,256,270–272</sup> A similar mechanism—that is, hydrolysis without a covalent enzyme intermediate—is posited for the enzymatic reaction. Solvent kinetic isotope effects for the enzymatic reaction implicate additional transition-state stabilization by proton flight.<sup>255,260</sup> The identity of the enzymatic proton donor/acceptor, however, remains a particular focus of mechanistic study. A notable commonality of the structure–kinetics analysis of the nonenzymatic solvolysis and the comparative enzymatic structure–kinetics is the particular susceptibility of the carbapenems to hydrolysis.<sup>34,229,269,270</sup>

This reality—the susceptibility toward metallo- $\beta$ -lactamase hydrolysis of nearly all (*vide infra*) of the serine  $\beta$ -lactamase inhibitors—is a growing concern. There are three reasons for this. The first is the burgeoning presence of metallo- $\beta$ -lactamases (first the IMP and now the VIM and SMP class B1 variants) as mobile plasmid-encoded (and often also as integron, or cassette) genes by the enterobacteria.<sup>273,274</sup> Second, these enzyme variants have a remarkable ability to alter their substrate capability (as can be accomplished by simple-point mutation, even remote to the active site), raising the possibility of rapid adjustment to encompass new substrates (and to thwart new inhibitors). Last, the metallo- $\beta$ -lactamase plasmids often encode additional (multi-substrate) resistance mechanisms.

The class B1 metallo- $\beta$ -lactamases are monomeric, binuclear zinc enzymes constituting the largest metallo- $\beta$ -lactamase subclass. Within this subclass the dominant subtypes are the IMP and VIM enzymes. While these subtype enzymes are defined by sequence, substantial diversity is found within each. For example, the recently observed IMP-12 variant has 89% sequence identity to its closest (IMP-8) relative but includes 10 amino acid changes at positions that are otherwise invariant among the 11 other subtypes.<sup>275</sup> The IMP enzymes are originally Asian (and the VIM enzymes originally European), but it is quite evident that geographical characterization of both of these enzymes is irrelevant. First observed in 1988, the IMP enzymes have a broad substrate (primarily cephalosporins and carbapenems, less so penicillins) acceptance, although at the level of specific  $\beta$ -lactam structure the  $k_{\text{cat}}/K_{\text{m}}$  variation can be substantial. Replacement of glycine-196 (a noncatalytic residue adjacent to His197, a zinc ligand) in the IMP-3 and IMP-6 variants with serine (as is found in IMP-1) results in a significant  $k_{\text{cat}}/K_{\text{m}}$  improvement toward certain cephalosporins and toward imipenem (IMP-1  $k_{\text{cat}}/K_{\text{m}}$  is 10–50-fold greater compared to IMP-3).<sup>272,276</sup> Nonetheless, a comprehensive in vitro substrate evaluation of IMP-6 and microbiological evaluation of *E. coli* possessing the TEM-1/IMP-6 plasmid indicate the IMP-6 to be the better enzyme in terms of extended carbapenemase activity.<sup>277</sup> Whereas Meropenem and imipenem are essentially equivalent (as measured by  $k_{\text{cat}}/K_{\text{m}}$ ) IMP-1 substrates, the IMP-6 glycine replacement results in a 6-fold improvement in the Meropenem  $k_{\text{cat}}/K_{\text{m}}$  (and a 2-fold loss for imipenem). This improved  $k_{\text{cat}}/K_{\text{m}}$  contributes to the significant difference in MIC (*E. coli* with the IMP-1/TEM-1 plasmid, 64  $\mu\text{g mL}^{-1}$  for Meropenem and 2–8  $\mu\text{g mL}^{-1}$  for imipenem; without, 0.25  $\mu\text{g mL}^{-1}$ ).<sup>277</sup> Efforts to correlate IMP sequence with altered substrate acceptance have been made by Oelschlaeger et al. (comparing G196 IMP-6 to S196 IMP-1)<sup>272</sup> and Moali et al. (evaluating the role of the distal 60–66 loop).<sup>271</sup> In the former study a favorable serine-196-lysine-33 interaction improves packing and rigidifies histidine-197; this rigidity propagates throughout the active site. A calculated enzyme–substrate stability index was found to correlate well to the experimental  $k_{\text{cat}}/K_{\text{m}}$ . In the latter study the loop was confirmed as nonessential for catalysis but contributed (especially tryptophan-64) to hydrophobic substrate binding. A mutagenesis study by Materon and Palzkill of IMP-1 active site proximal amino acids identified 52% of the IMP-1 amino acids as intolerant to substitution (by comparison, the TEM-1 value is 33%).<sup>278</sup> Materon and Palzkill<sup>278</sup> suggest that the IMP metallo- $\beta$ -lactamases may have a relatively more limited ability to adjust to “extended spectrum”  $\beta$ -lactam structure compared to the TEM enzymes (which have, of course, already done so). A related—and no less pertinent—question is whether the metallo- $\beta$ -lactamases have the capability to develop their catalytic apparatus to function at the substrate diffusion limit (that is, at full catalytic competence), as is already the case for the class A and C serine  $\beta$ -lactamases.<sup>115</sup> The extant IMP

