The β-lactamase cycle: a tale of selective pressure and bacterial ingenuity

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1 Introduction
1.1 DD-Transpeptidases and β-lactamases

The emergence of antibiotic resistant pathogenic bacteria is becoming an increasingly worrying clinical problem.1 It has been caused by the widespread use, and sometimes abuse, of antibiotics not only in medical practice but also in animal husbandry.2 The case of β-lactam compounds in particular is illustrative. They are the most widely used antibacterial agents and owe their success to their high efficiency and unusual absence of secondary effects in higher organisms. They inactivate specific bacterial targets (Fig. 1), the DD-transpeptidases, also called Penicillin Binding Proteins (or PBPs) which catalyse an essential step in the biosynthesis of the bacterial cell wall, a reaction without equivalent in the eukaryotic world.3–6 These enzymes are membrane-bound, but exert their activity on the outer face of the cytoplasmic membrane, so that potential inactivators can reach them without having to cross this protective and highly impermeable layer, which represents a clear advantage for their antibacterial efficiency.

Bacteria have developed several strategies for escaping the activity of lethal compounds;5,7–9 enzymatic destruction, decrease of the target sensitivity, modification of the diffusion barrier(s) and active efflux systems. They utilise them all to fight β-lactams but the most common of these resistance mechanisms is the synthesis of β-lactamases,1,10–12 enzymes which are usually secreted into the outer medium by Gram-positive species and into the periplasm by their Gram-negative counterparts. They hydrolyse the β-lactam ring (Fig. 1), thus precluding further reaction with the PBPs. Other bacteria, which appear to be unable to produce an adequate β-lactamase, have acquired penicillin-resistant DD-transpeptidases.3,5,13–15 Although these strains, including the (in)famous methicillin-resistant Staphylococcus aureus (MRSA), are often responsible for chemotherapeutic failures, mainly in the hospital environment, they remain a minority when compared to β-lactamase producers and will not be further discussed here (for more details, see references 3, 5, 13–15). In Gram-negative strains, modification of the outer membrane permeability16 and, more recently, efflux mechanisms9 have been shown to contribute to resistance phenomena. Their effects are strongly increased by the concomitant presence of one or several β-lactamases in the periplasm.17–19

Benzylenicillin, the first compound in clinical use, was active mainly against Gram-positive bacteria and very sensitive to the staphylococcal β-lactamases, so that the proportion of resistant S. aureus strains increased rapidly. New compounds were then introduced, either isolated from natural sources or synthesized de novo, with enlarged antibacterial activity spectra and increased resistance to the staphylococcal β-lactamases. Although S. aureus strains have failed, after 50 years of selective pressure, to acquire modified or new β-lactamases capable of hydrolysing methicillin or cephalosporins, the number of other pathogens producing one or more β-lactamases

has not ceased to increase over the years. The pharmaceutical industry responded by introducing new compounds resistant to the activity of the most common enzymes. This was consistently followed by the appearance of new, or modified β-lactamases exhibiting enlarged specificity spectra, thus initiating and fuelling the ‘β-lactamase cycle’ and leading to the present diversity of β-lactam structures (Fig. 2), recently and extensively reviewed by Burton et al. Presently, more than 200 different β-lactamases have been described, exhibiting a wide range of primary structures and catalytic properties. Despite this diversity, the enzymes can be divided on the basis of their amino acid sequences into only four classes, A, B, C and D. 

1.2 Active-site serine and metallo-β-lactamases

Class B β-lactamases are metallo-proteins. Although in vitro experiments have shown that the Co\(^{2+}\) and Cd\(^{2+}\) derivatives are enzymatically active, it seems that the naturally occurring cation is always Zn\(^{2+}\). For more than 20 years, these enzymes were considered as mere biochemical curiosities, since the only identified producer was *Bacillus cereus*, an organism of little clinical relevance. However, several pathogens are now known to synthesize members of this class which exhibit a very large activity spectrum. In particular, the metallo-enzymes are not sensitive to the common β-lactamase inactivators and always hydrolyse carbapenems, a family of β-lactams that most often escapes the activity of the more widespread class A, C and D enzymes. The fact that some of the metallo-β-lactamase genes are plasmid-encoded understandably represents an additional cause of concern.

The members of the three other classes, A, C and D, are active-site serine enzymes and can be distinguished on the basis of their primary structures. Their catalytic pathways involve the formation of acyl-enzymes, similar to those observed with the PBPs, according to a three-step model shown in eqn. (1): 

\[
E + P(s) \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EC \underset{k_2}{\rightarrow} EC* \underset{k_3}{\rightarrow} E + P(s)
\]

where E is the enzyme, C the antibiotic, EC a non-covalent Henri–Michaelis complex, EC* a covalent acyl-enzyme and P(s) the inactive degradation product(s) of the antibiotic (see Fig. 1).

However, these covalent intermediates are generally extremely unstable and are rapidly hydrolysed, regenerating the free enzyme (Fig. 1). Not surprisingly, their specificity profiles depend on the rates of acylation and deacylation and, with their best substrates, some of these enzymes seem to be close to catalytic perfection (see also section 2.1), the rate of reaction being limited only by that of diffusion of the β-lactam to the enzyme active site.

The three-dimensional structures of several PBPs and β-lactamases of classes A and C have been determined by X-ray crystallography (see references in the text). There are striking similarities in the organisation of the secondary structure elements, despite very low degrees of sequence similarity. Moreover, the residues which surround the active-site serine and exhibit similar chemical functionalities are located in corresponding positions. These structural and functional conserved elements, described in Table 1, are also found in the sequences of penicillin-recognizing enzymes of unknown threedimensional structures, including the class D β-lactamases and the large number of PBPs whose primary structures have been deduced from the corresponding gene sequences as will be seen below, however, it is not yet clear if these conserved elements play identical roles in the catalytic mechanisms of the various groups of enzymes.

2 Class A β-lactamases

Of all the active site serine β-lactamases, class A enzymes are the most numerous and the best studied. A very large number of these enzymes have been reported and more than 45 sequences determined. The structures of five class A enzymes (Fig. 3) have been obtained by X-ray crystallography and the function of many residues has been probed by site-directed mutagenesis. The catalytic properties and primary structures of class A β-lactamases differ considerably, making them a highly diverse class.

These medium-sized proteins (M, about 29 000) show a wide distribution of pl values, ranging from ~3.5 to ~10. Of their 260–280 residues, it appears today that only nine residues are strictly conserved. Four of them (Ser-70, Lys-73, Ser-130, Glu-166; Fig. 4) are essential residues for catalysis, whereas five other residues (Gly-45, Pro-107, Asp-131, Ala-134 and Gly-236) are conserved most probably for structural reasons. Additionally, residues at positions 132 (Asn in most sequences), 234 (Lys or Arg), and 235 (Ser or Thr) have also been shown to be important for the enzyme activity.
Table 1  The three equivalent structural and functional elements of penicillin-recognizing enzymes. The active-site serine is indicated by *

<table>
<thead>
<tr>
<th>Element 1</th>
<th>Element 2</th>
<th>Element 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class A</td>
<td>70</td>
<td>234</td>
</tr>
<tr>
<td>Ser*-Xaa-Xaa-Lys</td>
<td>Ser-Asp-Asn</td>
<td>Lys-Thr-Gly</td>
</tr>
<tr>
<td></td>
<td>Ser-Asp-Ser</td>
<td>Lys-Ser-Gly</td>
</tr>
<tr>
<td></td>
<td>Ser-Asp-Gly</td>
<td>Arg-Thr-Gly</td>
</tr>
<tr>
<td>Class C</td>
<td>64</td>
<td>314</td>
</tr>
<tr>
<td>Ser*-Xaa-Ser-Lys</td>
<td>Tyr-Ala-Asn</td>
<td>Lys-Thr-Gly</td>
</tr>
<tr>
<td></td>
<td>Tyr-Ser-Asn</td>
<td>Lys-Thr-Gly</td>
</tr>
<tr>
<td>Class D</td>
<td>70</td>
<td>214</td>
</tr>
<tr>
<td>Ser*-Xaa-Xaa-Lys</td>
<td>Tyr-Gly-Asn</td>
<td>Lys-Thr-Gly</td>
</tr>
<tr>
<td>S. R61 DD-peptidase</td>
<td>62</td>
<td>298</td>
</tr>
<tr>
<td>Ser*-Val-Thr-Lys</td>
<td>Tyr-Ser-Asn</td>
<td>His-Thr-Gly</td>
</tr>
<tr>
<td>Other known PBPs</td>
<td>Ser*-Xaa-Xaa-Lys</td>
<td>Lys-Thr-Gly</td>
</tr>
</tbody>
</table>

that are poorly inhibited by clavulanic acid; the very large group 2, which comprises ‘cephalosporinases’, ‘penicillinases’ and broad-spectrum β-lactamases that are generally well inhibited by specific mechanism-based inactivators; and group 3, which includes the 15 metallo-β-lactamases most of which hydrolyse a very broad spectrum of β-lactam antibiotics. The 32 enzymes of group 1 are most probably all class C enzymes, whereas the numerous (~140) enzymes in group 2 are subdivided into eight subgroups. The enzymes of known sequence belong to molecular classes A or D, with the 10 class D enzymes forming group 2d (cloxacillin-hydrolysing β-lactamases). The heterogeneity of group 2 in the Bush–Jacoby–Medeiros classification22 emphasizes the amazing diversity of the catalytic properties of class A β-lactamases.46

