

Designing New β -Lactams: Implications from Their Targets, Resistance Factors and Synthesizing Enzymes

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Abstract: Penicillins and cephalosporins are β -lactam antibiotics widely used to treat bacterial infectious diseases. They mainly target the cell wall biosynthesis pathway to inhibit bacterial growth. The targets, known as penicillin-binding proteins, are enzymes involved in the polymerization of glycan chains, cross-linking them during bacterial cell wall formation. However, the dispensation of these antibiotics has been concomitant with increasing incidence of resistance to them. Reportedly, this is due to the evolvement of two resistance mechanisms in the bacterial pathogens. One is the production of β -lactamases that cleave the β -lactam rings of penicillin and cephalosporin antibiotics, rendering them ineffective against the pathogens. Another is the modification of the targets, resulting in their inability to bind β -lactam antibiotics. Nevertheless, β -lactam antibiotics remain clinically relevant due to their high target specificity in bacteria and low toxicity to humans. Thus, to overcome the continuing emergence of resistance in pathogens, more efficacious β -lactams have to be developed and cephalosporins are often preferred over penicillins due to two alkyl sites in the cephalosporin core structure amenable for modification. Transformed β -lactams are expected to have improved antimicrobial spectra and pharmacokinetics. This is illustrated by the development of two cephalosporins, namely ceftobiprole and ceftaroline, which have shown good antimicrobial activities and are currently undergoing clinical trials. This review will discuss computer-aided studies of three enzymes closely related to cephalosporins: (1) its synthesizing enzyme, deacetoxycephalosporin C synthase, (2) its targets, the penicillin-binding proteins, and (3) its degrading enzyme, the β -lactamases, and their implications in the development of new cephalosporins.

Keywords: β -lactam, penicillin, cephalosporin, penicillin-binding proteins, β -lactamases, deacetoxycephalosporin C synthase.

INTRODUCTION

β -Lactam antibiotics or β -lactams, particularly the penicillins and cephalosporins, have been very successful as chemotherapeutics because of their high target specificity and low toxicity to humans [1]. Indeed, β -lactams specifically target penicillin-binding proteins (PBPs) resulting in the inhibition of bacterial cell wall synthesis. Since PBPs are not found in mammalian cells, inhibitory effects by β -lactams are evaded [2]. To broaden the application of β -lactams as antibacterial antibiotics, early penicillin- and cephalosporin-derivatives were developed *via* empirical genetic improvement of producing strains or semi-synthetic chemical synthesis [3, 4]. However, due to the prevalent use of these antibiotics, in time, resistant bacteria have emerged, possibly *via* selection of spontaneous mutants able to survive in β -lactam-infused environment, leading to increasing nosocomial infections [5, 6]. Thereafter, the development of new β -lactams was skewed towards overcoming antibiotic resistance and has driven many intensive studies in understanding the resistance mechanism responsible for nullifying their effects. Studies on the resistant bacteria have revealed two important strategies adopted to overcome the action of β -lactam antibiotics: (1) modification of their penicillin-binding proteins, resulting in a low affinity and/or low acylation rate for the antibiotics,

and (2) degradation by β -lactamases, which cleave the β -lactam ring of the antibiotics rendering them incapable of inhibiting their targets [7-9].

The discovery and development of penicillin and cephalosporin antibiotics has a long history since 1930's. Unlike some of the current drugs that were designed and developed using computer-aided methods after the birth of bioinformatics in the 1970s [10-13], the earlier β -lactams were mainly generated by biological and chemical means to obtain structural variation and were painstakingly tested against a range of microorganisms. Since protein structure determination methods were not easily available until recent years [14], β -lactams were developed without the knowledge of the molecular mechanisms involved in achieving drug target inhibition and inactivation by β -lactamases. With the advancement in the development of protein structure determination techniques and bioinformatics, many tertiary structures of PBPs (Table 1) and β -lactamases (Table 2) have been resolved and deposited in the Protein Data Bank [15] (PDB) (<http://www.rcsb.org>). These have allowed in-depth studies of the mechanistic reactivity of this class of antibiotics using various experimental techniques, including bioinformatics, in the hope of providing insights to the design of more efficacious derivatives for combating highly resistant bacteria.

In this review, a brief history of penicillin and cephalosporin structural development will be described. Recent computer-aided studies on the cephalosporin synthesizing enzyme, the penicillin-binding proteins and the β -lactamases will also be discussed. However, it is not the

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Table 1. Tertiary Structures and Classes of Resolved Bacterial PBPs

Bacterial Species	Nomenclature	Gene	Class [18]	PDB Accession No.*	Ligand	References
<i>Escherichia coli</i>	PBP1b	<i>ponB</i>	A2	3FWL	Moenomycin	Sung <i>et al.</i> , 2009 [248]
				3FWM	Moenomycin	
	PBP4	<i>dacB</i>	C type-4	2EX2		Kishida <i>et al.</i> , 2006 [134]
				2EX6	Ampicillin	
				2EX8	Penicillin G	
				2EX9	Penicillin V	
				2EXA	Faropenem	
				2EXB	Flomox (cefapeme pivoxil hydrochloride)	
	PBP5	<i>dacA</i>	C type-5	3BEB	Mimetic peptide penicillin	Sauvage <i>et al.</i> , 2008 [115]
				3BEC	Mimetic peptide cephalosporin	
				1Z6F	Boronic acid inhibitor	Nicola <i>et al.</i> , 2005 [124]
				1SDN	Mercury (II) ion	Nicola <i>et al.</i> , 2005 [249]
				1NZU	β -Mercaptoethanol	
				1NZO	β -Mercaptoethanol	Nicholas <i>et al.</i> , 2003 [122]
				1NJ4		Davies <i>et al.</i> , 2001 [123]
				1HD8		
	PBP6	<i>dacC</i>	C type-5	3IT9	Sucrose, sulphate ion	Chen <i>et al.</i> , 2009 [111]
				3ITA	Ampicillin, sulphate ion	
				3ITB	Substrate analogue, Sucrose, sulphate ion	
<i>Haemophilus influenzae</i>	PBP4	<i>dacB</i>	C type-4	3A3D	Glycerol	Kawai <i>et al.</i> , 2009 [250]
				3A3E	CMV	
				3A3F	FMZ	
				3A3I	Ampicillin	
	PBP5	<i>dacA</i>	C type-5	3A3J	Sulphate ion	
<i>Neisseria gonorrhoeae</i>	PBP2	<i>ftsI</i>	B3	3EQU	Glycerol, sulphate ion	Powell <i>et al.</i> , 2009 [251]
				3EQV	Glycerol, sulphate ion	
<i>Streptococcus pneumoniae</i>	PBP1a	<i>pbpA</i>	A3	2ZC5	Biapenem, zinc ion	Yamada <i>et al.</i> , 2008 [252]
				2ZC6	Tebipenem, zinc ion	
				2V2F	2-(N-morpholino)-ethanesulfonic acid, barium ion	Job <i>et al.</i> , 2008 [253]
				2C5W	Cefotaxime, 1,2-ethanediol, chloride ion, zinc ion	Contreras-Martel <i>et al.</i> , 2006 [137]
				2C6W	Chloride ion, zinc ion	
	PBP1b	<i>pbp1b</i>	A5	2JCI	N-benzoyl-D-alanine, chloride ion, sulphate ion	Macheboeuf <i>et al.</i> , 2008 [254]
				2JCH	Phenoxyacetyl-lactivicin, 1,2-ethanediol, chloride ion, sulphate ion	Macheboeuf <i>et al.</i> , 2007 [255]
				2JE5	Lactivicin, chloride ion, sulphate ion	
				2FFF	Nickel ion	Lovering <i>et al.</i> , 2006 [256]
				2UWY	Cefotaxime, sulphate ion	Macheboeuf <i>et al.</i> , 2005 [257]
				2UWX	1,2-ethanediol, chloride ion, sulphate ion	
				2BG1	chloride ion, sulphate ion	
	PBP2b	<i>pbp2b</i>	B5	2WAD	Zinc ion	Contreras-Martel <i>et al.</i> , 2009 [114]
				2WAE	Zinc ion	
				2WAF	Chloride ion	

(Table 1) contd.....

Bacterial Species	Nomenclature	Gene	Class [18]	PDB Accession No.*	Ligand	References
<i>Streptococcus pneumoniae</i>	PBP2x	pvpX	B4	2ZC3	Biapenem, sulphate ion	Yamada <i>et al.</i> , 2008 [252]
				2ZC4	Tebipenem, sulphate ion	
				2Z2L	Sulphate ion	Yamada <i>et al.</i> , 2007 [258]
				2Z2M	Cefditoren, sulphate ion	
				1RP5	Sulphate ion	Pernot <i>et al.</i> , 2004 [259]
				1PYY	(4S)-2-methyl-2,4-pentanediol, N-octanoyl sucrose, sulphate ion	Chesnel <i>et al.</i> , 2003 [260]
				1K25		Dessen <i>et al.</i> , 2001 [139]
				1QME	Sulphate ion	Gordon <i>et al.</i> , 2000 [119]
				1QMF	Cefuroxime	
				1PMD		Pares <i>et al.</i> , 1996 [138]
	PBP3	pbp3	C type-5	1XP4	Iodide ion, sulphate ion	Morlot <i>et al.</i> , 2005 [120]
<i>Staphylococcus aureus</i>	PBP2	pbp2	A3	2OLU	1,2-ethanediol	Lovering <i>et al.</i> , 2007 [261]
				2OLV	Moenomycin	
	PBP2a	mecA	B1	1VQQ	Chloride ion, cadmium ion	Lim & Strynadka, 2002 [131]
				1MWR	Chloride ion, cadmium ion	
				1MWS	Chloride ion, cadmium ion	
				1MWT	Chloride ion, cadmium ion	
				1MWU	Chloride ion, cadmium ion	
	PBP4	pbp4	C type-5	3HUM	Cefotaxime	Navratna <i>et al.</i> , 2009 [147]
				3HUN	Ampicillin	
				1TVF	Unknown ligand, sulphate ion	Rajashankar <i>et al.</i> , 2006 [unpublished]
<i>Bacillus subtilis</i>	PBP4a	dacC	C type-4	2J9P	Peptidoglycan mimetic peptide, D-alanine	Sauvage <i>et al.</i> , 2007 [125]
				1W5D	Calcium ion	

*http://www.rcsb.org

intention of this article to critically review the individual topics as they have been done so separately and extensively elsewhere [1, 16-22]. Nonetheless, updates and discussion on these topics are still necessary to understand their implications in the development of new β -lactam antibiotics, particularly the cephalosporins.

HISTORICAL DEVELOPMENT OF PENICILLIN AND CEPHALOSPORIN DERIVATIVES

Since the discovery of penicillin in *Penicillium* species by Alexander Fleming in 1928, many structurally related natural antibiotics e.g. cephalosporins, carbapenems, monobactams, have been discovered, studied and modified [1]. These structurally related antibiotics are collectively known as β -lactams because of the presence of a β -lactam ring in all these biomolecules. The first known property of penicillin was the inhibition of bacterial growth, which was then found to specifically target the bacterial cell wall synthesizing enzymes or PBPs. However, the structural relationship between penicillin and cephalosporin was not known initially until the chemical structure of cephalosporin C, produced by another fungus *Acremonium chrysogenum*,

was resolved [23, 24]. Subsequently, bacteria e.g. streptomycetes, were also found to produce β -lactams i.e. cephamycin C and cephabacins, and these initiated much interest in deciphering the β -lactam biosynthetic pathways and their regulation in these microorganisms [1, 16, 25].

β -Lactam Biosynthetic Pathway

Penicillin and cephalosporin are structurally related and their differences lie in the additional cyclic structure fused to the β -lactam ring i.e. penicillin has a thiazolidine ring and cephalosporin has a dihydrothiazine ring. Studies on penicillin- and cephalosporin- producing organisms have revealed a common biosynthetic pathway [25-28] (Fig. 1). Biosynthesis of both penicillin and cephalosporin begins with the fusion of three amino acids i.e. L-aminoadipic acid, L-cysteine and L-valine, into a tripeptide known as L-aminoadipyl-L-cysteinyl-D-valine (ACV), by ACV synthetase. ACV is cyclized to isopenicillin N by isopenicillin N synthase (IPNS). This is the formation of the first bioactive compound in the biosynthetic pathway. Hence, IPNS was proposed to be an important enzyme for industrial penicillin production and studies of its catalytic