kinetic data indicate that while certain cephalosporins have  $k_{\text{cat}}/K_{\text{m}}$  values that approach the diffusion limit of ( $10^7$ – $10^8$   $\text{M}^{-1} \text{s}^{-1}$ ), most substrates have lower values (typical carbapenem  $k_{\text{cat}}/K_{\text{m}}$  values are approximately  $10^6$   $\text{M}^{-1} \text{s}^{-1}$ ). Hall’s full mutagenesis in vitro evaluation of the IMP-1 structure, which failed to identify a mutant enzyme more capable of imipenem hydrolysis, is consistent with one (or both) of these possibilities.<sup>279</sup> As the diversity of known carbapenem structure is not nearly that of the penicillins and cephalosporins, cautious optimism may be entertained that newer generation  $\beta$ -lactams poorly capable of metallo- $\beta$ -lactamase hydrolysis may yet be made.

Two additional aspects may temper this conclusion. It is clearly not possible to determine, by evaluation of enzyme sequence or enzyme kinetics, a “direction” for metallo- $\beta$ -lactamase variant evolution (which variant arose from which variant). Hence, the apparent evolutionary limitation of IMP-1 with imipenem has no predictive value with respect to other metallo- $\beta$ -lactamase variants. As bluntly stated by Hall, “in order to understand the risks posed by metallo- $\beta$ -lactamases, it will be necessary to conduct similar studies on representative members of each of the three metallo- $\beta$ -lactamase subfamilies and to include all clinically relevant carbapenems”.<sup>279</sup> Second, it is evident that *incremental* changes in  $\beta$ -lactam fitness—in terms of PBP inactivation and competence as a substrate for  $\beta$ -lactamase hydrolysis—suffice to determine whether a bacterium is susceptible or resistant. An effect need not be dramatic to be important.

The VIM B1 subclass is newer (first observed in 1997) and biochemically less well studied.<sup>280</sup> A VIM sequence homology with IMP is recognizable (approximately 30–40%), and the overall pattern of  $\beta$ -lactam substrate recognition by the two enzymes is similar.<sup>229</sup> Seven VIM variants are extant.<sup>281</sup> In less than 7 years the VIM metallo- $\beta$ -lactamases have transitioned from chromosomal expression by nonfermenting Gram-negative bacteria (where it contributes to high-level antibiotic resistance in *P. aeruginosa* pathogenic strains)<sup>282,283</sup> to transferable plasmid expression in Gram-negative enterobacteria.<sup>262,273</sup> The presumptive circumstances leading to this change (evolutionary pressure for plasmid dissemination is not necessarily carbapenem but rather multidrug driven) and probable consequence of this change (likelihood of carbapenem clinical failure) underscore the caution expressed in the previous paragraph.