2.1 Kinetic characteristics

The high variability of the kinetic parameters of class A β-lactamases for a given β-lactam is clearly illustrated by Table 2. The values of both \( k_{cat}/K_m \) and \( k_{cat} \), which taken together give the genuine catalytic efficiency of the enzyme,40 are shown (note that high \( k_{cat}/K_m \) values can be due to very low values of both \( k_{cat} \) and \( K_m \)). Thus, if one excepts the TEM-7, Proteus vulgaris and IMI-1 enzymes, the \( k_{cat}/K_m \) values of class A (2800–84000 mM\(^{-1}\) s\(^{-1}\)) and class C (1200–75000 mM\(^{-1}\) s\(^{-1}\)) towards benzylpenicillin and ampicillin fall within the same range, but the \( k_{cat} \) values of the class C enzymes (0.5–75 s\(^{-1}\)) are substantially lower than those of class A enzymes (130–3900 s\(^{-1}\)), highlighting a major difference between these enzymes at the level of the deacylation step.46 A careful examination of these data reveals some deficiencies in the classification of Bush et al.22 For instance, the β-lactamase of S. aureus PC1 appears to be a very poor ‘cephalosporinase’, and displays a remarkably low \( k_{cat}/K_m \) value for oxacillin when compared with two enzymes (Bacillus licheniformis and Streptomyces albus G) classified in the same group (2a). Similarly, the Serratia fontalica and TEM-7 enzymes, both in group 2b, show markedly different values of the kinetic parameters obtained with most compounds, particularly cephalosporins. Finally, and as pointed out by Bush and co-workers,22 the class A β-lactamase of Actinomadura R39 had to be included in group 2d (class D ‘oxacillinases’) to account for its high activity against cloxacillin and oxacillin. Thus, although the Bush–Jacoby–Medeiros classification scheme is certainly useful for clinical purposes, it would appear that the catalytic properties of class A β-lactamases present a continuum, where only the extremes fall into clearly distinct groups.46

Table 2 indicates that with their best substrates, the interactions are characterized by very high values of both \( k_{cat}/K_m \) (close to the diffusion limit, i.e. 10 \( 8 \) M\(^{-1}\) s\(^{-1}\)), and \( k_{cat} \) (up

TEM-1 and lactamases are 'fully efficient enzymes'.

Avoid the activity of these enzymes by using the discovery or synthesis of mechanism-based inactivators of most class A clavulanic acid, sulbactam or tazobactam (Fig. 2), which behave to inhibit the catalytic activity of retaining antibacterial activity. The second strategy tries to compounds that are resistant to their hydrolytic action while by four different class A enzymes (I, B. cereus I, B. licheniformis, TEM-1 and S. aureus PC1), are similar. This, together with the high values for both kcat/Km and kcat, suggested that these β-lactamases are ‘fully efficient enzymes’.

2.2 Fighting the β-lactamases

Two fundamentally different strategies have been devised in the fight against β-lactamases. The first strategy attempts to avoid the activity of these enzymes by using β-lactam compounds that are resistant to their hydrolytic action while retaining antibacterial activity. The second strategy tries to inhibit the catalytic activity of β-lactamases, and this relies on the discovery or synthesis of β-lactam compounds such as clavulanic acid, sulbactam or tazobactam (Fig. 2), which behave as mechanism-based inactivators of most class A β-lactamases. Since they have little antibiotic activity per se, these compounds are administered in combination with classical β-lactamate-sensitive compounds. Thus, clavulanic acid has been widely used (Augmentin) to increase the efficiency of amoxyccilin against pathogenic strains producing the most common plasmid-encoded β-lactamases, i.e. the SHV and TEM class A enzymes. However, in the past two decades, the clinical use of such drugs has been responsible for the appearance of an increasing number of strains exhibiting resistance to the ‘β-lactamase-stable’ compounds (first strategy) and, more recently, to the β-lactamase inactivator-classical β-lactam combinations (second strategy).

2.3 Hydrolysis of the ‘β-lactamase-stable’ compounds

Several compounds such as cefotaxime, ceftazidime and aztreonam have been characterized as ‘β-lactamase-stable.’ Although this statement can often be misleading as their stability is neither absolute nor general, these compounds have been successfully used as antibacterial agents, displaying high efficiency against most strains producing either the chromosome-encoded class C enzymes, or the ubiquitous SHV-
1 and TEM-1 enzymes. However, within a few years of their introduction in the therapeutic arsenal, highly resistant strains were detected in the hospital environment. In most cases, this could be attributed to the production of new enzymes (usually referred to as ‘extended-spectrum’ β-lactamases) with modified substrate profiles, the large majority of which belong to the SHV and TEM families.5,54 At least 11 SHV and 29 TEM variants have now been identified, which differ from the parent enzymes by a very limited number of amino acid substitutions.5,54–57

Not surprisingly, most of the residues involved in the extended-spectrum properties of these enzymes were found to be located in close proximity to the active site cavity. However, none of the modified side chains appear to be directly involved in the catalytic mechanism of class A β-lactamases (section 2.5). Nevertheless, these mutations which extend the substrate profile of the variants often concomitantly lower the catalytic efficiencies against the classical, good substrates of the parent enzymes.48,58

Various attempts, resting on the known three-dimensional structures of related enzymes or, more reliably, of the TEM-1 parent enzyme, were made to explain the observed altered specificities of the mutants on the basis of structural variations. These studies (reviewed in refs. 40, 45, 59) drew attention to both the effect of individual mutations (particularly at positions 164 and 238) and the spectacular synergistic effects obtained by combining two or three mutations. For instance, molecular modelling studies of some of the TEM variants48 underlined the importance of the mobility of the Ω-loop. In most class A enzymes, this structural element is stabilized by a salt bridge between Arg-164 and Asn-179, thus ensuring the optimal positioning of the essential Glu-166 carboxylate, but also creating steric difficulties for the entry of cephalosporins with large oximino side chains (third-generation cephalosporins, e.g. cefotaxime and ceftriaxone; Fig. 2d,f) into the active site cavity.48 Substitution of Arg-164 by uncharged residues in some of the TEM mutants would be expected to increase the loop conformational freedom and hence allow more room for better accommodation of these compounds into the active site, but also to decrease their activity against the best substrates as a consequence of the poorer orientation of Glu-166.48

Several enzymes unrelated to the TEM and SHV families (e.g. Serratia fonticola, Proteus vulgaris and IMI-1) have been described which also exhibit relatively high activities against third generation cephalosporins (Table 2). Although the strategy is sometimes reminiscent of that observed in the TEM family (e.g. a hydroxylated residue at position 237 and, in two cases, at position 238), different modifications also appear to confer similar specificity profiles. A striking illustration of this is found in three recently reported enzymes, NmcA, IMI-1 and Sme-1, which share about 70% sequence identity61 and which are very broad spectrum β-lactamases, hydrolysing efficiently both classical penams and cephamems but also a wide range of β-lactam substrates usually considered as resistant to class A enzymes. In particular, these enzymes (often referred to as ‘carbapenemases’) confer resistance to imipenem and related carbapenem antibiotics (Fig. 2i), which are very efficient antibiotics, often used as a last resort for patients in intensive care units. The presence of a disulfide bridge between Cys-69 and Cys-238, which creates a new covalent bond between the two domains and thus significantly modifies the active site geometry (Fig. 5), is thought to be responsible, at least partially, for the high catalytic efficiency of these enzymes against imipenem.62