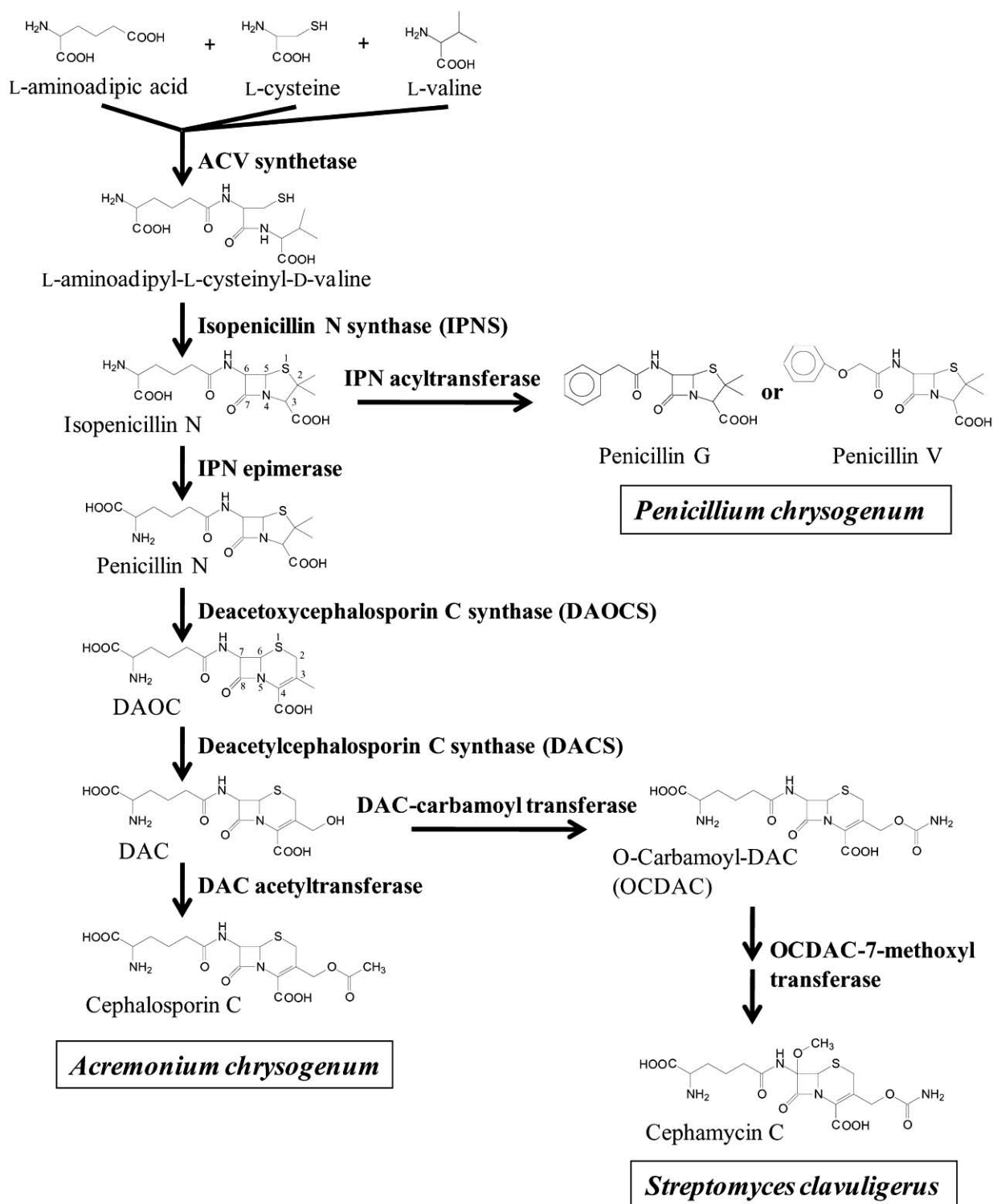


Fig. (1). β -Lactam biosynthetic pathways of *Penicillium chrysogenum*, *Acremonium chrysogenum* and *Streptomyces clavuligerus*. The β -lactam biosynthetic pathways in these microorganisms share the same initial steps, i.e., fusion of three amino acids and cyclization into isopenicillin N. However, the end-product in *P. chrysogenum* is either a penicillin G or V, depending on the type of substrate present in the fermentation medium. In *A. chrysogenum* and *S. clavuligerus*, the end-products are cephalosporin C and cephamycin C, respectively.

mechanism were actively pursued [29-31]. However, studies on this enzyme were not easy due to the need to synthesize the native substrate ACV, which is unstable and hygroscopic. Nevertheless, the crystal structure of *Aspergillus nidulans* IPNS (aIPNS) complexed with ACV

was resolved in the late 1990s [32, 33] and its catalytic mechanism has been probed with different ACV analogues [34-37]. In some of these studies, new β -lactam analogues were produced but their antimicrobial activities were not studied.

In penicillin-producing organisms e.g. *Penicillium chrysogenum*, the end-product of its β -lactam biosynthetic pathway can be altered by feeding phenylacetic acid or phenoxyacetic acid to the fermentation culture to yield penicillin G or penicillin V, respectively, which are the first clinically useful penicillins. This is achieved by isopenicillin N acyltransferase, which exchange the aminoacyl side chain of isopenicillin N to the respective feeding substrate [38] (Fig. 1).

In cephalosporin-producing organisms e.g. *A. chrysogenum*, penicillin N is ring-expanded to deacetoxycephalosporin C (DAOC) by DAOCS synthase (DAOCS), where the five-membered thiazolidine ring in penicillin N is converted to the six-membered dihydrothiazine ring in DAOC [39] (Fig. 1). This is a necessary step in the biosynthesis of cephalosporin. Hence, DAOCS was proposed to be an important enzyme for industrial production of cephalosporin [30, 31, 40]. Intensive biochemical and structural studies on DAOCS revealed similar catalytic mechanism to IPNS. When its crystal structure was first resolved, it was found to resemble that of IPNS with a similar structural fold [41, 42]. The identical spatial orientation of the catalytic HXDX_nH motifs, in IPNS and DAOCS, for the coordination of a co-factor iron (II) ion, and the RXS motifs for substrate and co-substrate binding, affirmed the earlier observation of the importance of these conserved amino acid residues for the enzymes' activities [41, 43].

Subsequently, with the aid of resolved crystal structure coordinates and bioinformatics, the catalytic mechanism and substrate specificity of DAOCS were figured out to facilitate the enzymatic conversion of penicillin G to a cephalosporin with improved efficiency. Since penicillin G can be easily extracted from fermentation cultures of *Penicillium*, it became an industrially-preferred substrate for the production of a cephalosporin precursor, 7-aminodeacetoxycephalosporanic acid (7-ADCA) [44-46]. However, the affinity of DAOCS for penicillin G is very low compared to penicillin N [47]. Thus, many studies have been attempted with the aim of improving the catalytic efficiency of DAOCS for penicillin G conversion [17]. Simultaneously, achieving an optimal enzyme assay condition for detecting ring-expansion activity of DAOCS was rigorously pursued over two decades. It was found that the order of adding the reagents is important [48] and some of the reagents could be omitted without affecting the overall enzyme reaction [49, 50]. Eventually, the optimized enzyme reaction condition also allowed a range of hydrophobic penicillins to be converted by DAOCS to their respective cephalosporin moieties [17, 49, 50].

The initial resolution of *Streptomyces clavuligerus* DAOCS (scDAOCS) crystal structure was without a coordinated penicillin substrate [41]. This made it difficult to identify the substrate binding pocket of scDAOCS. As DAOCS has a similar mode of catalysis and structural fold as IPNS, the superimposition of the crystal structure of scDAOCS onto that of aIPNS, using a structural visualization program, SwissPdb viewer, allowed identification of a putative substrate binding site of scDAOCS [42]. This prompted rational studies of the importance of its C-terminal tail in influencing substrate

recognition. It was shown that single mutations of amino acid residues N304, I305, R306 and R307 to different amino acids improved the conversion activity of scDAOCS to various extents [17, 47, 51, 52]. Indeed, single mutations of these four residues to leucine separately demonstrated improvements in the penicillin G conversion activity of scDAOCS by approximately 120% to 200%, compared to the wild-type enzyme under similar enzyme reaction conditions [47, 52]. Furthermore, N304L, R306L and R307L mutations also widened the substrate specificity of scDAOCS to include precursors with different side chains i.e. ampicillin, amoxicillin, carbenicillin and phenethicillin [17, 52, 53]. These improvements were initially attributed to better hydrophobic interaction of the substituted leucine with the hydrophobic penicillin substrates. The resolution of the crystal structure of scDAOCS complexed with penicillin G and ampicillin [54] revealed that only residues N304 and I305 were in direct contact with the penicillin substrates, in that, the side chain of residue N304 aligns with that of the penicillin substrates, and that of residue I305 protrudes towards the β -lactam ring. By assessing the effects of all possible amino acid replacements at the residue N304 [55], it became obvious that hydrophobic interaction at this position plays an important role in stabilizing the substrate in the active site of scDAOCS. This approach was also exploited to understand the role of residue R306 in the activity of scDAOCS. The results suggested that better hydrophobic interaction is possible with a hydrophobic cleft formed by residues Y184, L186, W297, I298, and V303 upon replacing arginine with either leucine, isoleucine or methionine [56].

Directed evolution studies of scDAOCS have also identified potential amino acid residues amenable to manipulation to improve the overall catalytic efficiency of the enzyme in converting penicillin G to a cephalosporin [51, 57]. These were at positions, T91, A106, C155, V275, C281 and L277, and are not in the substrate binding centre, thus highlighting the superficial understanding of an enzyme's architectural influence on its activity. Nonetheless, visual inspection of the spatial orientation of these residues allowed some hypotheses to be made and tested. For example, C281Y mutation conferred approximately 2-fold increment in the penicillin G conversion activity [47]. It was postulated that the *para*-hydroxyl group in the phenyl side chain of tyrosine would form a hydrogen bond with the backbone oxygen of residue V238 and might stabilize the polypeptide chain leading to residue H243, which is critical for iron (II) coordination [47, 58]. Since phenylalanine is structurally similar to tyrosine except that it lacks the hydroxyl group, C281F mutation was done to validate this hypothesis. Interestingly, a similar level of activity was obtained for C281F mutant, highlighting the importance of the phenyl side chain in enhancing the conversion activity of scDAOCS [58].

Although the potential of bioengineered DAOCS for industrial application and biosynthesis of novel cephalosporins has yet to be fully realized, the plasticity of its substrate binding pocket and enhanced prowess upon mutations support this possibility. As demonstrated by various studies, other than producing the cephalosporin precursor 7-ADCA from penicillin G, another potential industrial application of bioengineered DAOCS is the conversion of ampicillin and amoxicillin to their respective

useful cephalosporin moieties i.e. cephalexin and cefadroxil, respectively, using an enzymatic approach, which is probably cleaner and less hazardous [59].

Development of Penicillins

When penicillin G was first purified from a *Penicillium* fermentation culture and used as a chemotherapeutic, it was only effective in treating diseases caused by Gram-positive bacteria, and its instability to acid prevented its use *via* oral administration [60]. Conversely, penicillin V, which has a phenoxyacetyl side chain compared to penicillin G, was found to be acid-stable and could be administered orally but its narrow antimicrobial spectrum, which is similar to penicillin G, limited its clinical use [60]. Following the clinical use of penicillin G, the emergence of penicillin-resistant staphylococci became a serious clinical problem [61]. The implicated resistance factor was extracted from the pathogen and found to be an inactivator of penicillin, termed as penicillinase by Kirby in 1944 [62]. Thereafter, many studies followed with the intention of improving the antimicrobial spectrum of penicillin to include Gram-negative bacteria as well as penicillin-resistant staphylococci.

Recognizing the importance of the C6 side chain of penicillin for its antibacterial property, successes in the development of more efficacious penicillins were brought about by the discovery of 6-APA production in the fermentation culture of *P. chrysogenum*, and its potential as a precursor for the development of semi-synthetic penicillins [63] (Fig. 2). The semi-synthetic penicillins are mainly classified by structural similarity which also reflects their antimicrobial spectra. Methicillin is the first semi-synthetic penicillin that has an *ortho*-dimethoxyphenyl group as a side chain and was resistant to the action of staphylococcal penicillinase [60, 64]. It was clinically useful in inhibiting penicillin-resistant *S. aureus* in the early 1960s but was eventually replaced by other penicillinase-resistant penicillins e.g. cloxacillin, that have higher bioactivity against staphylococci [60]. However, strains of *S. aureus* that were resistant to methicillin and other penicillinase-resistant penicillins eventually emerged and till now, still pose a problem to the healthcare industries [65]. They are known as methicillin-resistant *S. aureus* (MRSA) and the resistance mechanism they possessed was due to the acquisition of a PBP with low affinity to β -lactam antibiotics known as PBP2a encoded by *mecA* gene [66-69].

Ampicillin is the first semi-synthetic penicillin that has a broader antimicrobial spectrum than penicillin G and its spectrum included Gram-negative bacteria [64]. It has a phenylglycyl side chain, which structurally differ from penicillin G by an amino group in the α -carbon of the side chain. Its poor absorption property in the guts led to the development of amoxicillin, which has a *para*-hydroxyl group in the phenyl side chain. This allowed better absorption from the gut and resulted in higher levels in blood than equivalent doses of ampicillin [60]. However, resistance to amoxicillin due to β -lactamases became wide-spread in the 1970s and the timely discovery of clavulanic acid as a β -lactamase inhibitor led to the combinatorial administration of amoxicillin and clavulanic acid [60, 70]. In the case of *P. aeruginosa*, which is one of the leading causes of

nosocomial infections, especially in immunocompromised and cystic fibrosis patients, typical broad spectrum antimicrobials i.e. ampicillin and amoxicillin were rendered useless [71, 72]. Instead, carbenicillin, a carboxypenicillin which has a carboxyl group instead of an amino group in the side chain of ampicillin, was found to have improved activity against *P. aeruginosa*. Moving forward, ticarcillin was found to possess even better anti-pseudomonal activity than carbenicillin by replacing the phenyl ring with a thiophenyl ring [64]. Subsequently, the addition of urea moieties to the α -amino group in the side chain of ampicillin yielded ureidopenicillins (azlocillin, piperacillin and mezlocillin), with even higher efficacies against *P. aeruginosa*, while retaining inhibitory activity against streptococci, enterococci and non- β -lactamase-producing staphylococci [60, 73, 74]. Eventually, the development of cephalosporin derivatives superseded that of the penicillins probably due to the amenability of the cephalosporin nucleus for multiple chemical modifications.

Development of Cephalosporins

The discovery of cephalosporin C led to the development of a series of cephalosporin derivatives. The synthesis of these derivatives was possible due to the production of the cephalosporin core structure, 7-aminocephalosporanic acid (7-ACA), from cephalosporin C by deacylation of its C7 side chain. 7-ACA was used as a precursor for the synthesis of many earlier semi-synthetic cephalosporins, a strategy similar to the development of semi-synthetic penicillins [75]. Other than having similar chemical structure and mode of action as penicillins, cephalosporins were found to be naturally resistant to penicillinase and hence, became more desirable in the development of new β -lactam antibiotics [76]. Furthermore, cephalosporins have two alkyl sites amenable to chemical modification compared to penicillins with only one (Fig. 3). This allows a larger variety of cephalosporin derivatives to be synthesized and assessed for suitable antimicrobial properties for clinical applications. Subsequently, a plethora of β -lactam derivatives were developed and marketed, with an estimation of at least 50 different cephalosporins available in the market by 2003 [75, 77, 78].