A consistent observation from the in vitro evaluation of the metallo- $\beta$ -lactamase substrate spectrum is the inability of these enzymes to hydrolyze the monocyclic *N*-sulfonyl  $\beta$ -lactam antibiotic aztreonam. Consequently, bacteria that have these metallo- $\beta$ -lactamases can retain aztreonam susceptibility (although moderate to substantial increases in the aztreonam MIC values, due to other plasmid-conferred resistance mechanisms or to the presence of aztreonam-capable serine  $\beta$ -lactamases, is common). As the intrinsic reactivity toward solvolysis of the aztreonam  $\beta$ -lactam is identical to that of the penicil-

lins,<sup>34,116</sup> the inability of the metallo- $\beta$ -lactamases to hydrolyze aztreonam must correspond to a failure either to bind to the enzyme or to bind but in a nonproductive orientation. Quite surprisingly, there do not appear to be enzymatic kinetic data on this matter. Diaz et al. predict, on the basis of computational study, the latter answer as correct.<sup>269</sup> Accordingly, aztreonam may represent an unusual example of what has emerged as a general strategy for metallo- $\beta$ -lactamase inhibition. Following the proven basis for zinc protease inhibitor design (that of inhibitors providing sulfur coordination to the thiophilic zinc), thiol-substituted carboxylic acids represent a general metallo- $\beta$ -lactamase inhibitor motif.<sup>248,259,284–286</sup> The carboxylate of these inhibitors occupies the identical site used in  $\beta$ -lactam carboxylate recognition and the thiol (as the thiolate) displaces the nucleophilic hydroxide from the zinc pair. When the juxtaposition is optimal (typically corresponding to  $K_i$  values of 0.1–1  $\mu$ M) a reorganization of the active site is seen that is believed to be similar to what occurs in substrate binding.<sup>248,259,287,288</sup> Given the exemplary quality of these inhibitor–enzyme crystallographic structures and the increasing sophistication with which proven  $\beta$ -lactam synthetic methodology is being applied to thiocarboxylate design, yet more potent inhibitors (approaching what will likely be required for clinical efficacy) are anticipated. Whether these will also possess the appropriate pharmacodynamics to effectively synergize with a  $\beta$ -lactam antibiotic remains to be seen.

## 4. Other Resistance Mechanisms

### 4.1. Porin Deletion

The temporal response of bacteria to antibiotics is both immediate (abrupt gene repression and gene activation) and evolutionary (empirical gene mutation and gene acquisition). Not surprisingly the increased sophistication with which these changes may be assessed and the dramatic breadth of change particularly in the immediate response<sup>289,290</sup> have led to justifiable optimism that understanding these responses will identify new targets for antibiotic design.<sup>291,292</sup> At a simpler level the comparison of bacterial protein expression before (susceptible) and after (resistant) antibiotic exposure has been the mainstay to the understanding of antibiotic resistance development, and it is these studies (as have been just described) that support the generalization that PBP alteration is a principle mechanism of Gram-positive resistance and  $\beta$ -lactamase expression is a principle mechanism of Gram-negative resistance. Nonetheless, these same studies indicate that other resistance mechanisms exist. In this section an overview of two of these other mechanisms—decreased antibiotic permeability and increased antibiotic efflux—is given.

It is axiomatic that a successful antibiotic has potency (the ability to incapacitate an essential target) and access (to its target). Indeed, PBP alteration is a strategy to render impotence and  $\beta$ -lactamase expression compromises access. A second method for controlling access is by changes within the outer