The genes coding for these enzymes can be considered as additions to the ‘pool of resistance genes’, some of which are expected to disseminate within the microbial populations by a variety of gene transfer mechanisms.1 Hence, following the introduction of a new compound, resistant strains can either recruit ‘original’ enzymes, quite different from those which are already well recognized, or ‘engineer’ variants derived from the latter by a few point mutations.59

2.4 Resistance to mechanism-based inactivators

The extended-spectrum β-lactamases described above fortunately remain sensitive to β-lactamase inactivators such as clavulanic acid, sulbactam and tazobactam (Fig. 2c,e). However, bacterial susceptibility to combinations of these compounds with potent β-lactam antibiotics has recently been challenged by the spontaneous appearance of TEM-type β-lactamases that are resistant to the mechanism-based inactivators. The inhibitor-resistant TEM β-lactamases differ from TEM-1/2 by one, two or three residue substitutions at new locations, which decrease the affinity for β-lactam substrates and alter the inhibitory action of suicide substrates such as clavulanic acid (see e.g. refs. 63, 64).

The important mutations (positions 69, 244 and 276; reviewed in refs. 40, 45, 60, 65) in these TEM variants significantly decrease the affinity for the inactivator. Moreover, replacement of Arg-244 whose guanidine side chain plays a critical role in the inactivation mechanism,66 or mutations at positions 69 or 276 which influence the positioning of Arg-244 in the active site, significantly hinder the inactivation process.

2.5 Catalytic mechanism

On the basis of the mechanism generally accepted for active site serine proteases,67 the active site of β-lactamases would be expected to contain both an ‘oxyanion hole’, involved in the stabilization of the tetrahedral intermediates, and a general base, which abstracts the proton from the serine hydroxy group. Although there is now a large body of evidence for similar oxyanion holes in all the active site serine penicillin-recognizing enzymes, formed by the main chain amino group of Ser-70 and Ala-237 in the class A enzymes, Ser-64 and Ser/Ala-318 in class C, and Ser-62 and Thr-301 in the Streptomyces R61 DD-peptidase,35,68–74 the nature of the specific residue that enhances the nucleophilicity of the active site serine hydroxy group remains unclear.

The most controversial situation prevails for class A \(\beta\)-lactamases, in which two distinct residues have been proposed as potential general bases (Fig. 6). In one hypothesis,79,75–77 this role is played by the conserved Glu-166. Its importance in the catalytic process was revealed by both crystallographic71,78 and mutagenesis75,79 studies. The detailed study of the E166D mutant of the \(B. cereus\) 569/H \(\beta\)-lactamase75 indicated that both the \(k_2\) and \(k_1\) first-order rate constants are decreased by the same (ca. 2000 fold) factor, hence suggesting a similar role for this residue in both the acylation and deacetylation processes. The crystallographic data however indicate that the Glu-166 and Ser-70 side chains are too distant to allow direct proton transfer between them. But the crystallographic and molecular modelling data76 have suggested that a conserved water molecule (W1), which forms a bridge between the Ser-70 hydroxy group and the Glu-166 carboxylate group (Fig. 4), might serve as a relay in this proton transfer. Alternatively, this might be achieved directly, as a result of the flexibility of the \(\Omega\)-loop.80

The activated \(O_{\gamma}\) of Ser-70 can then attack the \(\beta\)-lactam carbonyl carbon on the well exposed \(\alpha\)-face of the molecule and the proton would be delivered back to the leaving nitrogen atom of the \(\beta\)-lactam via a network of hydrogen bonds involving the \(\varepsilon\)-amino groups of Lys-73 and Lys-234, a second water molecule (W2), and the hydroxy group of Ser-130 which acts as the ultimate proton donor.76,77 This hypothesis concerning the role of Ser-130 has recently been further strengthened by the detailed analysis of the crystal structure of a phosphonate complex of the TEM-1 \(\beta\)-lactamase.81 Hydrolysis of the acyl-enzyme would subsequently occur according to a symmetrical mechanism where Glu-166 would activate the hydrolytic water molecule (W1) to attack the carbonyl carbon of the acyl-enzyme intermediate involves the transfer of a proton from the general base to the \(\beta\)-lactam nitrogens (dashed lines) via a pathway in which the Ser-130 hydroxy acts as the ultimate acceptor/donor. For detailed mechanisms, see references 76 (a) and 70 (b).

Fig. 6 Putative mechanisms for the formation of the tetrahedral intermediate in the acylation of class A \(\beta\)-lactamases. The role of general base is fulfilled by Glu-166 (a) or Lys-73 (b). In both cases, the breakdown of the tetrahedral intermediate involves the transfer of a proton from the general base to the \(\beta\)-lactam nitrogens (dashed lines) via a pathway in which the Ser-130 hydroxy acts as the ultimate acceptor/donor. For detailed mechanisms, see references 76 (a) and 70 (b).

The second hypothesis70 assumes an non-symmetrical mechanism, with two different general bases, Lys-73 and Glu-166, participating in acylation and deacylation, respectively. In this mechanism, the enzyme active site would provide a favourable local environment, namely a very positive electric field that strongly reduces the \(pK_a\) value of the alkylammonium group of Lys-73 (5–6 pH units), enabling the lysine to remain unprotonated at neutral pH. However, this electrostatic argument suffers from the fact that in class A enzymes the carbonylate group of Glu-166 is much closer to the \(\varepsilon\)-amino group of Lys-73 than is any positively charged side-chain.44 Moreover, NMR titration of the 13C-labelled lysine residues in the TEM-1 \(\beta\)-lactamase62 indicated a ‘normal’ \(pK_a\) value (\(\geq 10\)) for the Lys-73 side chain, making it a very unlikely candidate for proton abstraction in catalysis. These experimental findings have recently been further strengthened by a calculation of the \(pK_a\) values (\(\geq 10\)) for Lys-73 in two class A enzymes (TEM-I and \(B. licheniformis\)).83

Several kinetic studies performed with Glu-166 mutants of various class A \(\beta\)-lactamases (see e.g. refs. 75, 84) indicated that both acylation and deacylation rates appear to be decreased by the mutation, deacylation being sometimes more affected than acylation. Moreover, the K73R mutation in the the \(B. cereus\) enzyme causes a \(\sim 100\) fold decrease of the value of \(k_2\) for benzylpenicillin hydrolysis, while its E166D counterpart causes a \(\sim 2000\) fold decrease of the values of both \(k_2\) and \(k_3\), an observation which seems to indicate a more important role for Glu-166 than for Lys-73 in the acylation step.

The first hypothesis, where both acylation and deacylation involve Glu-166 acting as a general base via a conserved water molecule, is further strengthened by kinetic and modelling studies47,48,85–87 of \(\beta\)-lactam compounds bearing a methoxy (cefoxitin, moxalactam and temocillin; Fig. 2h,j) or a hydroxyl (imipenem; Fig. 2i) substituent side chain on the \(\alpha\)-face of the \(\beta\)-lactam ring, or also an oximino moiety on the C-7 side chain of cephalexinoros (ceftotaxime, cefazidime and cefepime; Fig. 2d,f). These molecules acylate the active serine of most class A enzymes with exceedingly poor efficiency (Table 2), and whenever acylation occurs, deacylation is generally even slower, both being several orders of magnitude below the rates observed with good substrates. Docking of all these molecules in the active site of class A \(\beta\)-lactamases indicates that they do not perturb the hydrogen bond between Lys-73 and Ser-70 but that their bulky substituents induce either a bad positioning of the Glu-166 carboxylate side chain or a displacement of the catalytic water molecule (both in some cases), thus making both acylation and deacylation very unlikely as expected from the symmetrical mechanism involving Glu-166.