The classification of cephalosporin derivatives is based on their antimicrobial properties, which is considered more useful for clinicians, and they are thus grouped into generations (Fig. 3). The first generation cephalosporins are made up of cephalosporins that have similar antimicrobial spectra similar to cephalexin. Cephalexin is one of the earlier semi-synthetic cephalosporin available and has the same side chain as ampicillin. Its antimicrobial spectrum encompasses that of ampicillin except that its resistance to penicillinase bestowed itself a wider spectrum than ampicillin [76]. The next two generations of cephalosporins have a wider spectrum against many Gram-negative bacteria and are more resistant to the evolving β -lactamases, but with decreasing inhibitory activity against Gram-positive bacteria. In contrast to the first three generations, the fourth-generation cephalosporins have a broader antimicrobial spectrum including those of the first generation cephalosporins and many Gram-negative bacteria, including pathogenic *P. aeruginosa*. They are also generally more resistant to the

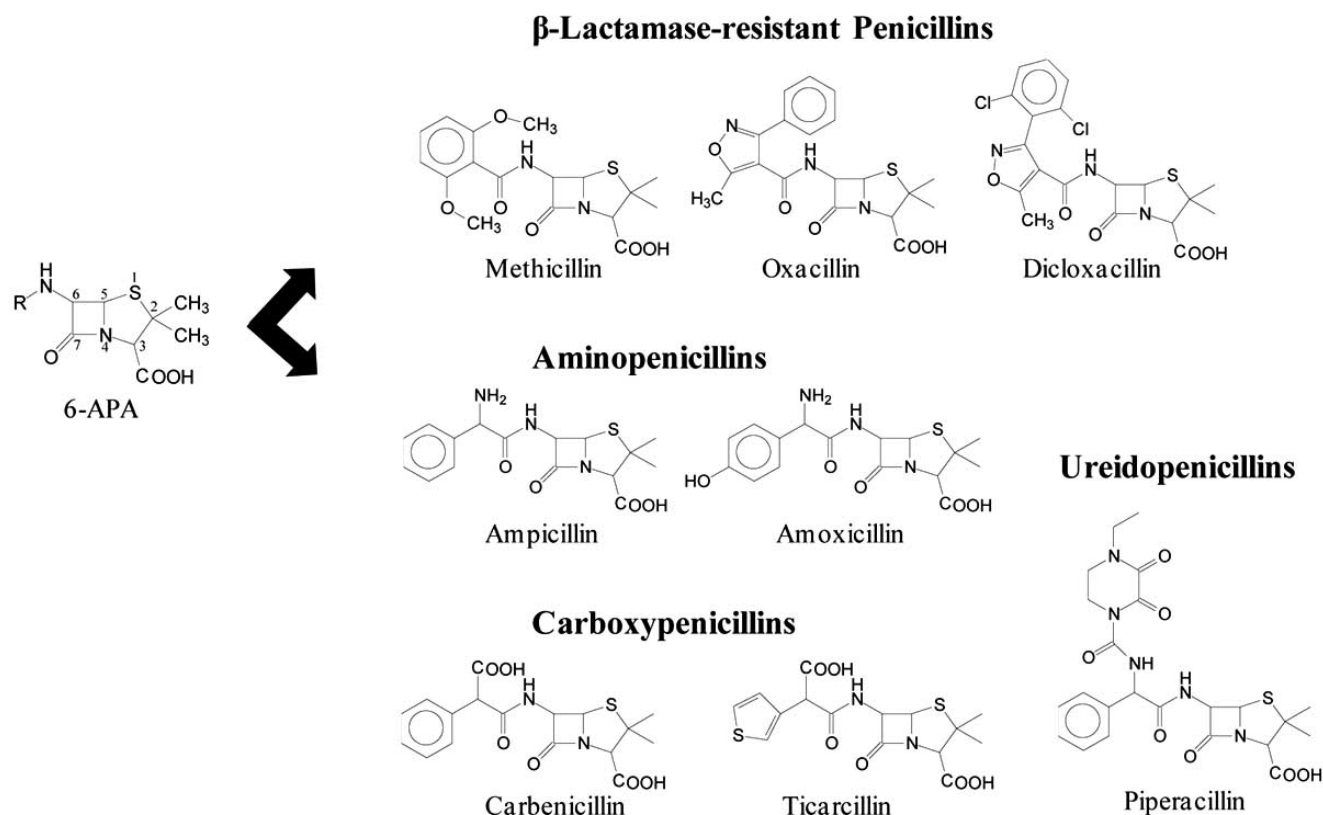


Fig. (2). Development of semi-synthetic penicillins using 6-aminopenicillanic acid (6-APA). The penicillin derivatives differ in their C6 (R) side chains.

evolved β -lactamases active against earlier generations of cephalosporins [79]. Selected examples of semi-synthetic cephalosporins from each generation are illustrated in Fig. (3). More examples of penicillins and cephalosporins, their therapeutic categories, structural development and relationship are available at the KEGG DRUG database [80] (<http://www.genome.jp/kegg/drug/>).

Historically, as soon as a new penicillin or cephalosporin antibiotic became available for clinical use, resistant bacteria would subsequently emerge. This has dampened the drive for many pharmaceutical companies to develop better antibiotics [3, 81]. However, recent efforts in modifying cephalosporin have continued to shed some hope in the fight against β -lactam-resistant bacteria (Fig. 4). Two cephalosporins, namely ceftobiprole [66, 82] and ceftaroline [66, 83], were developed and showed to be efficacious against MRSA. Ceftobiprole has also been shown to have activity against many Gram-negative bacteria *in vitro* including *P. aeruginosa* [84, 85]. Other cephalosporin derivatives with structurally different C3 side chains from ceftobiprole and ceftaroline were also synthesized and demonstrated to be effective against MRSA and vancomycin-resistant *S. aureus* [86]. By modifying the C3 side chain of ceftazidime, a new cephalosporin CXA-101 has been demonstrated to resist the action of the *P. aeruginosa* AmpC β -lactamase [87, 88]. Recently, it was tested to be effective against various β -lactam-resistant strains of *P. aeruginosa* [89-91]. In order to further understand the influence of the structural differences of these new cephalosporins in contributing to their improved efficacies, it

is necessary to next deliberate on the current knowledge of their drug targets and emergent resistance mechanisms.

TARGETS OF β -LACTAMS: PENICILLIN-BINDING PROTEINS

PBPs are a group of membrane-bound enzymes involved in bacterial cell wall synthesis. They are named as such due to their ability to bind penicillin although they play different roles in the cell wall synthesis process. Collectively, they are involved in the formation of peptidoglycans, which are important components of the bacterial cell wall in determining the final shape of the host bacteria. Peptidoglycan is made up of glycan chains, where each chain is composed of alternating *N*-acetylglucosamine and *N*-acetylmuramic acid, cross-linked by oligopeptides attached to the *N*-acetylmuramic acid [18].

PBPs are classified into three main classes, A, B and C, and are further sub-divided in each class based on sequence differences [18]. Class A and B PBPs, which are also known as high molecular mass PBPs, are multi-modular enzymes responsible for peptidoglycan formation and insertion into pre-existing cell wall. Generally, class A PBPs are bifunctional enzymes that possess an N-terminal glycosyltransferase domain, which catalyses the polymerization of glycan chains, and a C-terminal penicillin-binding domain, which possesses a transpeptidase activity responsible for peptide cross-linking between two glycan chains. The main function of class A PBPs is the synthesis of peptidoglycan. However, in a bacterium with more than one

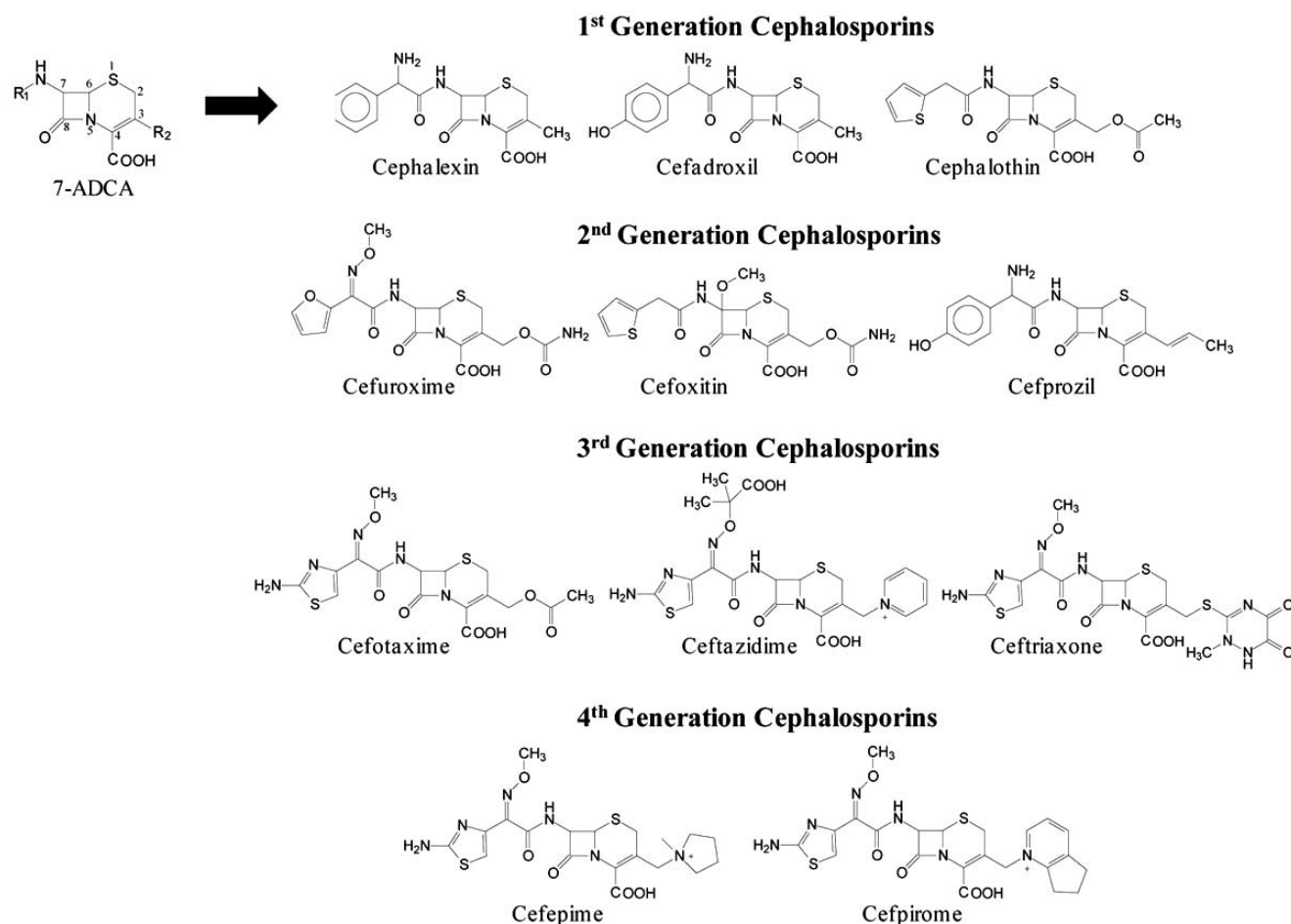


Fig. (3). Development of semi-synthetic cephalosporins using cephalosporin precursor 7-aminodeacetoxycephalosporanic acid (7-ADCA) or 7-aminocephalosporanic acid (7-ACA), which is derived from cephalosporin C and possesses an acetoxy group in its C3 position. The cephalosporin derivatives differ in their C3 (R₂) and C7 (R₁) side chains.

class A PBP, the exact role and significance of each class A PBP remains poorly understood. In *Bacillus subtilis*, mutation and deletion studies of the four class A PBPs, namely PBP1, PBP2c, PBP2d and PBP4, illustrated functional redundancy of these enzymes [92] but mutation of PBP1 alone increased its average cell length and decreased its cell diameter [93, 94]. Interestingly, with all the class A PBPs deleted, *B. subtilis* was still viable and has a relatively normal peptidoglycan structure [95]. In contrast, loss of both PBP1a and PBP1b in *Escherichia coli* is fatal even though it still has a third class A PBP i.e. PBP1c [96].

Class B PBPs also have a C-terminal penicillin-binding domain with transpeptidase activity but the functions of their N-terminal domains are still unclear although they are believed to be involved in cell morphogenesis by interacting with other proteins in the cell cycle [18, 97]. The function of class B PBPs in a bacterium is mainly cross-linking of the peptidoglycan chains via the stem pentapeptide covalently attached to *N*-acetylmuramyl portion of the glycan chain but the exact role of each class B PBP remains vague due to redundancy. Nevertheless, some studies have provided better understanding of class B PBPs in cell division. For example, in *E. coli* which possesses two class B PBPs, PBP2 has been shown to be important for cell elongation and may have a

role in the initiation of cell division [98, 99], whereas PBP3 is involved in septation [100-102]. In *B. subtilis*, there are six class B PBPs, and both PBP2a and PBP4 have been shown to play redundant roles in defining its shape [103, 104].

Class C PBPs are also known as low molecular mass PBPs, containing a penicillin-binding domain with either a DD-endopeptidase or DD-carboxypeptidase activity [93]. In general, class C PBPs are involved in the regulation of peptidoglycan formation [97, 105]. For example, PBP4 and PBP7 of *E. coli* are DD-endopeptidases that cleave the peptide cross-bridges between two glycan chains in the peptidoglycan cell wall [18, 106, 107]. *E. coli* PBP5 is a DD-carboxypeptidase involved in cleaving the terminal amino acid residue of the stem pentapeptide in a peptidoglycan chain, preventing peptidoglycan cross-linking [18, 108].