membrane (in Gram-negative bacteria) and cell wall (in Gram-positive bacteria). While the evaluation of these changes is among the most difficult tasks to accomplish at the microbiological level, the recent observations concerning several resistant *K. pneumoniae* species—those of an important Gram-negative pathogen—are revealing. The report by Bradford et al. is regrettably now typical.<sup>293</sup> An examination of 12 highly  $\beta$ -lactam-resistant *K. pneumoniae* strains and 6 *E. coli* strains from a single hospital showed that 17 possessed multiple  $\beta$ -lactamases. The three most resistant *K. pneumoniae* strains (as defined by resistance to imipenem, the prototypical carbapenem) achieved their resistance by the combined expression of an AmpC extended-spectrum  $\beta$ -lactamase and deletion of a major 42 kDa outer membrane protein (omp). A second report<sup>294</sup> likewise describes this same combination of the AmpC  $\beta$ -lactamase and the omp protein deletion giving an imipenem-resistant *K. pneumoniae*. The surmise that this omp protein—a porin, or nonspecific solute pore—is an important point of ingress for the  $\beta$ -lactam to the periplasmic space is supported by the subsequent studies of Nelson et al.<sup>295</sup> and Doménech-Sánchez et al.<sup>296</sup> Resistance derives from the synergistic combination of reduced permeability and the  $\beta$ -lactamase; the degree of resistance of these two mechanisms in concert exceeds that of each mechanism alone. Similar observations have been made recently (citing representative examples) with respect to  $\beta$ -lactam-resistant *E. coli*,<sup>216,297</sup> *Salmonella enterica*,<sup>298</sup> *Helicobacter pylori*,<sup>299</sup> *Acinetobacter baumannii*,<sup>300</sup> *E. aerogenes*,<sup>301,302</sup> *K. pneumoniae*,<sup>303</sup> and *P. aeruginosa*.<sup>304,305</sup>

A simple conclusion as to the importance of porin deletion (or modification) remains, however, elusive. It most certainly contributes for certain bacteria when selected for by certain antibiotics. The reasons why a broader generalization is not possible are straightforward. The variation in intrinsic antibiotic permeability among the bacteria is substantial. For example, Lakaye et al.<sup>306</sup> estimate that *E. coli* is approximately 20–1000-fold more permeable for a given  $\beta$ -lactam than *E. cloacae*. In addition, the variation in relative permeability as a function of  $\beta$ -lactam structure is no less variable: as assessed by Matsumura et al.,<sup>307</sup> the relative *E. coli* permeability of imipenem (most permeable) is approximately 60-fold greater than ceftazidime (the least permeable of six  $\beta$ -lactams evaluated). An appreciation for the basis of this variability is provided by Nikaido's superlative account<sup>45</sup> of the utter complexity of the Gram-negative outer membrane dynamics: an extraordinarily asymmetric bilayer dominated on the outside by the lipopolysaccharide surface, which itself exerts significant permeability selection particularly against hydrophobic solutes,<sup>308,309</sup> and punctuated on both surfaces by an array of nonspecific protein pores (porins) and transporters. Relative solute permeability is influenced by a nearly limitless number of variables. Porin deletion in a  $\beta$ -lactam-resistant *E. coli* is accompanied by (uncharacterized) changes in the outer membrane (and perhaps peptidoglycan) that could also contribute to resistance.<sup>297</sup>