Thus it seems that present evidence substantiates the view that Glu-166 is the genuine general base catalyst in both formation and hydrolysis of the acyl-enzyme intermediate formed with class A \(\beta\)-lactamases. A more detailed discussion of the many available data can be found in reference.40

3 Class C \(\beta\)-lactamases

The isolation and characterization of stable acylated-serine enzyme complexes by biochemical techniques88–90 and recently the direct observation of the acyl-enzyme intermediate with the help of electrospray mass spectrometry91 have firmly established class C \(\beta\)-lactamases as active-site serine enzymes.

They are monomeric proteins produced exclusively by Gram-negative bacteria and are usually located in the periplasmic space. Compared to the diversified class A enzymes, they form a more homogeneous family with a molecular mass of about 39 kDa. Class C enzymes represent the group I \(\beta\)-lactamases of the Bush–Jacoby–Medeiros classification.22

Class C \(\beta\)-lactamases confer resistance not only to classical compounds such as ampicillin and cephalothin but also to third generation cephalosporins when high-level enzyme production is achieved in derepressed mutants.92–94 Primary structure alignments show a high homogeneity with no less than 35% of identical residues between any two members and about 59 strictly conserved residues in the 22 known primary structures aligned.
3.1 Bacteria producing class C enzymes

With the exception of the Pseudomonas and Aeromonas species, they all belong to the Enterobacteriaceae family. Bacteria in this family include specific pathogens associated with a wide variety of human infectious diseases (Shigella, Salmonella, Citrobacter, Yersinia) while others are found among the natural colonizers of the human gastrointestinal tract (Escherichia, Klebsiella, Enterobacter) but can behave as dangerous opportunistic pathogens. Pseudomonas aeruginosa is sometimes found in the normal human microbial flora, but it usually colonizes the moist sites on the human body and is also a well-known factor in human diseases. All these organisms constitute the most prevalent causes of nosocomial infections.\(^9\)

Aeromonas sp. are environmental bacteria widely distributed in stagnant and flowing fresh waters, in fish tanks, in water supplies (even chlorinated ones) and in sewage. They are responsible for both human and animal infections.

The gene encoding class C enzymes was first found on the chromosome of Gram-negative bacteria,\(^24\),\(^96\)–\(^102\) but more recently many plasmid-mediated forms (MIR,\(^103\) MOX,\(^104\) BIL,\(^105\) FOX,\(^106\)–\(^108\) LAT,\(^109\),\(^110\) CMY,\(^111\),\(^112\) DHA,\(^113\) ACT\(^114\) and BlaMOR\(^115\)), have been isolated from geographically distant locations. This represents a serious clinical problem because plasmids can readily spread through pathogenic bacteria and, in addition, new variants of these enzymes are likely to appear under the selective pressure of the recently introduced \(\beta\)-lactam antibiotics.\(^116\) A similar phenomenon has already been observed with the emergence of the modified forms of plasmid-mediated class A (see above) and D \(\beta\)-lactamases which exhibit an extended spectrum of activity against aztreonam, cefotaxime, ceftazidime, ceftriaxone and lactamases which exhibit an extended spectrum of activity.

Table 3. Comparison of the kinetic parameters \(k_\text{cat}(s^{-1})\) and \(K_m(M^{-1} s^{-1})\) for the hydrolysis of some characteristic \(\beta\)-lactams by various class C \(\beta\)-lactamases

<table>
<thead>
<tr>
<th>Organism</th>
<th>PG(^a)</th>
<th>AMPI</th>
<th>CARB</th>
<th>OXA</th>
<th>CR</th>
<th>CT</th>
<th>CTX</th>
<th>CAZ</th>
<th>AZT</th>
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<th>CLA</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0.2</td>
<td>0.003</td>
<td>0.006</td>
<td>700</td>
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<td>0.0002</td>
<td>0.003</td>
<td>0.06</td>
<td>0.2</td>
</tr>
<tr>
<td>C. freundii</td>
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<td>6.5</td>
<td>0.002</td>
<td>0.005</td>
<td>700</td>
<td>210</td>
<td>0.017</td>
<td>ND</td>
<td>0.0002</td>
<td>0.016</td>
<td>0.32</td>
<td>ND</td>
</tr>
<tr>
<td>OS60</td>
<td>75</td>
<td>30.000</td>
<td>7000</td>
<td>12 000</td>
<td>20000</td>
<td>16 000</td>
<td>3400</td>
<td>ND</td>
<td>180</td>
<td>140</td>
<td>1300</td>
<td>ND</td>
</tr>
<tr>
<td>E. coli K12</td>
<td>45</td>
<td>4.2</td>
<td>0.004</td>
<td>0.003</td>
<td>700</td>
<td>300</td>
<td>0.17</td>
<td>ND</td>
<td>0.00016</td>
<td>0.01</td>
<td>0.2</td>
<td>0.15</td>
</tr>
<tr>
<td>S. marcescens</td>
<td>10</td>
<td>1200</td>
<td>230</td>
<td>ND</td>
<td>760</td>
<td>100</td>
<td>135</td>
<td>ND</td>
<td>135</td>
<td>11.2</td>
<td>300</td>
<td>0.017</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>76</td>
<td>4.4</td>
<td>0.005</td>
<td>0.004</td>
<td>110</td>
<td>430</td>
<td>0.15</td>
<td>ND</td>
<td>0.0023</td>
<td>0.03</td>
<td>0.12</td>
<td>ND</td>
</tr>
<tr>
<td>18SH</td>
<td>45</td>
<td>9000</td>
<td>240</td>
<td>1600</td>
<td>5000</td>
<td>17 000</td>
<td>7500</td>
<td>ND</td>
<td>58</td>
<td>88</td>
<td>2400</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(^a\) PG, benzylpenicillin; AMPI, ampicillin; CARB, carbenicillin; OXA, oxacillin; CR, cephaloridine; CT, cephalexin; CTX, cefotaxime; CAZ, ceftazidime; AZT, aztreonam; IMI, imipenem; COX, cefoxitin; CLA, clavulanic acid. All data are from Galleni and Frère\(^119\) and Galleni et al.\(^129\) ND, not determined.

3.2 Kinetic properties

Class C \(\beta\)-lactamases are often referred to as ‘cephalosporinases’ while class A are ‘penicillinases’, a distinction originally based on their relative hydrolytic activities (\(V\)) towards the various compounds. Today this distinction appears to be of little value as the large body of data available indicates that the kinetic parameters can vary largely within the same class, but also can be very similar between enzymes belonging to different classes.

With penicillins, class C enzymes show rather low values of both \(k_\text{cat}\) and \(K_m\), which means in practice that they are readily saturated to form acyl-enzymes which hydrolyse relatively slowly. Although the \(k_\text{cat}/K_m\) ratios are not very different from those observed with class A enzymes (see Tables 2 and 3), hydrolysis of penicillins is actually much more efficient with the latter, due to higher turn-over numbers.

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3.3 Inhibitors and inactivators

Class C \(\beta\)-lactamases are competitively inhibited by boronic acids\(^124\) with which they form a complex analogous to the catalytic pathway tetrahedral intermediate,\(^125\) a property which can be utilised to purify these enzymes by affinity chromatography.\(^126\)

The interactions of class A and C enzymes with the mechanism-based inactivator clavulanic acid are also significantly different. Although both types of enzymes react through a complex branched pathway\(^127\),\(^128\) class C enzymes are not irreversibly inactivated by clavulanic acid even though the reactivation rate appears to be very slow. The \(k_\text{cat}/K\) values are at least 3 orders of magnitude lower than those for most class A enzymes, in part due to a high \(K\) value (around 10 mM). Clavulanic acid is thus a poor inactivator of class C \(\beta\)-lactamases because of low affinity, poor acylation rate and non-negligible turnover.