Most bacterial species possess at least one PBP from each class and they are usually named using numbers, followed by an alphabet for sub-classes, if any. However, PBPs from two different bacterial species with the same numbering may not belong to the same class because they were initially numbered based on their migration patterns in a sodium dodecyl sulphate-polyacrylamide gel electrophoresis [18]. For example, *Staphylococcus aureus* PBP2 encoded by *pbp2* is a class A PBP while *E. coli* PBP2

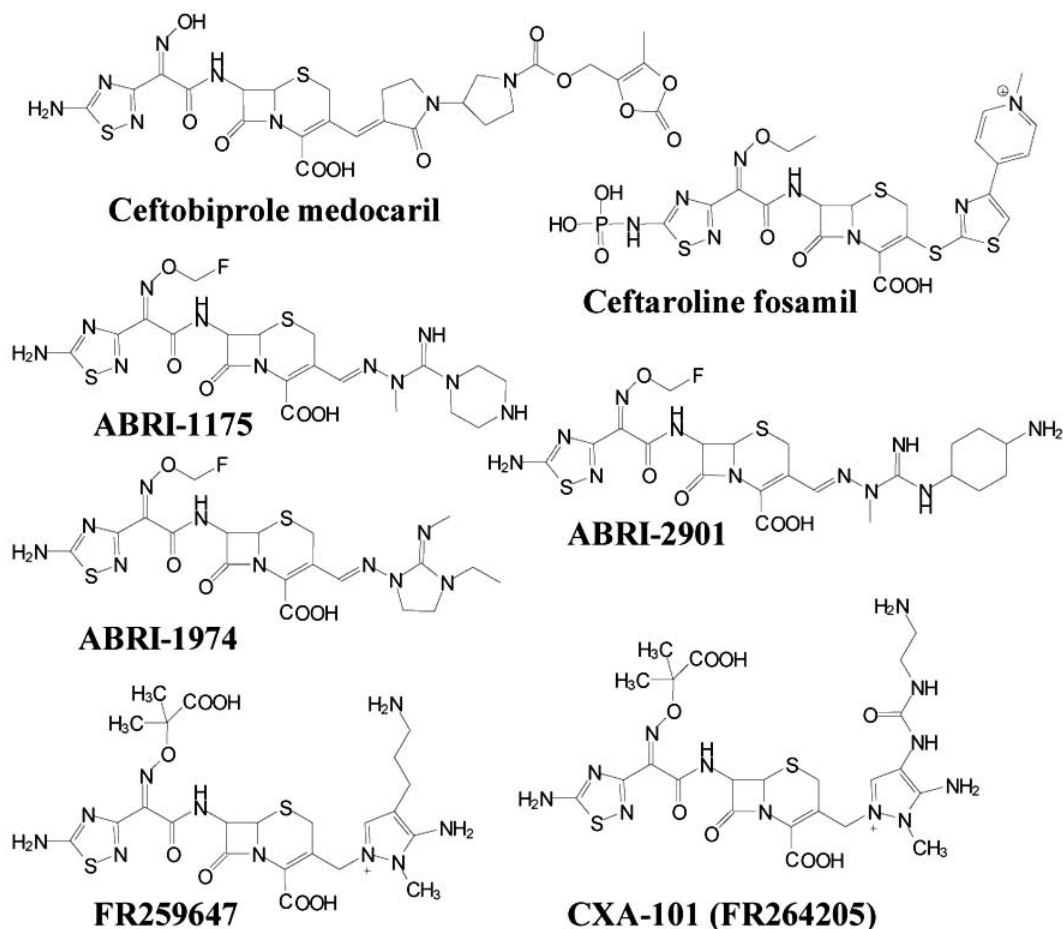


Fig. (4). Newly developed cephalosporins. Ceftobiprole medocartil, ceftaroline fosamil, ABRI-1175, ABRI-2901 and ABRI-1974 are anti-MRSA cephalosporins. FR259647 and CXA-101 have been shown to be effective against multi-drug resistant *P. aeruginosa* [87, 88].

encoded by *pbpA* is a class B PBP. Hence, this can be confusing and a standardized nomenclature will be useful, especially for phylogenetic studies. Interestingly, it has been noted that rod-shaped bacteria tend to have a higher number of PBPs compared to the cocci bacteria and bacteria of similar shapes tend to have similar sets of PBPs [109]. As such, efforts in understanding the role of each set of PBPs in different bacteria and their phylogenies may help in designing new drugs that are efficacious against multiple PBPs in closely-related bacteria, thus achieving more effective treatment.

Inhibitory Action of β -Lactam Antibiotics and Penicillin-Binding Domains of PBPs

Penicillin and cephalosporin antibiotics are able to inhibit the activity of PBPs because of their structural similarity to the terminal D-Ala-D-Ala stem peptide of peptidoglycan precursor and they bind to the penicillin-binding domain of PBPs [110]. This subsequently leads to the deregulation of bacterial cell wall synthesis, followed by activation of cell lysis. This hypothesis is strengthened by the recent crystallization of *E. coli* PBP6 with a peptidoglycan substrate fragment containing the full pentapeptide, *N*-acetylmuramyl-(L-Ala-D-isoGlu-L-Lys-D-Ala-D-Ala) [111]. Superimposition of this structure and a *Streptomyces* strain R61 DD-peptidase complexed with a non-covalently bound

β -lactam (1PW1) revealed a similar spatial orientation of the C3 carboxylate of the β -lactam with that of the terminal D-Ala of the peptidoglycan substrate fragment, which forms hydrogen bonds with residues S106 and T210 of *E. coli* PBP6. This study provided empirical evidence for the theory of molecular mimicry of β -lactam antibiotics to the terminal D-Ala-D-Ala moiety of the peptidoglycan in inhibiting bacterial PBPs [111].

The inhibitory action of β -lactam antibiotics occurs in three steps and this can be described using the kinetic model depicted in Fig. (5) [112]. The formation of a non-covalent complex between a PBP and a β -lactam is described by the dissociation constant K_d , followed by the formation of the acylated PBP at the rate k_2 . Subsequently, deacylation occurs at the rate k_3 to yield the PBP and an inactivated β -lactam. Often, k_3 is slower than k_2 and this leads to inhibition of the PBP by the β -lactam since the active site of the PBP is occupied.

The penicillin-binding domain of PBPs bears the three conserved motifs i.e. SXXX, SXN and K(S/T)G, present in the penicilloyl serine transferase superfamily, which includes class A, C and D β -lactamases [97, 113]. Although the primary sequences of the penicillin-binding domain of PBPs are very diverse, the structural folds of these domains are much conserved (Fig. 6) [18]. The penicillin-binding domain is made up of two sub-domains i.e. one is an anti-parallel

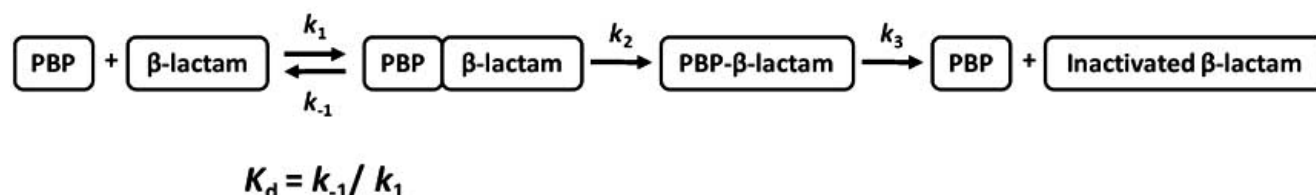


Fig. (5). A reaction scheme for the kinetic interaction of a PBP with a β -lactam [112]. The dissociation constant for the reversible formation of the non-covalent PBP- β -lactam complex is described by K_d . The acylation rate is described by k_2 and the deacylation rate is described by k_3 .

five-stranded β -sheets surrounded by three α -helices and another is an all-helical domain [18]. The SXXK motif bears the active site serine residue responsible for catalysis and is positioned at the beginning of helix α_2 . The SXN motif is situated in a loop between helices α_4 and α_5 , and the K(S/T)G motif is localized in β_3 (Fig. 8A) [18].

Many structural and molecular dynamic simulation studies have been attempted to understand the catalytic mechanism of PBPs upon binding of substrate analogues or β -lactam antibiotics [114-118]. The activation of the active site serine by abstraction of a proton is achieved by either the lysine residue of the SXXK motif or the carboxylate group of the β -lactam [119]. Recent studies on *E. coli* PBP5, which is a class C PBP, suggested that the former hypothesis is more likely [116]. The reaction begins with the binding of penicillin to the penicillin-binding domain followed by the nucleophilic attack of the deprotonated active site serine on the β -lactam ring. This results in the formation of a stable acyl-enzyme complex. In contrast to class A TEM-1 β -lactamase, PBPs generally lack the equivalent residue to the conserved residue E166 in TEM-1 responsible for deacylation of β -lactams. This provides an explanation for the inability or extremely slow deacylation rate of PBPs in hydrolyzing β -lactams [120]. This is further supported by approximately 10-100 fold increased in the deacylation rate of penicillin G when residue F450 of *Streptococcus pneumoniae* PBP2x was mutated to either an aspartic acid or a glutamic acid to mimic the corresponding position of residue E166 in TEM-1 β -lactamase [121].

Retrospectively, this is inadequate to explain the deacylation of peptidoglycan stem peptide by class C PBPs,

which is a native reaction in these enzymes (usually DD-carboxypeptidase or DD-endopeptidase activity). Structural studies using a deacylation-defective G105D mutant of *E. coli* PBP5 suggested the involvement of the SXN motif in deacylation activity of PBPs for its native substrate [122, 123]. Further studies using a substrate analogue inhibitor covalently bound to the active site of *E. coli* PBP5 suggested a hydrogen-bonding network involving residues S110 of the SXN motif, K213 of the KTG motif, and a bridging water molecule to activate the hydrolytic water molecule as a mechanism of deacylation of native substrate by class C PBPs [124]. Since some class C PBPs are strictly carboxypeptidases, while others may possess endopeptidase activities, there remains the question of what determines their functional differences. This is even more puzzling for transpeptidase, which links two stem peptides together. This could be inferred by the substrate preference of carboxypeptidase for linear stem peptide in peptidoglycan, that of endopeptidase for interlinked stem peptide between two glycan chains of the peptidoglycan, and that of transpeptidase for two stem peptides to be interlinked. Hence, it is likely that the active site of carboxypeptidases could not accommodate a bulky structure due to steric hindrance [125]. Structural studies on *B. subtilis* PBP4a, which is a class C PBP, led Sauvage and co-workers (2007) to propose the plausible role of residue T394 in determining its activity. This residue is hydrogen-bonded to residue T412 and indirectly contributes to the hydrogen-bond network that strengthens the active site. Interestingly, in DD-carboxypeptidases e.g. *E. coli* PBP5, PBP6 and PBP6b, a bulkier arginine residue is located at the analogous position of residue T394 in *B. subtilis* PBP4a, whereas in DD-

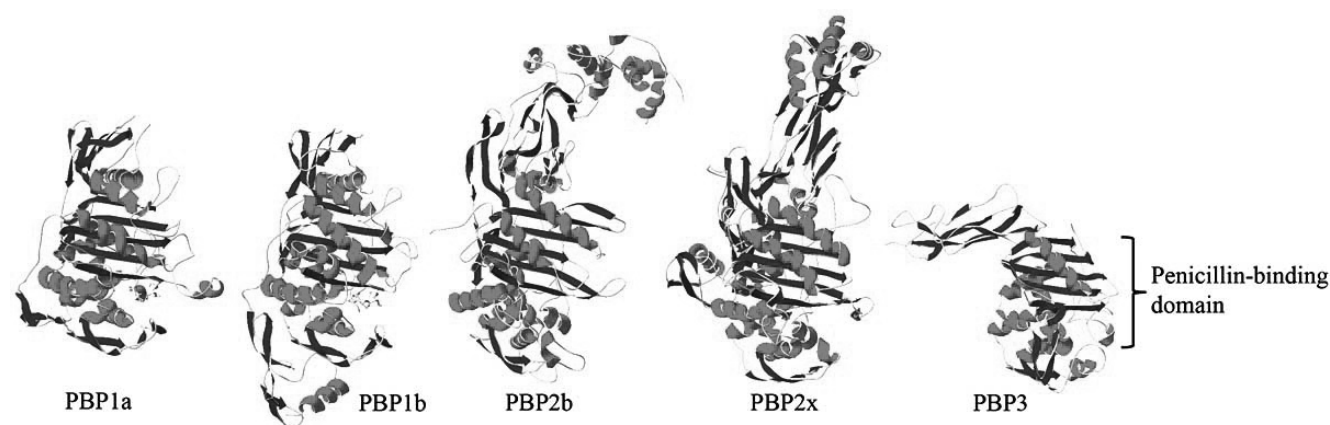


Fig. (6). Crystal structures of *S. pneumoniae* penicillin-binding proteins (PBPs), depicting the common penicillin-binding domain in PBPs of class A (PBP1a and PBP1b), B (PBP2b and PBP2x) and C (PBP3). (PDB accession numbers: PBP1a – 2C5W; PBP1b – 2UWY; PBP2b – 2WAF; PBP2x – 1RP5; PBP3 – 1XP4).

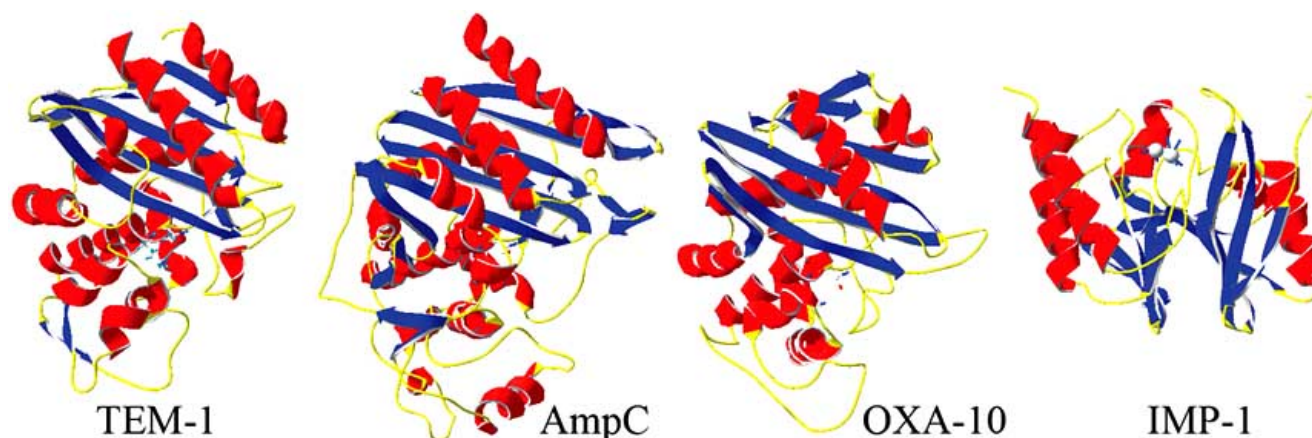


Fig. (7). Crystal structures of β -lactamases representing the four Ambler classes. Serine β -lactamases TEM-1, AmpC and OXA-10 are class A, C and D β -lactamases, respectively, while metallo- β -lactamase IMP-1 is a class B β -lactamase. (PDB accession numbers: TEM-1 – 1M40; AmpC – 2BLS; OXA-10 – 1FOF; IMP-1 – 1DDK).

endopeptidases e.g. *E. coli* PBP4 and PBP7, it is either a threonine or a serine residue. Furthermore, majority of classes A and B PBPs have a threonine or a serine in the analogous position. Hence, it is likely that the type of residue at this analogous position of 394 in *B. subtilis* PBP4a plays an important role in determining the substrate specificity, leading to the type of peptidase activity using similar catalytic platform [125]. Nevertheless, the exact mechanism of native substrate processing by various classes of PBPs remains to be revealed. It has also been suggested that a different acylation-deacylation mechanism may be used by PBPs in interacting with their native substrates for cell-wall formation and with β -lactam antibiotics, their inhibitors [124].