The presumption that compensatory responses such as porin deletion exact a fitness cost is almost certainly correct.<sup>5,91,310</sup> In this regard bacteria are little different from other organisms: to the extent that a choice is possible between death and discomfort, the latter is chosen. An example of accommodation between survival and vastly decreased solute permeability is provided by the mycobacteria. The mycobacteria are notable (and also opportunistic) human pathogens that are suggested evolutionarily to bridge the Gram-negative and -positive bacteria. Their high intrinsic resistance to chemotherapy—including complete  $\beta$ -lactam resistance—is believed to result from a combination of restricted porin ingress (either by limited abundance or by pore character) and an impenetrable exoskeleton (consisting of a thick peptidoglycan and an outer membrane having on its outer leaflet an additional barrier of long mycolic fatty acids attached an arabinogalactan chain).<sup>45,311</sup> It may therefore be surmised that exoskeleton adjustment, so as to limit  $\beta$ -lactam exposure, is also a strategy exploited by the Gram-positive bacteria (the more so because they are regarded as more solute permeable than either the Gram-negative or mycobacteria). This surmise is correct. However, the astonishing complexity of these adjustments is just now being appreciated. While it has been known for some time that the cell wall composition of both Gram-negative and -positive bacteria changes in response to  $\beta$ -lactam exposure,<sup>26,312,313</sup> the presumption with respect to the Gram positives is that these changes reflected the direct (for want of a better term) resistance response: the peptidoglycan is altered by compensatory overexpression of a PBP, or as a result of differential inactivation by the  $\beta$ -lactam of a PBP from among the ensemble, or the differential recognition of the selected low-affinity PBP for the biosynthetic cell wall precursors results in the modified cell wall. That a more complex stratagem was in play was discovered by Filipe and Tomasz<sup>314</sup> from the observation that inactivation of the *murMN* operon, encoding the *murM* and *murN* cell wall cross-bridge biosynthesizing enzymes, abolished  $\beta$ -lactam resistance in low-affinity PBP containing (and thus previously) highly  $\beta$ -lactam-resistant *S. pneumoniae*.<sup>315</sup> The indisputable correlation of *murM* enzymatic activity (*murN* deletion has little effect) with robust penicillin resistance is discussed by Fiser et al.<sup>316</sup> and Rohrer and Berger-Bächi.<sup>317</sup> The facile conclusion—that the *murM* cross-link-enabling reaction, the acylation by serine of the lysine  $\epsilon$ -amino of Lipid II, is a necessary event in the construction of a  $\beta$ -lactam-impermeable peptidoglycan—may indeed prove correct. Fiser et al. suggest a more intriguing possibility based on homology modeling of the *murM* sequence with FemA and myristoyl transferase: a winged coiled-coil helical DNA-binding domain structure similar to the bacterial transcription factors known to control multidrug exporter expression. Their conjecture that *murM* additionally serves to regulate bacterial gene expression following  $\beta$ -lactam exposure, such as by exporter expression, is consistent with the appearance of high-level  $\beta$ -lactam resistance. We know this conjecture to be credible

since the operation of a drug transporter is already known to confer high-level  $\beta$ -lactam resistance to another insidious bacterial pathogen, *P. aeruginosa*.

## 4.2. Transporter Expression

The *P. aeruginosa* species is a particular contributor to highly drug-resistant biofilm infections in cystic fibrosis patients for which carbapenem therapy is often the only recourse. The breadth of its resistance is believed to result from the combination of overall membrane impermeability (especially porin deletion and thickened peptidoglycan) and the action of active transporter-catalyzed drug efflux (typically the RND MexAB–OprM transporter).<sup>304,305,318,319</sup> The bacterial transporters (of which five families are now recognized, abbreviated as MFS, SMR, MATE, RND, and ABC) have become a central issue to the understanding of overall bacterial vitality. Simply put, the volume of regulated (such as by transporters) and unregulated (such as by porins) molecular traffic in and out of the bacterium is astonishing; it is now believed that up to 20% of the *E. coli* genome encodes transporters of one variety or the other.<sup>320</sup> The importance of this phenomenon, especially in relation to drug resistance and virulence factor release,<sup>321,322</sup> is attested to by the volume of exemplary reviews that describe the rapidly changing status of this field.<sup>320,323–328</sup> What is particularly disconcerting is the appearance of highly resistant bacteria wherein the operation of these transporters, as part of an ensemble of resistance mechanisms including porin loss and  $\beta$ -lactamase acquisition, suffice to compromise the carbapenems as an effective therapy. Moreover, bacteria that operate these transporters coincide to a multidrug-resistant phenotype. Also, while imipenem is understood not to be an efficient substrate of the *P. aeruginosa* transporter, the increasing appearance of imipenem-resistant *P. aeruginosa* indicates that refinement of the panoply of *P. aeruginosa* resistance mechanisms to embrace imipenem is in progress. Whether antibacterial design of new  $\beta$ -lactam structures that evade these structures is possible remains to be seen. There is decreasing room for optimism: imipenem is an exceptionally small and permeable antibacterial. The strategy in medicinal chemistry design is invariably that of increased functional-group complexity and concomitant increased molecular mass, entirely in the wrong direction for transporter evasion. Perhaps more promising is the concept of synergism via co-administration of transporter inhibitors, which has emerged as an active area of drug design.<sup>329–332</sup> Even more intense interest in these approaches is likely as the role of these transporters in Gram-negative<sup>333</sup> and Gram-positive<sup>316</sup> drug resistance is clarified.