Another important difference is observed with cephemycins (e.g. cefoxitin, Fig. 2j) which contain a 7\(\alpha\)-methoxy substituent. Generally class A enzymes do not interact efficiently with this compound, because the \(\alpha\)-methoxy group displaces the catalytically important W1 water molecule in the active-site cavity.\(^125\) Conversely class C enzymes are readily acylated by cefoxitin, which they slowly hydrolyse.\(^129\) These differences in the catalytic properties of class A and C enzymes suggest somewhat different ‘catalytic machineries’

3.4 Catalytic mechanism

In 1990, Oefner and co-workers,\(^68\) on the basis of the refined three-dimensional structures of the class C \(\beta\)-lactamase from...
Citrobacter freundii and of the acyl-enzyme formed with aztreonam (Fig. 2g), hypothesized that Tyr-150 (Fig. 7) could function as the general base during catalysis. Phosphonate monoesters are specific inhibitors of serine \( \beta \)-lactamases.\(^{130,131} \) They react through the phosphorylation of the active-site serine to form a tetrahedral intermediate analog.\(^{132} \) Inactivation of class C enzymes is relatively rapid (\( \sim 10^2 \text{M}^{-1} \text{s}^{-1} \)) whereas reactivation is very slow (\( \sim 10^{-6} \text{s}^{-1} \)).\(^{130} \) The crystal structure of a phosphonate derivative of the Enterobacter cloacae P99 \( \beta \)-lactamase was determined at 2.3 Å resolution.\(^{133} \) Careful examination of this structure also suggested that the phenolate form of Tyr-150 is the most likely candidate for the activation of the active site Ser-64 hydroxy group (Fig. 7).

Fig. 8 depicts the activating role played by Tyr-150 during the acylation (a) and deacylation (b) steps. In this model the carbonyl oxygen sits in the oxyanion hole,\(^{134} \) where it is hydrogen-bonded to the main-chain NH groups of Ser-64 and Ser-318. During catalysis, the alkylammonium group of Lys-315 would play an important role, its positive charge contributing to depress the pK\(_a\) of the Tyr-150 phenol which acts as the general base during catalysis. Modifications of this residue resulted in severe impairment of the enzyme activity suggesting the different, and more important, role played by this residue in class C enzymes. In turn, Lys-67, which is closer to the Ser-64 O\(_{\gamma}\), seems to play a mainly electrostatic and orientating role.\(^{139} \) Electrostatic calculations showed a net positive potential in the catalytic site, especially in the area where the carboxylic group of the \( \beta \)-lactam is expected to bind.\(^{133} \) The positive charges carried by the lysine side-chains and that due to the dipole of the \( \alpha \)-helix bearing the Ser-64 residue (Fig. 3B) may also serve to provide electrostatic stabilization of the tetrahedral oxyanion intermediate and assist in lowering the pK\(_a\) of Tyr-150.

Finally, mutagenesis experiments\(^{140} \) and close examination of the crystal structure of the P99 enzyme\(^{69} \) indicated that no equivalent of the class A Glu-166 residue could be found in class C \( \beta \)-lactamases. In conclusion, as in class A, it appears unlikely that the unprotonated amino group of Lys-67 acts as a general base.

![Simplified stereo view of the active site of the class C E. cloacae P99 \( \beta \)-lactamase. The important catalytic residues Ser-64, Lys-67, Tyr-150 and Lys-315 are numbered. Besides those of the backbone NH groups, the protons on the following groups are shown: the hydroxy groups of Ser-64, Lys-315, and the amide of Asn-152.](image)

3.5 Evolutionary considerations

These observations, added to the facts that class C \( \beta \)-lactamases catalyze acyl transfer reactions (a catalytic property more common to DD-peptidases) and that their deacylation mechanism is rate-limiting with many substrates, suggest that compared to class A, class C enzymes are a ‘primitive’ form of \( \beta \)-lactamases, evolutionarily closer to DD-peptidases than to other serine \( \beta \)-lactamases. Comparison of their overall three-dimensional structures also reveals some additional similarities, which tends to strengthen this concept.\(^{69} \) Surprisingly, replacement of the strictly conserved Thr-316 by Ala in a class C enzyme\(^{143} \) had little influence on the penicillinase activity, and only the activity against cephalosporins was significantly decreased. This bias was even more marked with the equivalent Ser-235 to Ala mutation in the TEM-1 enzyme.\(^{141,142} \) The corresponding mutation (Thr-299 to Val) in the S. R61 DD-peptidase\(^{143} \) resulted in an enzyme with markedly decreased activity towards peptide substrates and the transpeptidation reaction was totally abolished. Remarkably, the enzyme was again more affected in its interaction with cephalosporins than penicillins.\(^{143} \) This would suggest that during their evolution, the natural selective pressure exerted on the \( \beta \)-lactamase-producing organisms has been due to cephalosporins rather than penicillins.

4 Class D \( \beta \)-lactamases

Class D \( \beta \)-lactamases are monomeric proteins of 27 to 31 kDa, which are located in the periplasm of Gram negative bacteria.
No three-dimensional structure is available yet. Although they are generally plasmid-mediated, chromosome-mediated forms are also found in Aeromonas.\textsuperscript{144} Their existence has been known for a long time,\textsuperscript{145} but their identification as a new distinct molecular class came with the determination of the first primary structures in the late 1980s.\textsuperscript{146–148,25} Presently, at least nine primary structures have been determined and their phylogenetic relationship with the other classes of β-lactamases has been discussed.\textsuperscript{37,149}

In contrast to most class A and to all class C enzymes, class D β-lactamases present very different substrate profiles since oxacillin and cloxacillin are very efficiently hydrolysed, and hence they are usually considered as ‘oxacillins’. A comparative study of three members of this class showed that this behaviour was their most remarkable common feature.\textsuperscript{150} The discrepancy observed between the mass estimated by gel filtration and that calculated for the OXA-2 enzyme was not due to dimerization, as originally suggested.\textsuperscript{145}

Class D β-lactamases exhibit ‘burst’ or biphasic kinetics with many substrates. An extensive study\textsuperscript{149} of this phenomenon with the representative OXA-2 enzyme demonstrated this behaviour with most penicillins, cephalosporins, flomoxef and imipenem. Only oxacillin, ampicillin and cefaclor appeared to follow the classical Henri–Michaelis model. Mechanisms generally accepted as accounting for reversible partial inactivations by branches at either the free enzyme or the acyl-enzyme level failed to explain the dependence of the rate of substrate-induced inactivation upon substrate concentration.

Another distinct feature of these enzymes is their high homology with the membrane bound signal-sensing BLAR protein,\textsuperscript{26,37} an essential component of the β-lactamase-induction system in B. licheniformis (see section 6.3).

In terms of mechanistic characterization, very little work has been devoted to these enzymes and no site-directed mutagenesis study has been performed. In consequence, hypotheses about their catalytic mechanism rest on comparisons with those of the other serine β-lactamases. In this regard it is interesting to note that primary structure alignments highlight a conserved Glu/Tyr-Gly-Asn motif at locations equivalent to the Glu-166 and Tyr-150 of class A and C β-lactamases, respectively.\textsuperscript{151,26} However, only site-directed mutagenesis experiments and three-dimensional structure determination could confirm the putative catalytic role of these conserved residues.

A plasmidic extended-spectrum variant, OXA-15, with increased activity towards ceftazidime, cepirole, ceftriaxone, moxalactam and aztreonam was recently isolated and shown to arise from OXA-2 through a single amino-acid replacement.\textsuperscript{152} This represents another illustration of the adaptability of β-lactamases and of the continuous challenge they pose to the clinical world.

5 Class B β-lactamases

Zinc β-lactamases constitute a rather small group when compared to their much more numerous active-site serine counterparts. The first of these enzymes was isolated in 1966 from Bacillus cereus (BcII).\textsuperscript{153} At that time and during the two following decades, it was the only known example of a metallo-β-lactamase and it was considered as a biochemical curiosity. Unfortunately, the situation changed in the eighties. Indeed, similar Zn\textsuperscript{2+}-requiring enzymes were found in Stenotrophomonas maltophilia (Xanthomonas maltophilia)\textsuperscript{154} and in an increasing number of nosocomial strains such as Pseudomonas aeruginosa,\textsuperscript{30} Serratia marcescens,\textsuperscript{29} Klebsiella pneumoniae,\textsuperscript{155} Bacteroides fragilis,\textsuperscript{156,157} Aeromonas hydrophila\textsuperscript{158} and Chryseobacterium meningosepticum.\textsuperscript{159} To worsen the situation, many of these bacteria produce more than one type of β-lactamase.