Low Affinity PBPs as a Resistance Mechanism to β -Lactams

One of the resistance mechanisms adopted by some bacteria in resisting the effect of β -lactam antibiotics is to possess one or multiple PBPs with low affinity for these antibiotics. Probably the most notorious of all infectious bacteria is MRSA. *S. aureus* has acquired an additional class B PBP2a encoded by *mecA* which is intrinsically resistant to the action of β -lactam antibiotics [126]. This gene is localized in the staphylococcal chromosome cassette which is a large mobile element integrated into the chromosome of *S. aureus* at a site-specific location near the origin of replication [127, 128]. The origin of this mobile element is still not clear but some evidence has pointed to *Staphylococcus sciuri* as a likely host [129, 130]. Importantly, the structural basis of *S. aureus* PBP2a resistance to β -lactam antibiotics was revealed via structural studies of *S. aureus* PBP2a using its apo- and β -lactam-acylated enzymes [131]. The overall fold of the *S. aureus* PBP2a structure resembled that of other transpeptidases and the serine β -lactamases, with all the active residues intact. Based on the comparison of the kinetic parameters of PBP2a with β -lactam-sensitive PBPs, the resistance of PBP2a to β -lactam antibiotics is due to inefficient formation of the acyl-PBP complex (k_2) and is neither a consequence of the β -lactam's poorer fit in the active site (K_d) nor from a more

faster turnover of the acyl-PBP complex (k_3) [112, 131-133]. Hence, as suggested by the comparative superimposition analysis of the apo- and nitrocefin-acyl-enzyme structures of *S. aureus* PBP2a, this low acylation rate is likely due to a displaced active site, which must undergo conformational changes at strand β_3 and N-terminus of helix α_2 for acylation to occur [131]. In contrast, as reported by Lim and Strynadka (2002), there was no equivalent conformational difference observed between the apo- and acyl-enzyme structures of *Streptomyces* strain R61 PBP and *S. pneumoniae* strain R6 PBP2x, further supporting this mechanism. This was similarly observed in the structural studies of *E. coli* PBP4 in its apo- and acylated states with various β -lactam antibiotics [134].

Unlike *S. aureus*, *S. pneumoniae* has yet to be reported to possess additional low affinity PBPs or to harbor β -lactamases as its means of penicillin resistance [19]. Instead, *S. pneumoniae* overcomes the action of β -lactam antibiotics by modifying its PBPs via genetic recombination of PBP-encoding genes, resulting in mosaicism [19]. Among the six PBPs in *S. pneumoniae*, PBP1a, PBP2b and PBP2x were often reported to be modified in resistant strains [114, 135].

Mosaic PBP1a has been implicated in high level of resistance to penicillins and cephalosporins [136]. Mapping PBP1a sequence of penicillin-resistant clinical strains onto a recent crystal structure of PBP1a from a penicillin-sensitive strain R6 revealed the location of a mutational hotspot at the entrance to the catalytic cleft [137]. These mutations are (1) T574N, S575T, Q576G and F577Y localized on the loop region between strand β_3 and β_4 , (2) L606I, N609D, L611F and T612L localized at the N-terminus of helix α_{11} , which is in close proximity to β_3 - β_4 loop, and (3) N546G located in the loop between α_9 and β_3 . These mutations probably can modify the surface polarity of the active site and affect the recognition of β -lactam antibiotics, which are negatively charged. There is another mutation that is commonly found in PBP1a sequences of highly resistant strains but it is not localized within the hotspot region. This mutation, T371A, is the second residue of the SXXK motif of PBP1a and is localized at the N-terminal of α_2 . The hydroxyl group of T371 forms a hydrogen bond with the main chain carbonyl

group of W368 and stabilizes $\alpha 2$. The loss of this hydrogen bond accompanied by L583M/S and A585V mutations could probably affect the position of the catalytic serine residue. This form of resistance is similar to that described for PBP2a of *S. aureus* [131].

S. pneumoniae PBP2x is a class B PBP and its sequences from different species and strains have also been reported to display mosaicism [19]. Its first crystal structure from a penicillin-sensitive strain R6, which is also the first high molecular mass PBP to be resolved, was reported in 1996 [138] and one complexed with cefuroxime was later reported in 2000 [119]. Another crystal structure from a highly penicillin-resistant clinical isolate strain Sp328 was reported in 2001 [139]. Strain Sp328 harbors 92 mutations in its PBP2x compared to strain R6 and most of these are concentrated in the penicillin-binding and C-terminal domains [139]. Importantly, strain Sp328 PBP2x has a T338A mutation that resembles the corresponding T371A mutation in *S. pneumoniae* PBP1a and has been demonstrated to be one of the determinants in influencing its affinity for β -lactam antibiotics [140]. The results from structural comparison of PBP2x from strains R6 and Sp328 suggested a likelihood of T338A mutation conferring the PBP with a mechanism similar to that of *S. aureus* PBP2a and *S. pneumoniae* PBP1a. Furthermore, the SXN motif of this resistant PBP2x was shown to be displaced with reference to the crystal structure of strain R6 PBP2x. This was postulated to be the effect of S389L and N514H mutations leading to a more flexible loop region formed from amino acid residues at positions 365 to 394. Interestingly, resistant strains of *S. pneumoniae* have been reported to possess branched stem peptides in their peptidoglycan cell wall, while sensitive strains exhibit linear forms of stem peptides [141-143]. Hence, this flexible region leading to an exposed active site in PBP2x was suggested as a possible explanation for *S. pneumoniae* PBPs to accept alternate branched substrate in bridging peptidoglycan chains during cell wall synthesis [139]. A more recent study using another resistant strain 5204 further supported the importance of this flexible loop [144]. Using tryptic digestion, PBP2xs of strain Sp328 and 5204 were cleaved into two fragments while that of strain R6 remained intact and the cleavage site was determined to be after residue R372. However, mutational studies vindicated positions 371 and 384 to be important in determining the conformation of this region instead of positions 389 and 514 [144].

A similar strategy in understanding the resistance mechanism of PBP2x was adopted for PBP2b by resolving the crystal structures of PBP2b from strain R6 and resistant strain 5204 [114]. Comparison between these two structures revealed distinct differences and offered probable explanation for changes in the affinity of PBP2b for β -lactams. This highly resistant strain 5204, which bears a total of 58 mutations, has two outstanding mutations, A619G and D625G, in the $\beta 3$ - $\beta 4$ interconnecting region and this probably conferred high flexibility to this region. These two mutations, including Q628E and T630N, are often found in high drug-resistant strains [145]. There is also a concentration of four mutations around the substrate-entry site. These four mutations S412P, N422Y, T426K and Q427L are located on the loop that precedes helix $\alpha 4$ and they probably modify the polar characteristic of the site,

particularly T426K mutation, which adds bulk and positive charge to the region. This study also highlighted T446A mutation as a potential determinant of resistance since it has been constantly observed in many drug-resistant isolates [145, 146]. Residue T446 is located after SXN motif on $\alpha 4$ - $\alpha 5$ and its side chain interactions with residues D410, Q411 and I413 probably serves to stabilize the $\alpha 4$ - $\alpha 5$ loop. Hence, the loss of these interactions will impart instability to this region of the active site and affect β -lactam recognition. Interestingly, there are other mutations that are not in the vicinity of the active site e.g. F502L and S508N, and they are often observed in the sequences of PBP2b from drug-resistant strains. However, their roles in the alteration of the affinity of PBP2b for β -lactams remain unclear. Nevertheless, these structural studies of *S. pneumoniae* PBPs led Contreras-Martel and co-workers (2009) to suggest active site flexibility as the key β -lactam-resistance mechanism adopted by *S. pneumoniae* PBPs in countering the inhibitory effects of β -lactam antibiotics [114].

Other Resistance Mechanisms Involving PBPs

Interestingly, other than expressing low-affinity PBPs to substitute the functions of β -lactam-inhibited PBPs, two different resistance strategies involving PBPs have been reported. *S. aureus* PBP4, a class C PBP with carboxypeptidase activity, has been reported with β -lactamase activity [147]. Although *S. aureus* PBP4 has been implicated in the resistance of *S. aureus* to β -lactam antibiotics [148], its significance to resistance has yet to be fully comprehended. Similarly, deletion of *E. coli* PBP5 was shown to result in higher susceptibility of the host organism to β -lactams, indicating a role of PBP5 in conferring a basal level of resistance to β -lactam antibiotics [149]. These findings highlighted the lack of complete understanding of the role of these non-essential class C PBPs in the host organisms in conferring resistance to β -lactam antibiotics although extremely low deacylation rate of β -lactam-acyl-PBPs *in vitro* (a difference of five-order of magnitude in activity) has suggested their improbable involvement in resistance [119, 120, 140, 150].

Yet, another resistance mechanism has been found to involve the regulatory effect of a PBP. This was implicated from a recent study on *P. aeruginosa*, in which overproduction of AmpC β -lactamase was triggered by the inactivation of a non-essential PBP4 [151]. In addition, inactivation of PBP4 also led to the specific activation of the CreBC two-component regulator and this was found to play a major role in conferring resistance to *P. aeruginosa*, independent of AmpC overproduction. However, the underlying mechanism is still not clear. Nonetheless, this again indicates the need to fully understand the functionalities of all PBPs and the different resistance mechanisms present in a pathogen, which otherwise may jeopardize the chemotherapeutic use of newly-developed antibiotics.

DEGRADATION OF β -LACTAMS BY β -LACTAMASES

Probably the most important resistance mechanism to β -lactam antibiotics is the inactivation of β -lactams by β -lactamases. These enzymes are divided into two main groups

based on their catalytic mechanisms, (1) serine β -lactamases, which catalyze the reaction using a nucleophilic serine like PBPs, and (2) metallo- β -lactamases (MBLs), which are zinc-dependent hydrolases. However, there are two main classification systems. One is the Ambler's classification, which is based on the molecular structures of β -lactamases [152] and the other is Bush's classification, which is based on their functional properties [153, 154].

In Ambler's classification, the β -lactamases are divided into classes A, B, C and D. Classes A, C and D β -lactamases are the serine β -lactamases while class B β -lactamases are MBLs [152, 155, 156]. The main difference between class A, C and D β -lactamases lies in their structures (Fig. 7). The structures of class A β -lactamases resemble those of PBPs in that they possess the conserved SXXK, SDN and K(T/S)G motifs. Hence, it has been speculated that class A β -lactamases might have evolved from PBPs [113]. However, class A β -lactamases possess a conserved glutamate residue in their Ω -loop, which is essential for their activities [21]. Class A β -lactamases were previously known as penicillinases. As many class A β -lactamases have their substrate specificities evolved to include a wider range of β -lactam antibiotics [157, 158], this name is now obsolete. Importantly, this group of enzymes is generally susceptible to serine β -lactamase inhibitors (clavulanate, tazobactam and sulbactam) although inhibitor-resistant class A β -lactamases have been reported [158-160].

In class C β -lactamases, the enzymes comprise of mainly *ampC*-encoded β -lactamases, which are also known as cephalosporinases due to their higher activities against cephalosporins [161]. Generally, the β -lactamases in this class degrade penicillins and cephalosporins readily, and are resistant to clavulanate. However, they can be inhibited by cloxacillin and aztreonam [154]. Most of these genes are chromosomally-encoded in Gram-negative bacteria and are often repressed. They can be derepressed by induction with certain β -lactams, particularly cefoxitin [161-163]. This form of regulation has been intensively studied and three genes, *ampR*, *ampD* and *ampG*, have been identified. *AmpR* is a transcriptional regulator responsible for the expression of *ampC* while *AmpD* is an amidase responsible for the removal of stem peptides from the precursors of peptidoglycan synthesis. These two enzymes are metabolically related where the peptidoglycan precursor turnover will affect the conformational changes of *AmpR* leading to the altered expression of *AmpC* [162, 163]. *AmpG* is an inner membrane permease that transports the oligopeptides generated from the cell wall recycling back into the cytosol. Although it is seldom implicated in the resistance of the host organism to β -lactams, it has been shown to be essential for the high-level expression of *AmpC* β -lactamase in *Enterobacter cloacae* [164]. Overexpression of *AmpC* in clinical isolates has often been implicated with mutations of *ampD* [165]. However, mutations in *ampR* have also been reported to result in *AmpC* overexpression phenotypes [162, 166, 167].

Class D β -lactamases are enzymes that are known to hydrolyze oxacillin in addition to penicillins, cephalosporins and carbapenems [168, 169]. Hence, they were named as oxacillinases. They are also resistant to the inhibition of clavulanate, sulbactam and tazobactam, with some

exceptions [20, 154, 169]. Although they have similar structural folds as the class A and C β -lactamases, distinct structural differences do exist, which probably account for their substrate preferences [170].

Class B β -lactamases are MBLs with zinc-dependence. They have broad substrate specificities and are not inhibited by serine β -lactamase inhibitors [20, 171, 172]. Currently, there are no clinically useful inhibitors against MBL-producing pathogens. Generally, MBLs use a water molecule coordinated by zinc ion to hydrolyze the amide bond of a β -lactam, which is a different catalytic mechanism from serine β -lactamases. They are further subdivided into three classes based on their zinc ion dependency [173, 174]. Subclass B1 enzymes are fully active with either one or two zinc ions while subclass B3 enzymes require two ions to be active. Subclass B2 enzymes are those that are active in the presence of one ion and become inhibited by binding an additional ion. Importantly, structural and biochemical studies have shown the significance of the zinc ion centre in interacting with the carboxylate group present in all penicillins, cephalosporins and carbapenems, contributing to the broad substrate specificity of the MBLs [172, 175-177]. Hence, it is unlikely that any β -lactam-based inhibitor will be effective against the MBLs.