## 5. Envoi

That the thoughtless use of antibiotics is reckless is an opinion that will fail to provoke dispute from any reader of these words. Indeed, the issue is no longer whether a clinical problem exists (the statistical data would be deemed indisputable even by Samuel Clemens in his retelling of Disraeli's quote

about statistics)<sup>6,334–338</sup> nor largely what could (or should) be done to forestall the inevitable crisis but rather how to invest *today* in the scientific and medical strategies that will provide preparedness *tomorrow*. Jefferson's insightful connection between vigilance and liberty is no less appropriate to parlous geopolitical circumstances as it is the inexorable progression of resistant human pathogens. Two questions address the relationship of scientific vigilance to the future role of the  $\beta$ -lactam antibacterials in the treatment of infections. Do the  $\beta$ -lactams remain a viable template for drug discovery? Do their targets—the enzymes of cell wall biosynthesis—remain a viable target for antibacterial design?

The former question is unquestionably the more contentious, especially given the precipitous decline in pharma investment in antibacterial discovery.<sup>2,339–343</sup> The choice of template, always paramount to ultimate success in drug discovery, is yet more so when resources are limited. Notwithstanding the historical dominance of the  $\beta$ -lactams, which continues to this very day, the trend toward greater mass and functional-group complexity in successive  $\beta$ -lactam generations strongly suggests that design limits are being approached. In the past these conceptual limits have been breached by revelation from Nature—a vastly more imaginative engineer of chemical structure than man—but here as well pharma investment is in decline.<sup>344,345</sup> It is evident that the era of medicinal chemistry manipulation of the  $\beta$ -lactam, guided by the paradigm of iterative optimization of MIC values, is coming to an end. This is not to say, however, that future medicinal chemistry efforts toward  $\beta$ -lactam optimization are exercises in futility. Rather, future antibacterial discovery (not just  $\beta$ -lactams) will follow the much more complicated process wherein chemical structure is evaluated in terms of the interrelationships among bacterial genomics and proteomics relating to *several* antibacterial targets.<sup>290–292,346,347</sup> As has been noted on several occasions within this review, bacterial resistance as a phenotype is the result of multiple compensatory adjustments, many of which are incremental. An example of the possible fragility of this accommodation (and as well the depth of our ignorance as to these compensatory adjustments) is the synergistic and compensatory relationship among the bacterial genetic background,  $\beta$ -lactamase expression, and the maintenance and expression of plasmid-carried *mecA* in  $\beta$ -lactam-resistant *S. aureus*, as discussed by Katayama et al.<sup>348</sup> This observation would immediately suggest the beneficial combination of a  $\beta$ -lactam targeting the PBPs with a  $\beta$ -lactam targeting the  $\beta$ -lactamase, which has been of course a mainstay of clinical therapy for some two decades. The conclusion that this combination therapy would prove successful is arguably, in retrospect, facile. Whether future antibacterial therapy of multidrug-resistant microorganisms will also require complex drug combinations (as is already the case for anti-HIV therapy) is not nearly as obvious. This is, nonetheless, a likely course of events. Fortunately, the experimental resources needed to accomplish this task are coming into place. Single-cell microscopy, for example, may