5.1 Multiple β-lactamase production

The production of more than one β-lactamase represents a tremendous advantage for the strain which thereby becomes resistant to nearly all known β-lactam antibiotics. Various Aeromonas species such as A. hydrophila, A. jandei, A. salmonicida and A. sobriae, produce three different enzymes, class A, class D and class B β-lactamases.\textsuperscript{61,158,160,161} Other organisms, \textit{i.e.} B. cereus, B. fragilis and S. maltophilia, produce a chromosomal metallo-β-lactamase together with a serine β-lactamase.\textsuperscript{28} Thus, B. cereus secretes a class A β-lactamase (BcI) together with the metallo BcII enzyme.\textsuperscript{27} Furthermore, the production of the different enzymes can be induced by the presence of β-lactams in the media.\textsuperscript{160}

5.2 Activity profiles

A major characteristic of most metallo-β-lactamases is that they catalyse the hydrolysis of nearly all β-lactam antibiotics used for therapeutic purposes (see refs. 162–169; N. Laraki, M. Galleni and J. M. Frère, unpublished work). The $k_{cat}/K_m$ values observed with penicillins and cephalosporins are quite similar.\textsuperscript{153,162} With the latter, this was found to be independent of the nature of the C-3 substituent. However, esterification of the carboxylic acid group at position C-3 in penicillins and C-4 in cephalosporins decreases the catalytic efficiency of the B. cereus II β-lactamase by two orders of magnitude.\textsuperscript{170} The same phenomenon is observed when the carboxylic group of cephalosporins is modified in the corresponding lactone. Most class B enzymes exhibit high activity towards the ‘β-lactamase-stable’ compounds carabepenam, cephemycins and third-generation cephalosporins (Table 4). Monobactams are the sole β-lactam antibiotics to be poorly recognized. By contrast, the Zn\textsuperscript{2+}-β-lactamase produced by A. hydrophila (CphA) exhibits a very distinct activity profile. It specifically hydrolyses carbapenems but its catalytic efficiency towards all other β-lactams is rather poor; thus, it acts as a specific ‘carbapenemase’.

Moreover, cefoxitin and moxalactam behave as inactivators of the CphA enzyme, another unique observation among class B β-lactamases.\textsuperscript{162–164}

The β-lactamases produced by \textit{B. fragilis} cfaA (CfIA),\textsuperscript{166} S. maltophilia ULA511 (L1)\textsuperscript{162–164} and \textit{S. marcescens} TN9011 (IMP-1)\textsuperscript{29} are the most efficient enzymes. Their $k_{cat}/K_m$ values for penicillins and cephalosporins are of the same order of magnitude as those observed with BcII ($k_{cat}/K_m = 10^{-5}–10^{-6}$ M\textsuperscript{-1}·s\textsuperscript{-1}). They are the most active enzymes against compounds of the cephemycin (Fig. 2j) and oxacephemycin (Fig. 2h) families ($k_{cat}/K_m = 10^6$ M\textsuperscript{-1}·s\textsuperscript{-1} for L1 vs. 100 M\textsuperscript{-1}·s\textsuperscript{-1} for BcII). Tazobactam poorly inactivates these enzymes at very high molar ratios (&gt;10,000).\textsuperscript{171}

The presence of chelating agents, such as EDTA, EGTA, dipicolinic acid and o-1,10-phenanthroline not surprisingly inhibits the metallo-β-lactamases.\textsuperscript{153,172} The interactions between the enzymes and the chelating agent lead to the formation of ternary enzyme–metal–chelatant complexes, which slowly decay into apoenzyme and chelatant-metal complexes. In the case of the \textit{A. hydrophila} AE036 β-lactamase, EDTA is the most effective inactivating agent with a second order rate constant of 10000 M\textsuperscript{-1}·s\textsuperscript{-1}.\textsuperscript{172} The formation of the ternary complexes increases the difficulty of obtaining good preparations of apoenzymes. Preparation of the \textit{A. hydrophila} and \textit{B. fragilis} apoenzymes requires extensive and repeated dialysis steps of the protein solution against a ‘metal-free’ buffer, most often containing an immobilized complexing agent such as IDA-agarose.

Alignment of the amino-acid sequences of \textit{B. cereus} 569H BcII,\textsuperscript{173} \textit{B. fragilis} CfIA,\textsuperscript{157} S. maltophilia,\textsuperscript{174} \textit{A. hydrophila} AE036\textsuperscript{158} and \textit{S. marcescens} TN9011\textsuperscript{29} indicates a rather low degree of isology (Fig. 9). In this comparison, it should be kept in mind that the \textit{S. marcescens}, \textit{P. aeruginosa} (N. Laraki, M. \textit{Nat. Prod. Rep.}, 1999, \textit{16}, 1–19

9
Table 4 Comparison of the kinetic parameters \( k_{cat}/(s^{-1}) \) and \( k_{cat}/K_{m}(M^{-1} s^{-1}) \) for the hydrolysis of some characteristic \( \beta \)-lactams by various class B \( \beta \)-lactamases

<table>
<thead>
<tr>
<th>PG</th>
<th>AMPI</th>
<th>CARB</th>
<th>OXA</th>
<th>CR</th>
<th>CTX</th>
<th>COX</th>
<th>AZT</th>
<th>IMI</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. cereus</td>
<td>( k_{cat} )</td>
<td>680</td>
<td>1100</td>
<td>16</td>
<td>325</td>
<td>25</td>
<td>60</td>
<td>0.2</td>
</tr>
<tr>
<td>(BclI)</td>
<td>( k_{cat}/K_{m} )</td>
<td>720</td>
<td>760</td>
<td>16</td>
<td>540</td>
<td>15</td>
<td>55</td>
<td>1.5</td>
</tr>
<tr>
<td>B. fragilis</td>
<td>( k_{cat} )</td>
<td>190</td>
<td>190</td>
<td>190</td>
<td>ND</td>
<td>48</td>
<td>92</td>
<td>10</td>
</tr>
<tr>
<td>(CflA)</td>
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<td>4800</td>
<td>1600</td>
<td>800</td>
<td>ND</td>
<td>7000</td>
<td>3600</td>
<td>90</td>
</tr>
<tr>
<td>C. menigosepticum</td>
<td>( k_{cat} )</td>
<td>280</td>
<td>850</td>
<td>ND</td>
<td>ND</td>
<td>34</td>
<td>55</td>
<td>10</td>
</tr>
<tr>
<td>(Blab)</td>
<td>( k_{cat}/K_{m} )</td>
<td>620</td>
<td>4800</td>
<td>20</td>
<td>ND</td>
<td>2400</td>
<td>350</td>
<td>200</td>
</tr>
<tr>
<td>S. marcescens</td>
<td>( k_{cat} )</td>
<td>320</td>
<td>950</td>
<td>ND</td>
<td>ND</td>
<td>34</td>
<td>15</td>
<td>35</td>
</tr>
<tr>
<td>(IMP-1)</td>
<td>( k_{cat}/K_{m} )</td>
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<td>4800</td>
<td>20</td>
<td>ND</td>
<td>2400</td>
<td>350</td>
<td>200</td>
</tr>
<tr>
<td>A. hydrophila</td>
<td>( k_{cat} )</td>
<td>I</td>
<td>I</td>
<td>I</td>
<td>0.75</td>
<td>0.12</td>
<td>0.07</td>
<td>I</td>
</tr>
<tr>
<td>(CphA)</td>
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<td>I</td>
<td>I</td>
<td>I</td>
<td>0.75</td>
<td>0.12</td>
<td>0.07</td>
<td>I</td>
</tr>
<tr>
<td>X. maltophilia</td>
<td>( k_{cat} )</td>
<td>280</td>
<td>175</td>
<td>280</td>
<td>285</td>
<td>28</td>
<td>66</td>
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</tr>
<tr>
<td>(ULAS11)</td>
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<td>1400</td>
<td>1400</td>
<td>1400</td>
<td>1100</td>
<td>93</td>
<td>2600</td>
<td>550</td>
</tr>
</tbody>
</table>

\( ^{a} \) PG, benzylpenicillin; AMPI, ampicillin; CARB, carbenicillin; OXA, oxacillin; CR, cephaloridine; CTX, cefotaxime; COX, cefoxitin; AZT, aztreonam; IMI, imipenem. The data are from \(^{b}\) Felici et al.\(^{162}\); \(^{c}\) Felici et al.\(^{163}\); \(^{d}\) Felici et al.\(^{164}\); \(^{e}\) Rossofoni et al.\(^{172}\); \(^{f}\) Watanabe et al.\(^{50}\); \(^{g}\) Hernandez Valladares et al.\(^{172}\). (--) no activity detected. ND, not determined. I, inactivation.