In contrast to Ambler's classification, Bush's classification has more clinical relevance since it is based on the functional characteristics of β -lactamases [154]. With the significant increase in the number of β -lactamase sequences due to global effort in genome sequencing of numerous pathogens, functional classification of β -lactamases has been shown to correlate well with their molecular structures [154]. There were initially four groups described in Bush's classification where group 4 consists of β -lactamases that are not inhibited by clavulanic acid and are placed together because of incomplete characterization [154]. Recently, this group was removed in the updated system because the authors believed that those enzymes would most likely be included in one of the existing groups when their structural and functional characteristics are available [169]. Briefly, the β -lactamases are grouped based on their substrate preferences and inhibitor profiles. Group 1 comprises of cephalosporinases that are not well inhibited by clavulanic acid while those β -lactamases that are generally inhibited by serine β -lactamase inhibitors are in group 2. Group 2 is further subdivided based on more specific substrate profiles e.g. Group 2a are the classical penicillinases, Group 2b are the broad-spectrum β -lactamases, Group 2be are the extended-spectrum β -lactamases, Group 2c enzymes have higher activities for carbenicillin than penicillin G and Group 2d are the oxacillinases. Hence, Ambler class C β -lactamases are in Bush group 1 and Ambler class A and D β -lactamases are in Bush group 2. Group 3 comprises of MBLs, which are the Ambler class B β -lactamases.

Active Site of Serine β -lactamases

Class A, C and D β -lactamases are structurally related despite low homology at primary sequence level and they share a similar hydrolytic mechanism as the penicillin-binding domain of PBPs in that they all used deprotonated nucleophilic serine for catalysis. However, activation of nucleophilic serine remains elusive as different experimental

evidence point towards either lysine residue of the SXXX motif or conserved residue E166 (standardized ABL numbering for Ambler class A β -lactamases) [178] in the Ω -loop as the general base in deprotonating active site serine in class A β -lactamases (Fig. 8B) [179]. This is somewhat analogous to the debatable mechanism of the activation of nucleophilic serine of PBPs in binding β -lactams described earlier. Nevertheless, the role of the conserved ABL E166 in the Ω loop as the general base for proton abstraction in the deacylation step of β -lactam inactivation has been widely accepted [179, 180].

Generally, the structure of AmpC is similar to that of class A β -lactamases except that its substrate binding pocket is more open, which probably helps to accommodate the bulkier structure of cephalosporins [161, 181, 182]. Interestingly, in class C β -lactamases, instead of the conserved SXN motif in class A β -lactamases, the serine residue is replaced by a tyrosine (Fig. 8C) [170]. Studies have suggested that this tyrosine residue plays the role of a general base in activating nucleophilic serine for the acylation reaction and may also be involved in the deacylation step [183, 184]. However, structural studies of the *E. coli* AmpC complexed with a deacylation transition state analogue suggested that it is unlikely to be involved in deacylation as it remained protonated throughout the reaction [185]. Instead, deacylation of β -lactam by AmpC may proceed by a substrate-activated catalysis mechanism although the role of residue K67 of the SXXX motif in the coordinate base mechanism cannot be ruled out [185, 186].

Interestingly, for class D β -lactamases, despite having similar structural folds, they possess distinct regional variations and biochemical differences. Most of these enzymes can dimerize and this could almost double their activities compared to those monomeric forms [170, 187]. In addition, this class of enzymes has a hydrophobic character in the active site region, which is believed to confer the ability to accommodate a wide range of β -lactams with varying side chains [170, 187-189]. The lysine residue of the SXXX motif has been suggested to be the general base in both acylation and deacylation steps since there is no conserved glutamate residue in the shortened Ω -loop [170]. The ability of this residue to be involved in both acylation and deacylation of β -lactams can probably be explained by the lysine residue being carbamylated (Fig. 8D) [187, 188]. Structural studies of *P. aeruginosa* OXA-10 has also revealed hydrogen bonding of the carboxylate group of the carbamylated lysine side chain with hydroxyl group of residue S67 side chain and nitrogen atom of residue W154 side chain [188]. In addition, the recent mutational and structural studies on residue W154 of OXA-10 revealed the importance of tryptophan at this position in influencing the carbamylation state of residue K70 and deacylation of penicillin G [190].

Mutations in β -Lactamases Leading to Altered Properties

Since the discovery of β -lactamases, studies of these enzymes have diverged from understanding their catalytic mechanisms and substrate specificities to application for

scientific research e.g. as a selection factor for molecular cloning, as a tool in protein-protein interaction studies [191], and as a model for understanding protein evolution [192-194]. Nevertheless, studies on the enzymatic properties of clinically important β -lactamases remain intensive and have shed light on the mechanism involved in altering their substrate specificities [158, 195-199]. With approximately 700 β -lactamases reported so far, naming them has become more difficult [169, 200]. Some names were given based on their sources, e.g. TEM, which is named after the patient Temoneira, who provided the first sample and some based on their functionalities e.g. CTX, which refers to those that hydrolyze cefotaxime, and OXA, which refers to those that hydrolyze oxacillin [200]. Hence, efforts in standardizing the nomenclature and collation of structural information of various β -lactamases identified were initiated by Lahey Clinic. This database is now publicly available online (<http://www.lahey.org/studies>) allowing free access for investigators to assess the novelty of any recent isolation of β -lactamases. This also allows further research into the effects of various mutations to the functionality of different classes of β -lactamases.

Class A β -lactamases form the largest group among the serine β -lactamases and are well-characterized, especially the TEM and SHV enzymes. Earlier enzymes characterized were initially known to hydrolyze mainly penicillins. Eventually, mutants of this group of enzymes were found to have wider substrate specificity including cephalosporins [158]. Extensive studies using saturation mutagenesis on TEM-1 β -lactamase and sequence comparison among 20 class A β -lactamases indicated that over 200 out of the 263 amino acid positions could tolerate mutations although some mutations might require the co-existence of other mutations to preserve functionality [201]. Those mutations that alter the substrate specificity are mainly situated near the active site [158]. For example, in TEM-1 β -lactamase, mutations at positions E104, R164, D179, A237, G238 and E240 have been reported. Structural inspection of TEM-1 revealed that these positions are mainly situated at the β_3 strand and the Ω -loop, which surround the side chain of penicillin in the acyl-enzyme intermediate (Fig. 9A) [158, 202]. This has significant implication on the plasticity of the enzyme active site in accommodating the β -lactam substrates, which differ structurally in their side chains. These mutations also displayed functional trade-offs where improvement in the hydrolysis of one substrate is often accompanied by a drop in another substrate [195, 203]. In an earlier study on the substrate specificity determinants of TEM-1 β -lactamase for cephalosporin, A237T and E240C mutations were identified as cephalosporin-specific [198]. However, the exact catalytic efficiencies of these mutants for cephalosporins, especially the third generation cephalosporin like cefotaxime, were still low compared to those for penicillins. This indicates that there are other structural requirements for specific recognition and efficient hydrolysis of each substrate [195]. Furthermore, sequence comparison of TEM-1 mutants selected for wild-type or higher hydrolytic activities against ampicillin and cephaloridine suggested a greater restriction in sequence variation in TEM-1 for maintaining cephalosporinase activity compared to maintaining penicillinase activity [198]. Hence, it is likely that the

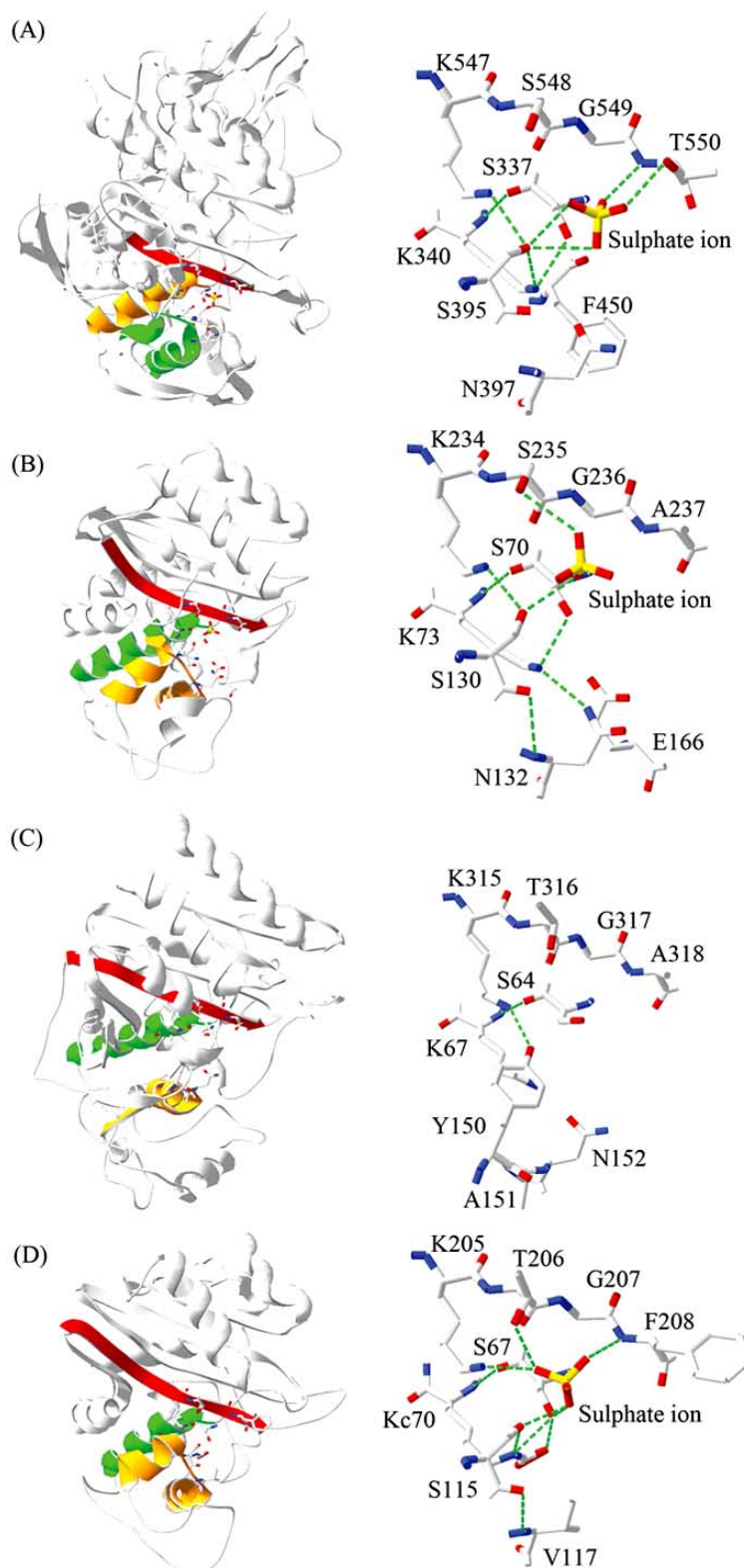


Fig. (8). Crystal structures and active site residues of (A) *S. pneumoniae* PBP2x (1QME), (B) *E. coli* TEM-1 β -lactamase (1BTL), (C) *E. coli* AmpC (2BLS) and (D) *P. aeruginosa* OXA-10 (1E4D). The crystal structures and the active site residues of these proteins are depicted in the left column and right column, respectively. The $\alpha 2$ helix, the loop between $\alpha 4$ and $\alpha 5$ helices, and the $\beta 3$ strand containing the SXXK, SXN and K(T/S)G motifs are colored green, orange and red, respectively, in *S. pneumoniae* PBP2x. The corresponding folds in serine β -lactamases TEM-1, AmpC and OXA-10 are similarly colored. The loop harboring the conserved residue E166 in TEM-1 is colored blue. The atoms in the right column are in CPK colors; red - oxygen atom, blue - nitrogen atom, yellow - sulphur atom, white - carbon atom. The hydrogen bonds are depicted as green dotted lines. Carbamylated lysine residue in OXA-10 is labeled as Kc70 (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper).

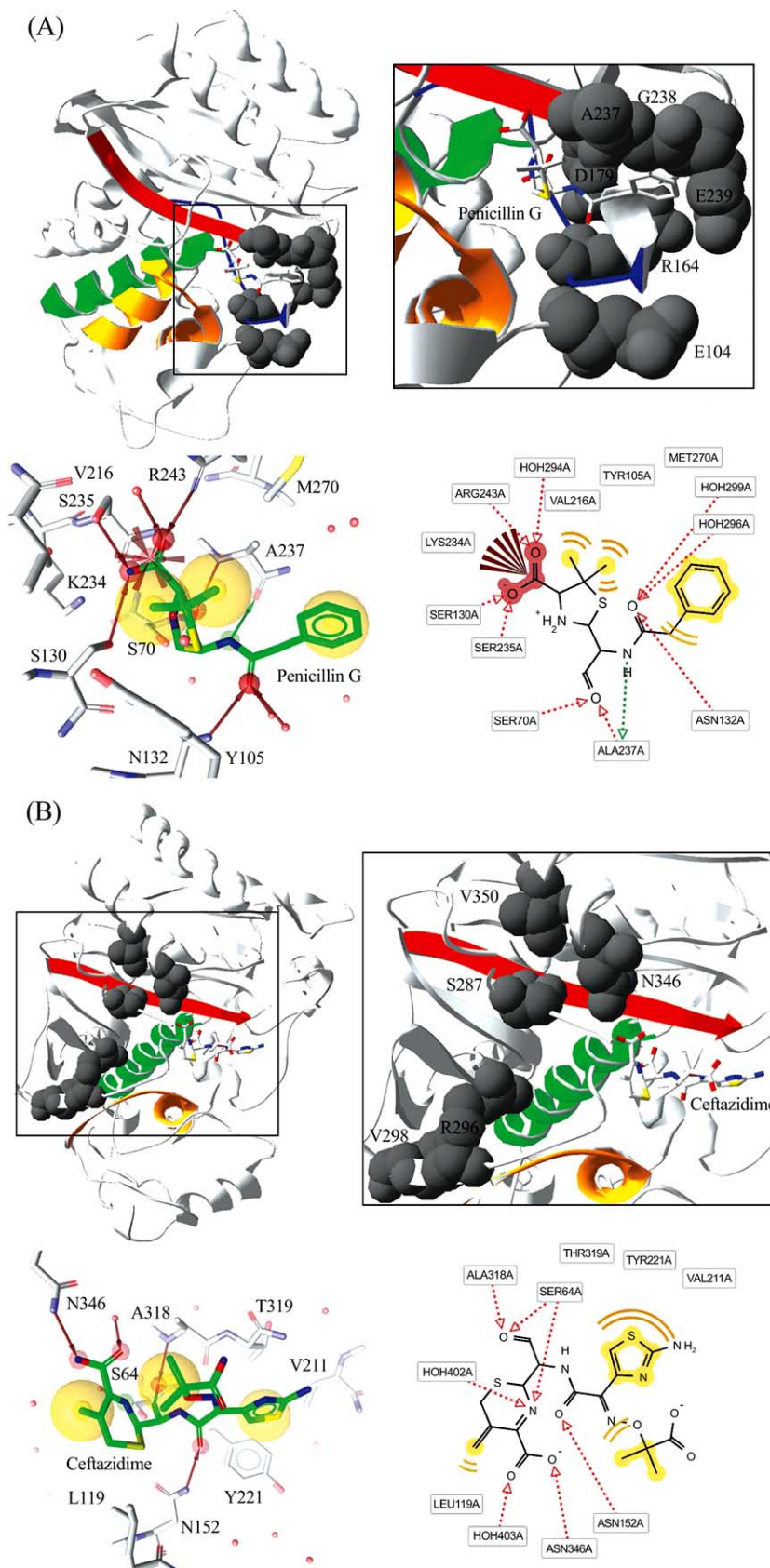


Fig. (9). Mutational hotspots in (A) TEM-1 (1FQG) and (B) AmpC (1IEL) β -lactamases. The amino acid residues that are often mutated in both enzymes leading to altered substrate specificity are presented as grey spheres. The remaining color codes are similar to those in Fig. (8). The figures below are the pharmacophores, where red and green arrows refer to the hydrogen bond acceptors and donors, respectively, yellow and red spheres represent hydrophobic interactions and water molecules, respectively, and red beams represent negative ionizable areas (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper).