allow one to identify the role of the target in cell wall biosynthesis as the cell is seen to respond to drug exposure.<sup>62,349–352</sup> This response can be correlated to proteomic analysis.<sup>289,291,353</sup> The understanding of the structural basis for  $\beta$ -lactam induction of the bacterial SOS response<sup>354</sup> and for creation of persister bacteria populations<sup>290,355,356</sup> are but two examples of how future  $\beta$ -lactam SAR may be guided.<sup>357</sup> Screening to identify drug combinations that synergize with  $\beta$ -lactams (as is currently being done with efflux pump inhibitors), with respect to target pairings, can be done on a high-throughput scale. Old  $\beta$ -lactams, with proven safety and performance, can be given continued clinical relevance.<sup>358</sup> Nonetheless, the likelihood that future antibacterial chemotherapy will be a multidrug regimen is real.<sup>359</sup>

The task of identifying and then optimizing multidrug safety and efficacy is daunting. Drug discovery is already the zenith of the collaboration between human scientific and engineering ingenuity, and the emerging anticipation that this may now need to be done on a multifactorial scale is part of the reason that the economics for future antimicrobial drug discovery are so dismal. While these economics may change after the crisis (as only a crisis galvanizes consensus of opinion), the prudent and sobering reminder is that successful drug discovery is emphatically not instantaneous. Should our vigilance in antibacterial drug discovery falter, the length and depth of the crisis may be unlike anything modern man has experienced.

There remain, of course, the companion questions as to what should be done *now* to preserve  $\beta$ -lactam efficacy for future generations. There is no other antibacterial class that can substitute for  $\beta$ -lactam antibiotics in the foreseeable future, and none are in the pipeline.<sup>4,360</sup> These are questions that involve the entire breadth of clinical practice, including minimizing global environmental antibiotic exposure (the reduction of antibiotics in animal feeds is likely a step in the right direction), re-appreciating the value of hospital quarantine, and re-emphasizing the incredible importance of proper hygiene to minimizing infection. At the level of drug therapy, additional possibilities are emerging. We now appreciate that the gut is, to use the delectable phrasing of Courvalin and Davies,<sup>4</sup> a “veritable microbial bordello” with extensive capacity for genetic exchange.<sup>361,362</sup> Exploratory therapies that include  $\beta$ -lactamases (to destroy nonabsorbed  $\beta$ -lactams, either *in situ* or *post facto*) have shown promise.<sup>363–366</sup> The interrelationship of community- and hospital-resistant microorganism reservoirs now is recognized.<sup>367</sup> Judicious use of early generation  $\beta$ -lactam therapy can mitigate resistance development against later generation  $\beta$ -lactams.<sup>358,368–371</sup>

The central importance of the cell wall biosynthetic enzymes as antibacterial targets is irrefutably validated by the  $\beta$ -lactams themselves. Not only are these enzymes accessible and essential, but also these are enzymes with a demonstrated commonality for inhibitor (and substrate) recognition at their active sites. The proposal that the  $\beta$ -lactams constitute the *only* motif for inhibitor design is not merely unproven but largely untested.<sup>372</sup> Antibacterial screening is

only now transitioning from classical broth MIC identification to discrete enzyme (target) screening evaluation. As this is accomplished and as we more clearly understand the relative importance of (say) one specific PBP over another, opportunities for structure-based  $\beta$ -lactam design (a remarkably open frontier!)<sup>14,28,373–376</sup> and for new template identification will be created. The role and identity of each enzyme contributing to the assembly of the cell wall are only now emerging. As each falls into place, yet another discrete target for antibacterial discovery is acquired.

Nonetheless, the long-term future of the  $\beta$ -lactams is uncertain. While their widespread clinical use is certain to continue for the foreseeable future, as with all classes of anti-infective drugs continued efficacy is the difference between chemical innovation and clinical erosion by resistance development. The latter is not merely certain but is both irreversible and progressive. Also, while one should underestimate neither human resilience nor human innovation, the confluence of these attributes to accomplish drug discovery has always required the context of need and reward. While the imperfections of this system have long been evident (look no further than the obliviousness of major pharma to third-world disease), until now a demonstrable connection between anti-infective need and reward has existed. This is no longer true. Until society understands the difference between chemicals that are commodities and chemicals that are creations, the investment of human intellect in the  $\beta$ -lactams may soon extinguish. Sheehan's description of the  $\beta$ -lactams as "the enchanted rings" was a tribute to their intricacy, safety, and efficacy. There is no reason to believe that the burgeoning microbial resistance to their efficacy is anything other than opportunity for further enchantment, unless we choose otherwise.