Galleni and J. M. Frère, unpublished work) and \( K. pneumoniae\)\(^{155}\) \( \beta \)-lactamases are identical. For the same reason, we only take account of the \( B. fragilis\) CflA and \( A. hydrophila\) CphA sequences, and discard those of \( B. cereus\) CcrA, CcrA3 and CcrA4,\(^{161,164,157}\) \( A. veronii\) Imi\(^{161}\) and CphA2. \(^{165}\) This comparison (Fig. 9) indicates that the \( S. maltophilia\) enzyme is only remotely related to the five other ones, which appear to constitute a more homogeneous group. Only seven residues are strictly conserved in the six sequences, namely His-90, Asp-92, Gly-95, Leu-117, His-168, Gly-204, and His-236.

Fig. 9 Sequence alignment of all class \( B \) \( \beta \)-lactamases. The side-chains which act as ligands for the \( Zn^{2+}\) ions are in bold-face types. First \( Zn^{2+}\) site: His(Asn)-88, His-90 and His-168. Second \( Zn^{2+}\) site: Asp-92, Cys-193 and His-236. BcII: \( B. cereus\) 569/H; Blab: \( C. meningosepticum\); CflA: \( B. fragilis\); IMP-1: \( S. marcescens\) TN9011; CphA: \( A. hydrophila\) AE036; L1: \( S. maltophilia\) ULAS11.
This number increases to 15 when the L1 enzyme is excluded. Note that His-88 is replaced by Asn in the case of the A. hydrophila enzyme and that Cys-193 is substituted by Ser in the S. maltophilia β-lactamase. It seems that class B comprises two sub-classes, one containing the only tetrameric enzyme and the second the six monomeric β-lactamases. This distinction, however, does not reflect the substrate profiles of the various enzymes.

5.3 Genetic organization

The genes encoding the zinc β-lactamases of B. cereus,173,175,176 B. fragilis,156, 157 S. maltophilia,174 C. meningo- septicum179 and A. hydrophila182 are chromosome-encoded. The bla-IMP gene encoding for the IMP-1 enzyme produced by S. marcescens is found on a large plasmid, which can be transferred to other bacterial strains. Today, the same gene is transferred to other bacterial strains. Today, the same gene is found in at least four other nosocomial microorganisms, i.e. P. aeruginosa, P. putida, Alcaligenes xylosidans and K. pneumoniae.177 In P. aeruginosa 101/477, bla-IMP constitutes a small gene cassette inserted in a new integron element (In101), located on a defective transposon (N. Laraki, M. Galleni, G. Rossolini and J. M. Frère, unpublished work). The spreading of the plasmid between the different nosocomial strains might be mediated by conjugation between the different bacteria.

Surprisingly, isolates of B. fragilis and A. hydrophila which do not produce the metallo-β-lactamase nonetheless contain a chromosomal copy of the corresponding gene which thus remains silent.178 Successive culture of these bacteria in the chromosomal copy of the corresponding gene which thus mediated by conjugation between the different bacteria.

5.4 Three-dimensional structures and mechanisms

The three-dimensional structures of the metallo-β-lactamases of B. cereus 569H181 and B. fragilis cfiA182,183 have been solved (Fig. 10). The folds of these two proteins are similar. They consist of a ββ sandwich with two α-helices on each external face (αββα). The ββ sandwich structure is also found in DNAse I, in the N-terminal domain of glutamine 5-phosphoribosyl-1-pyrophosphate aminotransferase and in the proteasome subunits, but the αββα organisation represents an original structure in the vast family of metalloproteins. The two β-sheets can be superimposed by a rotation around a 2-fold axis. In the case of the B. cereus (BcII) enzyme, two different structures have been solved at 2.5 and 1.85 Å,184 respectively. The first structure determined at pH 5.5 and at room temperature contains one zinc ion. The second, solved at the same pH but at low temperature (−180 °C) contains two zinc ions in the active site (Fig. 10). The active site is located at the bottom of a groove running between the two β sheets. Six of the nine strictly conserved residues are located in the active site: His-90, Asp-92, Leu-117, His-168, Gly-204 and His-236. The active site groove is open at both ends. When compared to the shape and size of the class A and C β-lactamase active sites, the catalytic cavity of the metallo-β-lactamases is wider. It can thus better accommodate β-lactam antibiotics with large side-chain substituents. In the monozinc structure, the zinc ion is liganded to three protein side-chains (His-88, His-90 and His-168) and a water molecule. The four Zn2+-ligands are disposed in a distorted tetrahedral shape. A hydrogen-bonded network involving the Zn2+ ion, the water molecule, Asp-92 and Cys-193 anchors the water molecule in the active site. Finally, Gly-204, Asn-205 and His-236 are too far from the active site to participate directly in the enzymatic mechanism. However, they may be responsible for specific interactions with the β-lactam substrate.

As observed in other zinc hydrolases, the metal ion can play a dual role in catalysis. Firstly, the Zn2+-bound water molecule is activated to perform a nucleophilic attack on the carbonyl of the β-lactam ring. The interaction with the zinc ion can decrease its pKₐ value from 14 to 7, as shown for carbolic anhydrase.185 At physiological pH, a non-negligible proportion of the water molecules would be present as hydroxide ions. Secondly, the Zn2+ ion can bind and polarise the carbonyl group and the tetrahedral intermediate would be stabilized by an interaction between the oxyanion and the zinc ion. The hydrogen-bonded network between the water molecule, Asp-92 and Cys-193 would contribute to the activation of the water molecule. The docking of a cephalosporin molecule in the active site of BcII revealed two other potential interactions: the Asn-205 side-chain would interact with the C-7 substituent of the antibiotic whereas that of His-236 would interact with the antibiotic carboxylic group. The structure containing the two zinc ions indicates that the first zinc remains coordinated by the three histidine residues and a water molecule, as described for BcII. In the second zinc, Cys-193, His-236 and a water molecule are the ligands for the second zinc. In that situation, the zinc ion ligands are arranged in two distorted tetrahedral shapes. The distance between the two zinc ions is 3.7 Å.181

The apoenzyme has the same overall structure as the zinc-containing enzyme. Differences are observed near the active site which is more open in the apoenzyme. The positions of the metal ligands His-88, His-90, Asp-92 and Asn-205 are significantly affected. These residues move 1 Å away from their positions in the mono-Zn2+ enzyme.184 Moreover, the absence of the active site metal ion results in a rotation of the side chain of His-88. These results indicate that the metal at the first binding site in BcII has not only a catalytic but also a structural role.

The crystal structure of the B. fragilis cfiA metallo-β-lactamase indicates that it contains a binuclear zinc centre.182,183 The two zinc ions are located as in BcII and separated by a distance of 3.5 Å. The first zinc is tetrahedrally coordinated by three histidines residues (His-88, His-90 and His-168) and a water molecule (W1). The second zinc is coordinated to five ligands in a distorted trigonal bipyramidal geometry: Asp-92,
Cys-193, His-236 and two water molecules (W1 and W2). One of these (W1) is shared by the two zinc ions and interacts with the Asp-92 side-chain. As in BcII, the hydrogen-bond network would decrease the pK_a value of W1, resulting in the appearance of a hydroxide ion. On the basis of the CfiA β-lactamase structure, an alternative mechanism has been proposed in which the nucleophilic attack on the carbonyl of the β-lactam ring would be performed by the shared hydroxide ion. Interaction with Asp-103 would appropriately orient the hydroxide moiety and reduce its interaction with the zinc ions. The reaction yields a charged tetrahedral intermediate, which is stabilized by the zinc ions. The second water molecule then donates a proton to the amide nitrogen of the β-lactam bond. W2, converted into a hydroxide ion, would then move to occupy the position of W1 while the tetrahedral intermediate decomposes and the hydrolysed β-lactam diffuses away from the active site. Finally, a solvent water molecule would enter in the active site and occupy the apical coordination site of the pentacoordinated zinc.

A third mechanism can be proposed, in which the W2 water molecule is the nucleophilic agent and attacks the carbonyl moiety of the β-lactam. However, this appears rather unlikely since no protein ligand or water molecule would contribute to the stabilization of the reaction intermediate and the generation of the nucleophilic reagent.