requirement for class A β -lactamases to evolve from a wild-type sequence to one with high activity against a different class of β -lactam antibiotics involved more than just point mutations. The existence of structurally different classes C and D β -lactamases with higher preference for cephalosporins and oxacillin-related penicillins, respectively, supported this notion.

Most penicillins and cephalosporins are susceptible to class C AmpC β -lactamases, except ceftazidime and cefepime, which are hydrolyzed slowly [204]. Similarly, deletion, insertion and point mutations have been reported to elevate the hydrolytic activity of AmpC β -lactamases against them. These mutations are mainly located at H-9 (residue S287), H-10 (residues H296 and V298) and H-11 helices (residues N346 and V350), which made up the region of the active site interacting with the C3 side chain of a cephalosporin (Fig. 9B) [196, 205-208]. This is expected since ceftazidime and cefepime differ mainly in their C3 side chains while bearing similar C7 side chains from other third generation cephalosporins (Fig. 3).

NOVEL CEPHALOSPORINS THAT TARGET LOW-AFFINITY PBPs

MRSA and penicillin-resistant *S. pneumoniae* have been a threat to human health due to their high level of resistance towards many clinically available antibiotics [209]. However, new cephalosporins have been developed and validated to be effective against these pathogens, targeting their low-affinity PBPs (Fig. 4). Ceftobiprole has been approved by the United States Food and Drug Administration for the treatment of skin and skin structure infections [210]. Studies have shown that it is effective in binding *S. aureus* PBP2a with an IC_{50} value of 0.9 $\mu\text{g/mL}$ compared to ceftriaxone and ceftazidime, which both have IC_{50} values of more than 50 $\mu\text{g/mL}$ [211]. The minimum inhibitory concentration (MIC) value of ceftobiprole against MRSA strain OC 3726 was 2 $\mu\text{g/mL}$, which is at least 32 fold lower than that of ceftriaxone and ceftazidime. Ceftobiprole is also effective in inhibiting *S. pneumoniae* PBP2x with an IC_{50} value of 1 $\mu\text{g/mL}$ compared to ceftriaxone, which has an IC_{50} value of 8 $\mu\text{g/mL}$. It was able to inhibit penicillin-resistant *S. pneumoniae* strain OC 8819 with a MIC value of 1 $\mu\text{g/mL}$, which is 8 fold lower than that of ceftriaxone [211]. This efficacy is due to the ability of ceftobiprole to form a stable acyl-enzyme complex with low-affinity PBP via its C3 side chain [82, 212]. Furthermore, ceftobiprole was also tested against other PBPs in *S. aureus*, *S. pneumoniae*, *E. coli* and *P. aeruginosa*, and showed that it was able to inhibit them equally well or in some cases, better than ceftriaxone, ceftazidime and cefepime [211]. A recent study on the interaction of ceftobiprole with selected Ambler classes A to D β -lactamases showed that it was refractory to hydrolysis by the common staphylococcal PC1 β -lactamase, the class A TEM-1 and SHV-1 β -lactamases, and the class C AmpC β -lactamases but was labile to hydrolysis by class B, class D, and class A extended-spectrum β -lactamases e.g. CTX-M, K1 and TEM-26 [213].

Another cephalosporin, ceftaroline, has also been demonstrated to be effective against MRSA and penicillin-resistant *S. pneumoniae* [214-216]. Similar to ceftobiprole, its effectiveness is due to its ability to inhibit their low-

affinity PBPs [217, 218] and this is also due to its C3 side chain that probably acts in a similar fashion as ceftobiprole in binding low affinity PBPs. As discussed earlier, the resistance mechanism of *S. aureus* PBP2a is probably due to its displaced active site and the low acylation rate of existing β -lactams. Importantly, it has been demonstrated that ceftaroline and other anti-MRSA cephalosporins (ABRI-1175, ABRI-2901 and ABRI-1974) although with similar acylation rate constants as other β -lactams, their acylation rates to *S. aureus* PBP2a were significantly enhanced in the presence of a peptidoglycan fragment analogue [83, 86]. This suggests that these cephalosporins might be able to compete with PBP2a for the interactions of the cell wall *in vivo* to gain access to the active site of this enzyme, leading to acylation [83, 86].

NOVEL CEPHALOSPORINS THAT EVADE β -LACTAMASE DEGRADATION

Other than ceftobiprole and ceftaroline that have been successfully designed and tested to be effective against β -lactam-resistant Gram-positive pathogens, there are other cephalosporins that have been synthesized and shown to be effective against *P. aeruginosa*. Earlier clinical studies have shown that ceftazidime is efficacious against *P. aeruginosa* but becomes ineffective when *P. aeruginosa* overproduces AmpC β -lactamase [87, 219, 220]. A new cephalosporin FR259647 (Fig. 4), which is structurally similar to ceftazidime except for its C3 side chain of the cephalosporin nucleus, has been tested to be effective against AmpC overproducing strains of *P. aeruginosa* [87]. These results indicated that the C3 pyrazole side chain of FR259647 might have contributed to its stability to AmpC by obstructing the entry of this cephalosporin into the active site of AmpC enzyme. In order to test this hypothesis, Murano and co-workers (2008) assessed and predicted the probability of cephalosporin entry into the binding pocket of AmpC by conformational analysis. Using six derivatives of FR259647 with variations in the 3- and 4-substituent on the pyrazole ring, they demonstrated that the calculated probability of entry of these derivatives correlated well with their index of stability, which is reflected by the MIC ratio of AmpC overproducing strain to a wild-type strain [87]. This study subsequently led to the synthesis of CXA-101 (previously known as FR264205) [89, 221], which has good antibacterial activity with weak convulsion-inducing effect [88], and has proceeded to Phase 2 clinical trials [222, 223]. This study has provided a good platform for future drug designs where *in silico* prediction of antibiotic "fitness" to the active site of β -lactamases would be useful in reducing the number of potential compounds to be synthesized and evaluated.

IMPLICATIONS FOR FUTURE DRUG DESIGN

The recognition of side chain variation in determining the efficacies of penicillin and cephalosporin has marked the beginning of β -lactam antibiotic design and development. From exploiting the biochemical feasibilities to modify the side chains of penicillins and cephalosporins to the understanding of the efficacy of side chain variations in inhibiting bacterial PBPs, the amount of information generated has been tremendous and yet, there is no antibiotic

that is able to fully address the problem of spontaneous antibiotic resistance in emerging pathogens [224]. From the vicious cycle of new antibiotic development to the emergence of new resistant pathogens, it is likely that the problem of antibiotic resistance is here to stay and perhaps pose even more challenges to future drug discovery and design. Hence, there is a need to speed up the understanding of the evolution of antibiotic resistance for designing competent antibiotics rather than surrender to the problem of finding new drugs to combat elusive pathogens with endowed capabilities [225].

Although the continuous emergence of resistant pathogens may dampen the enthusiasm in β -lactam antibiotic development [81, 226], the synthesis of new cephalosporin derivatives effective against MRSA and retaining relatively wide spectra has given new hope to the pharmaceutical industries [227]. By understanding the binding mechanisms of both susceptible and resistant PBPs and β -lactamases, cephalosporins can be further modified to better inhibit resistant PBPs and evade degradation by β -lactamases [228]. However, we should not become complacent by the recent development of effective cephalosporins against MRSA and *P. aeruginosa* because there is still a possibility that resistance to these new cephalosporins may emerge as resistant bacteria will be selected under the selective pressure of antibiotics.

Efforts in searching and predicting resistance to these newly developed cephalosporins should continue. It will be beneficial if the type of resistance that may emerge when the newly developed antibiotic is used clinically could be predicted. For example, in the directed evolution experiment of TEM-1 β -lactamase, a triple mutant E104K/M182T/G238S was generated, which is equivalent to the clinically isolated TEM-52, under the selection of cefotaxime [229]. This illustrated the relevance of laboratory-directed evolution in predicting possible emergence of resistance to newly developed antibiotic. Furthermore, by combining with structural analysis, M182T mutation was revealed to reverse the destabilizing effects of antibiotic- or inhibitor-resistant mutations by forming hydrogen bonds with residues E63 and E64, which probably reduced the conformational flexibility of the hinge region between the two domains of the active site [229]. Hence, this co-application of *in vitro* and *in silico* techniques could be used to provide insights for selecting the compound candidates that are less likely to generate resistance or to anticipate the emergence of new resistance for newer drug development.

Genomic sequencing of many infectious bacteria has provided a wealth of genetic information for identification of new targets and design of new drugs [230, 231]. Structural determination of drug targets and resistant factors has also contributed to our understanding of the mechanistic effectiveness and failures of antibiotics [21, 232]. With the development of various structural modeling, docking and molecular dynamic simulation programs, drug discovery and design could be realized in a more rational and probably more economical manner [233-236]. For example, virtual screening for novel inhibitors of *S. pneumoniae* PBP2x was performed using the National Cancer Institute open database

compound library and a homology model of resistant PBP2x [237]. Of the approximately 260,000 compounds screened, 160 hits were found and 55 were available for experimental testing. Finally, three compounds with inhibitory activity (IC_{50}) in the micromolar range were identified [237]. This has dramatically reduced the number of potential candidates for empirical biological assessment, which can be tedious, time-consuming and expensive. Another example is the use of homology model of *P. aeruginosa* AmpC in designing β -lactamase-resistant cephalosporins, which has been discussed earlier [87].

In the development of β -lactams, clinical failures of earlier and existing antibiotics towards resistant bacteria have provoked many structural and mechanistic studies of PBPs and β -lactamases, and their interaction with various β -lactams. These studies have provided valuable insights and implications for future designs of β -lactam antibiotics [20, 87, 88, 131], and are summarized as follows:

Increase the Binding Affinity of β -Lactam Antibiotics to the Active Site of PBPs

This can be achieved by improving the non-covalent interactions between β -lactams and the active site of PBPs. As illustrated by the development of ceftobiprole and ceftaroline, the binding affinity of these two cephalosporins were significantly improved by modifying the C3 of the cephalosporin nucleus [83, 212]. In the design of more efficacious β -lactam antibiotics, reduction of the dissociation constant (K_d) is expected to play a larger part in improving overall acylation efficiency (k_2/K_d) [131]. However, other factors e.g. presence of peptidoglycan fragment, may play a role in determining the ultimate effectiveness of the antibiotic [83, 86]. With the availability of crystal structures of various PBP classes deposited in the open access PDB, better shape complementarity of the antibiotic to the active site of PBPs could be designed.

Stabilize the Interaction of β -Lactam Antibiotics to PBPs Using Natural Substrate Mimicry as an Approach

Studies using compounds that mimic the natural substrate of PBPs have provided clues to the interaction mechanism presence in the active sites of these PBPs [111, 115, 125]. Since β -lactam binds to the active site of PBPs by mimicking the terminal D-Ala-D-Ala of the peptidoglycan stem peptide, any further resemblance to this stem peptide should be able to enhance interaction between the antibiotic and the PBP. As demonstrated by the widening of the antimicrobial spectra of ampicillin and ceftazidime *via* the addition of an amino and oxyimino group in the α -position of their side chain, respectively, it appears that this position has a significant effect in interacting with the active site of PBPs. By superimposing the crystal structures of *E. coli* PBP6 complexed with ampicillin and peptidoglycan substrate fragment, this position was found to correspond to the branching point of the third amino acid in the peptidoglycan stem peptide (Fig. 10), which varies the most between different bacterial species [238]. Hence, this position poses a great potential for modification to improve the binding affinity of β -lactam antibiotics to various bacterial PBPs.