## 6. Acknowledgments

The constructive comments of the referees are appreciated. This work was supported by Grant AI33170 from the National Institute of Allergy & Infectious Disease and by Grant GM61629 from the National Institute of General Medical Science.

## 7. Abbreviations

ESBL	extended spectrum $\beta$ -lactamase
IRT	inhibitor-resistant TEM (class A) $\beta$ -lactamase
MBL	metallo- $\beta$ -lactamase
MIC	minimal inhibitory concentration of an antibacterial
MRSA	methicillin-resistant <i>S. aureus</i>
PBP	penicillin-binding protein
rms	root-mean square

## 8. Note Added in Proof

Levy and Marshall<sup>377</sup> and Payne and Tomasz<sup>378</sup> offer perspectives on the phenomenon of bacterial resistance, whereas Poole<sup>379</sup> provides a complementary review of bacterial  $\beta$ -lactam resistance. Mal-lorqui-Fernandez et al.,<sup>380</sup> Walsh and Amyes,<sup>381</sup> and Gotz<sup>382</sup> review aspects of the molecular basis for MRSA/VRSA and possible new strategies for anti-

bacterial intervention. Two counterpoints on the structure of the murein polymer are presented.<sup>383,384</sup> Arbeloa et al. make the significant discovery that *S. aureus* PBP2a can confer  $\beta$ -lactam resistance to other Gram-positive bacteria, with the synthesis of mosaic peptidoglycan cross-bridges.<sup>385</sup> Gardete et al. provide further insight to the role of murE in *S. aureus* resistance by control of PBP2 and PBP2a expression.<sup>386</sup> The Class B (monofunctional) PBP2b transpeptidase from resistant *S. pneumoniae* has a T446A mutation that reduces penicillin affinity by 99%.<sup>387</sup> The crystal structures are disclosed of the R61 D,D-peptidase (inactivated with a peptidoglycan-mimetic penicillin),<sup>388</sup> of a truncated *S. pneumoniae* Class A cell division PBP1b enzyme (inactivated with nitro-cefin and cefotaxime),<sup>389</sup> and of the *S. pneumoniae* PBP3 peptidoglycan synthesis regulatory factor.<sup>390</sup> Labia reviews the structural evolution of the TEM and SHV Class A  $\beta$ -lactamases.<sup>391</sup> Computational modeling of the Class A  $\beta$ -lactamase acylation supports Glu166 as the general base activating Ser70.<sup>392</sup> An engineered cystine in the Toho-1 ESBL alters the active site, reducing activity toward third-generation cephalosporins.<sup>393</sup> The sequence requirements of the IMP-1 and FEZ-1 metallo- $\beta$ -lactamases,<sup>394,395</sup> evidence for direct  $\beta$ -lactam-metal contact,<sup>396</sup> and the crystal structure of the CphA carbapenemase metallo- $\beta$ -lactamase (complexed with biapenem)<sup>397</sup> are discussed. Two reports evaluate the structural basis for the high affinity  $\beta$ -lactamase-BLIP ( $\beta$ -lactamase inhibitory protein) protein-protein complex.<sup>398,399</sup> Freiberg et al. discuss the impact of transcriptome and proteome analysis on antibacterial drug discovery.<sup>400</sup> The inactivation mechanism of broad-spectrum methyldene penem  $\beta$ -lactamase inhibitors<sup>401</sup> is revealed by the structure of the inactivated  $\beta$ -lactamase.<sup>402</sup> A series of reviews update recent progress in  $\beta$ -lactam medicinal chemistry.<sup>403–409</sup>

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CR030102I