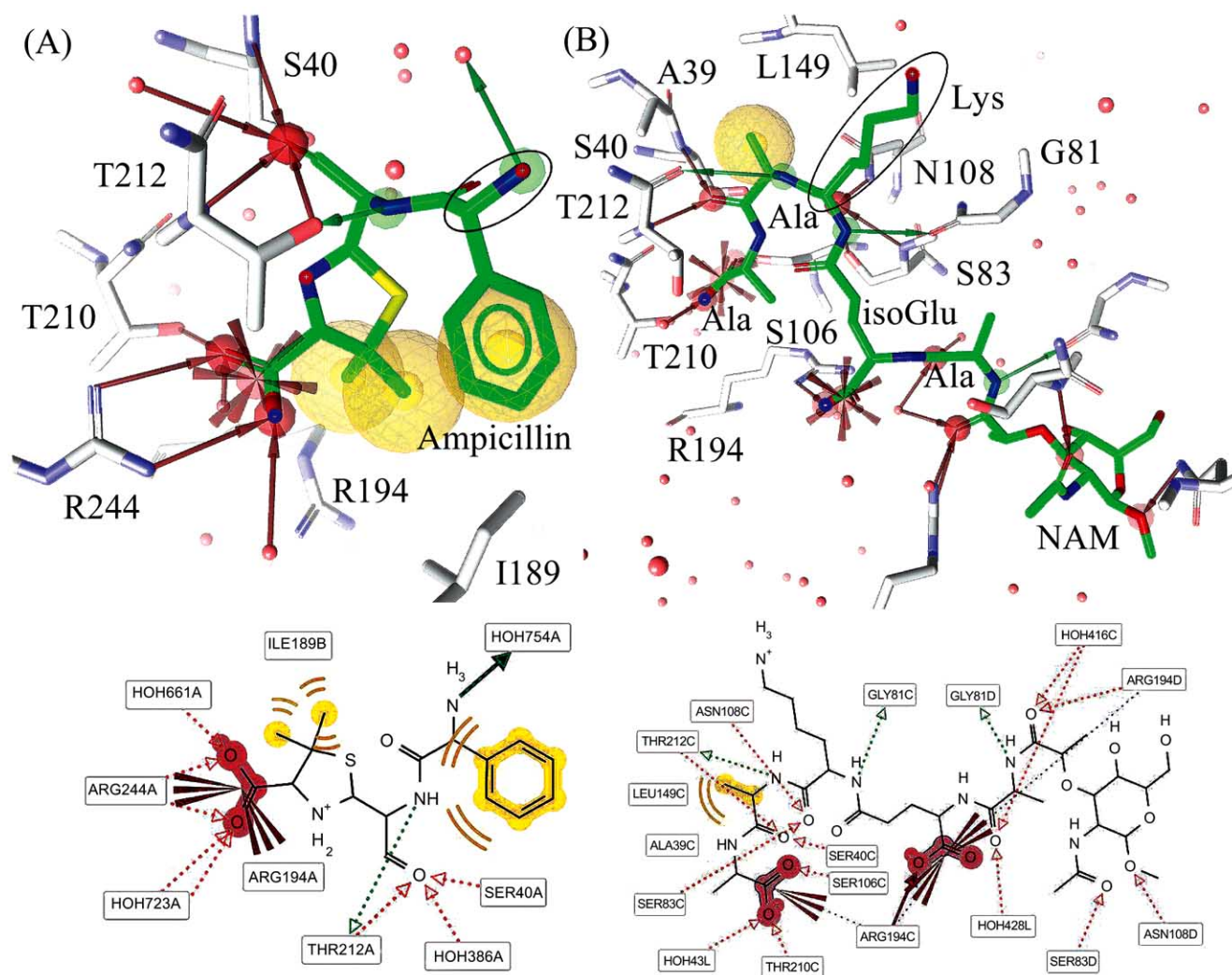


Fig. (10). Active sites of *E. coli* PBP6 complexed with (A) ampicillin (3ITA) and (B) peptidoglycan substrate fragment, NAM-(L-Ala-D-isoGlu-L-Lys-D-Ala-D-Ala) (3ITB). The amino group at the α -position of ampicillin side chain coincides with the side chain of lysine residue in the peptidoglycan substrate fragment (framed). Red and green arrows refer to the hydrogen bond acceptors and donors, respectively, yellow and red spheres represent hydrophobic interactions and water molecules, respectively, and red beams represent negative ionizable areas (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper).

Lower the Binding Affinity of β -Lactam Antibiotics to β -Lactamases

Other than using β -lactamase inhibitors, designing β -lactamase-resistant cephalosporin is also a feasible approach in circumventing the problem of resistance due to intervention by the action of β -lactamases. By understanding the substrate binding mechanism of AmpC β -lactamase, Murano and co-workers (2008) have proposed steric hindrance of the C3 substituent of cephalosporin as a disturbance to the entry of the AmpC active site. Hence, variation in the C3 substituent of a cephalosporin could also achieve resistance to β -lactamase other than enhancing the interaction with PBPs. This has led to the development of CXA-101 [88, 89, 221], which is currently in a Phase 2 clinical trial.

Target More than one PBP in the Same Organism, Especially the Essential Ones, and Include All the β -Lactamases they Produced

This strategy is significant as simultaneous inhibition of various PBPs and β -lactamases should be efficient to impede the growth of the bacteria and will diminish the likelihood of rapid point mutation-based resistance development [2, 4]. In fact, studies have shown the multi-targeting capabilities of various penicillins and cephalosporins in inhibiting various PBPs in the same bacterium [211, 218, 239]. Since structural information of various classes of PBPs (Table 1) and β -lactamases (Table 2), and the genomic sequences of many infectious bacteria are available, tertiary structures of many yet-to-resolved bacterial PBPs and β -lactamases could be easily modeled and exploited in the design of species-

Table 2. Tertiary Structures and Classes of Resolved Bacterial β -Lactamases

Nomenclature	Host Organism	PDB Accession No.*	Ambler's Class [152, 155, 156]	Bush's Group [§] [153, 154, 169]	References
PC1	<i>Staphylococcus aureus</i>	3BLM	A	2a	Herzberg, 1991 [262]
TEM-1	<i>Escherichia coli</i>	1M40	A	2b	Minasov <i>et al.</i> , 2002 [263]
TEM-30	<i>Escherichia coli</i>	1LHY	A	2br	Wang <i>et al.</i> , 2002 [264]
TEM-32	<i>Escherichia coli</i>	1LI0	A	2br	
TEM-34	<i>Escherichia coli</i>	1LI9	A	2br	
TEM-52	<i>Klebsiella pneumoniae</i>	1HTZ	A	2be	Orencia <i>et al.</i> , 2001 [229]
TEM-64	<i>Escherichia coli</i>	1JWZ	A	2be	Wang <i>et al.</i> , 2002 [265]
TEM-76	<i>Escherichia coli</i>	1YT4	A	2br	Thomas <i>et al.</i> , 2005 [266]
SHV-1	<i>Klebsiella pneumoniae</i>	1SHV	A	2b	Kuzin <i>et al.</i> , 1999 [267]
SHV-2	<i>Klebsiella pneumoniae</i>	1N9B	A	2be	Nukaga <i>et al.</i> , 2003 [268]
PER-1	<i>Pseudomonas aeruginosa</i>	1E25	A	2be	Tranier <i>et al.</i> , 2000 [269]
CTX-M-9	<i>Escherichia coli</i>	2P74	A	NA [†]	Chen <i>et al.</i> , 2007 [270]
CTX-M-14	<i>Escherichia coli</i>	1YLZ	A	NA	Chen <i>et al.</i> , 2005 [271]
CTX-M-27	<i>Escherichia coli</i>	1YLP	A	NA	Chen <i>et al.</i> , 2005 [272]
Toho-1 (CTX-M-44)	<i>Escherichia coli</i>	1IYS	A	2be	Ibuka <i>et al.</i> , 2003 [273]
PSE-4	<i>Pseudomonas aeruginosa</i>	1G68	A	2c	Lim <i>et al.</i> , 2001 [274]
BS3	<i>Bacillus licheniformis</i>	1I2W	A	NA	Fonze <i>et al.</i> , 2002 [275]
L2	<i>Stenotrophomonas maltophilia</i>	1O7E	A	2e	Petrella <i>et al.</i> , 2004 [unpublished]
KPC-2	<i>Klebsiella pneumoniae</i>	2OV5	A	2f	Ke <i>et al.</i> , 2007 [276]
SME-1	<i>Serratia marcescens</i>	1DY6	A	2f	Sougakoff <i>et al.</i> , 2002 [277]
NMC-A	<i>Enterobacter cloacae</i>	1BUE	A	2f	Swaren <i>et al.</i> , 1998 [278]
GES-1	<i>Klebsiella pneumoniae</i>	2QPN	A	NA	Smith <i>et al.</i> , 2007 [279]
OXY-1-1	<i>Klebsiella oxytoca</i>	3BYD	A	NA	Liang <i>et al.</i> , 2009 [unpublished]
SED-1	<i>Citrobacter sedlakii</i>	3BFE	A	NA	Pernot <i>et al.</i> , 2007 [unpublished]
BCII	<i>Bacillus cereus</i>	2BFK	B1	3a	Davies <i>et al.</i> , 2005 [280]
CcrA	<i>Bacteroides fragilis</i>	2BMI	B1	3a	Carfi <i>et al.</i> , 1998 [281]
IMP-1	<i>Serratia marcescens</i>	1VGN	B1	3a	Kurosaki <i>et al.</i> , 2005 [282]
SPM-1	<i>Pseudomonas aeruginosa</i>	2FHX	B1	NA	Murphy <i>et al.</i> , 2006 [283]
VIM-2	<i>Pseudomonas aeruginosa</i>	2YZ3	B1	3a	Yamaguchi <i>et al.</i> , 2007 [284]
CphA	<i>Aeromonas hydrophila</i>	3F9O	B2	3b	Bebrone <i>et al.</i> , 2009 [285]
BJP-1	<i>Bradyrhizobium japonicum</i>	2GMN	B3	NA	Stoczko <i>et al.</i> , 2006 [286]
FEZ-1	<i>Fluoribacter gormanii</i>	1K07	B3	3a	Garcia-Saez <i>et al.</i> , 2003 [287]
L1	<i>Stenotrophomonas maltophilia</i>	1SML	B3	3a	Ullah <i>et al.</i> , 1998 [288]
AmpC	<i>Escherichia coli</i>	3GQZ	C	1	Teotico <i>et al.</i> , 2009 [289]
ACT-1	<i>Klebsiella pneumoniae</i>	2ZC7	C	1	Shimizu-Ibuka <i>et al.</i> , 2008 [290]
CMY-2	<i>Klebsiella pneumoniae</i>	1ZC2	C	1	Bauvois <i>et al.</i> , 2006 [unpublished]
CMY-10	<i>Enterobacter aerogenes</i>	1ZKJ	C	1	Kim <i>et al.</i> , 2006 [291]
GC1	<i>Enterobacter cloacae</i>	1RGZ	C	1e	Nukaga <i>et al.</i> , 2004 [292]
P99	<i>Enterobacter cloacae</i>	1BLS	C	1	Lobkovsky <i>et al.</i> , 1993 [182]
OXA-1	<i>Escherichia coli</i>	1M6K	D	2d	Sun <i>et al.</i> , 2003 [189]
OXA-2	<i>Salmonella typhimurium</i>	1K38	D	2d	Kerff <i>et al.</i> , 2003 [unpublished]
OXA-10	<i>Pseudomonas aeruginosa</i>	1FOF	D	2d	Paetzel <i>et al.</i> , 2000 [170]
OXA-13	<i>Pseudomonas aeruginosa</i>	1H8Y	D	NA	Pernot <i>et al.</i> , 2001 [293]
OXA-24	<i>Acinetobacter baumannii</i>	2JC7	D	NA	Santillana <i>et al.</i> , 2007 [294]
OXA-46	<i>Pseudomonas aeruginosa</i>	3IF6	D	NA	Docquier <i>et al.</i> , 2010 [295]
OXA-48	<i>Klebsiella pneumoniae</i>	3HBR	D	2df	Docquier <i>et al.</i> , 2009 [187]

*http://www.rcsb.org, [§]http://www.lahey.org/studies, [†]NA: not available.

specific or wide spectrum β -lactam antibiotics. Thereafter, the prediction of the efficacy of these newly designed antibiotics could be virtually assessed against modeled structures *via* molecular docking.

It is likely that PBPs and β -lactamases will continue to develop resistance to newly-developed β -lactams. This widely acknowledged perception therefore warrants interest in understanding their mechanisms of resistance. It is also important to consider the possible changes in the functions of these enzymes when they evolve to circumvent the effect of new antibiotics [203, 240]. For example, studies on the resistant strains of *S. pneumoniae* showed that their resistance to β -lactams was attributed to modified PBPs, and this resulted in the synthesis of abnormal peptidoglycan cell wall [143]. It was postulated that the altered active sites of these PBPs might have changed their substrate preferences, leading to a different peptidoglycan structure in the host organism. It would be conspicuous to probe into the molecular changes in the PBPs which eluded inhibition by newly developed β -lactams, yet retained the ability to synthesize peptidoglycan, albeit with unusual performance. In turn, β -lactams in the next generation would have to be designed to fit into the catalytic grooves of the altered PBPs in the emerged resistant strains, in order to inhibit the pathogen's cell wall synthesis. In another example, studies on β -lactamases showed that mutations in these enzymes could result in the alteration of their substrate preferences or inhibitor susceptibilities [21, 158]. Therefore, it may also be possible to use earlier β -lactams to treat infectious bacteria harboring mutated β -lactamases. In time, new therapies using β -lactam antibiotics can exploit "old" β -lactams to treat highly resistant pathogens, targeting their evolved resistance factors.

Since the introduction of β -lactam antibiotics as chemotherapeutics, the emergence of β -lactam-resistant pathogens has been a grave concern in the clinical treatment of infectious diseases [241]. Nevertheless, continued efforts in the synthesis of new cephalosporins effective against such evolving and highly resistant pathogens have again illustrated the usefulness of this five decade-old compound. Other than using cephalosporins as antibacterial drugs, a recent screening effort has also identified ceftriaxone and other β -lactams as activators for the expression of the excitatory amino acid transporter in humans, which is important for preventing glutamate neurotoxicity [242]. This has invoked intensive discussion on the "re-discovery" of other possible applications of β -lactam antibiotics. The obvious advantage is that many of these β -lactam antibiotics have already been demonstrated to be non-toxic to humans [243-247]. Another application of cephalosporins is to use them as substrates for the detection of β -lactamase reporter activity in protein-protein interaction studies. This can be achieved by attaching fluorogenic or chromogenic moieties to the C3 or C7 positions of the cephalosporin core structure [191]. Summarily, the usefulness and wide applications of cephalosporins in healthcare and scientific research have been well illustrated over 50 decades since its discovery, and deserves continued support and exploitation.

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