5.5 Role of the metal
As shown by X-ray crystallography, the B. cereus 569/H (BcII) and B. fragilis CfiA β-lactamases contain two metal ion binding sites. In the case of CfiA, both zins appear to be tightly bound. The enzyme can bind either two Zn^{2+} or two Cd^{2+} ions per mole. Electron paramagnetic resonance studies of the Co(t) derivatives indicated that the ions are independent and five to six coordinated. Analysis of the UV-visible spectra revealed the presence of a strong S–Co(t) interaction suggesting that Cys-193 is one of the ligands. Substitution of the cysteine by a serine abolished the typical d–d transition of the Co–S interaction. The crystal structure suggested that Asp-92 is involved in the second binding site and plays the role of a general base. The enzyme can bind either two Zn^{2+} or two Cd^{2+} ions per mole. Electron paramagnetic resonance studies of the Co(t) derivatives indicated that the ions are independent and five to six coordinated. Analysis of the UV-visible spectra revealed the presence of a strong S–Co(t) interaction suggesting that Cys-193 is one of the ligands. Substitution of the cysteine by a serine abolished the typical d–d transition of the Co–S interaction. The crystal structure suggested that Asp-92 is involved in the second binding site and plays the role of a general base. The substitution of Asp-92 by Val leads to a protein which is 6000-fold less active against benzylpenicillin than the wild type enzyme. Similar results were obtained with the D205V mutant. Moreover, only 0.5 equivalent of zinc ion are found in the D92V mutant. Interestingly, the D62V and D171V mutants, which can only bind one zinc, retain a high catalytic activity. Indeed, with both mutants, the K_cat values for the hydrolysis of benzylpenicillin are only two-fold lower than that of the wild type, which contains two zinc ions per enzyme molecule. These results suggest that the presence of only one zinc is essential for the β-lactamase activity; the second zinc would thus play an accessory function in the catalytic process.

In the case of the BcII enzyme, the formation of the di-zinc form has little effect, if any, on the catalytic activity. Kinetic studies of the hydrolysis of benzylpenicillin by the Co(II)–β-lactamase II and of nitrocefin by Zn^{2+}–lactamase II at sub-zero temperature indicated the prevalence of a branched mechanism with two non-covalent intermediates ES1 and ES2. Spectroscopic studies of these complexes suggested that the metal is pentacoordinated in ES1 and tetracoordinated in ES2. These studies also suggested that the cysteine residue (Cys-193) is directly interacting with the metal during the catalytic cycle.

In the A. hydrophila enzyme, whose structure remains to be solved, the binding of a second zinc ion results in pure non-competitive inhibition. Fluorescence emission and circular dichroism spectra reveal a small conformational change upon titration of the apoenzyme by zinc ions, resulting in the successive saturation of the first and second binding sites. Moreover, the catalytic zinc strongly stabilizes the conformation of the enzyme, and the di-zinc form is even more resistant to thermal denaturation than the monozinc enzyme.

Despite the general structural similarities, the three metallo-β-lactamases exhibit very distinct behaviours when the second zinc binds to the active site, a surprising and apparently unique situation in Zn^{2+} hydrolases.

5.6 Inhibitors of class B β-lactamases
Various compounds with inhibitory activity against metallo-β-lactamases have been reported. Two metabolites (SB212021 and SB212305), members of the phenazine group, have been isolated from a culture broth of A Streptomyces strain. Studies of their mode of action indicated that these molecules inhibit the β-lactamase by chelation of the active site metal ion. Some synthetic mercaptoacetic acid thiol esters also act as metallo-β-lactamase inhibitors. Interestingly, these compounds are inactivators of CphA, BcII and L1 but have no effect on the CfiA enzyme. These compounds behave as suicide-substrates. The hydrolysis of the thioester bond by the enzyme releases a molecule of mercaptoacetic acid, which in turn reacts with the free cysteine residue in aerobic conditions and yields a covalent complex. Trifluoromethyl alcohols and ketones also constitute potential inhibitors of the zinc-enzymes. Thus, trifluoromethyl ketones derived from t-alanine, β-alanine or d-phenylalanine display significant inhibition of the L1 and CphA metallo-β-lactamases, but are less active against the enzymes from B. cereus and P. aeruginosa. Interestingly, the CphA enzyme is irreversibly inactivated by these compounds. Recently, some biphenyl tetrazoles have been described as potent inhibitors of the B. fragilis enzyme.

6 Genetic support and induction
Chromosome and plasmid-encoded proteins are found in all four classes of β-lactamases. Similarly, production can be both constitutive or inducible (Table 5). The class A plasmid-encoded enzymes of the TEM and SHV families are constitutively produced. In class C, AmpD^{-} constitutive over-

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Table 5 Genetic support and mode of synthesis of some characteristic β-lactamases

<table>
<thead>
<tr>
<th></th>
<th>Constitutive</th>
<th>Inducible</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Class A</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>plasmid</td>
<td>TEM, SHV</td>
<td>S. aureus PC1 (6.3)</td>
</tr>
<tr>
<td>chromosome</td>
<td>K. oxytoca, K. pneumoniae, S. albus G</td>
<td>B. licheniformis (6.3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S. cacao (6.2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P. vulgaris (6.1)</td>
</tr>
<tr>
<td><strong>Class C</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>plasmid</td>
<td>BIL, CMY, FOX</td>
<td>M. morganii (6.1)</td>
</tr>
<tr>
<td>chromosome</td>
<td>AmpD mutants</td>
<td>All other Enterobacteria (6.1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E. coli Pseudomonas sp. (6.1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A. jandaei AsB1A (6.4)</td>
</tr>
<tr>
<td><strong>Class D</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>plasmid</td>
<td>All OXA but OXA-12</td>
<td>A. jandaei AsB1B (OXA-12) (6.4)</td>
</tr>
<tr>
<td>chromosome</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Class B</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>plasmid</td>
<td>K. pneumoniae, B. fragilis cfiA, S. marcescens, . . . (see text)</td>
<td>A. jandaei AsB1M (6.4)</td>
</tr>
<tr>
<td>chromosome</td>
<td>B. fragilis, B. cereus 569/H, S. maltophilia, B. cereus 5/B/6, B. cereus 569, A. hydrophila</td>
<td>A. jandaei AsB1M (6.4)</td>
</tr>
</tbody>
</table>

The numbers in parentheses (6.1) to (6.4) refer to the sections where induction mechanisms are described.
producers represent a major threat, because they appear with a high frequency.\textsuperscript{195}

Presently, four distinct induction mechanisms have been identified.

6.1 The AmpG-AmpD-AmpR-AmpC system
With the exception of \textit{E. coli}, naturally occurring chromosome-encoded class $\beta$-lactamases are inducible, but all of the highly resistant clinical isolates exhibit a derepressed constitutive phenotype which results in the expression of very large quantities of enzyme in the periplasm.\textsuperscript{192,194}

This acute clinical problem has fuelled active interest in the induction of class C $\beta$-lactamases which has been extensively studied\textsuperscript{194} and it has been found that a simple disrupting mutation in the $\text{ampD}$ gene could result in the constitutive, overproducing phenotype.\textsuperscript{195}

Rather than detecting the presence of the $\beta$-lactam in the medium, the induction mechanism of class C $\beta$-lactamases is sensing the delicate balance between intracellular pools of degradative and synthetic intermediates of the peptidoglycan cell-wall metabolism. By inhibiting the enzymes responsible for the ultimate steps and the continuous remodelling of the peptidoglycan (the PBP$s$), $\beta$-lactams modify this balance and trigger the induction mechanism.\textsuperscript{196}

The $\text{ampC}$ gene encodes the $\beta$-lactamase.\textsuperscript{24} It is under the control of the product of the nearby $\text{ampR}$ gene, a DNA-binding protein of the LysR family which also represses its own expression.\textsuperscript{197} When the cells are growing in a normal environment, a large proportion of the peptidoglycan degradation products, mainly anhydromuramyl-tripeptides produced in the periplasm by autolytic enzymes, re-enter the cell via the AmpG permease.\textsuperscript{198} The AmpD enzyme, an intracellular amidase, then liberates the tripeptide which is reintroduced in the biosynthetic pathway.\textsuperscript{199} The free AmpR protein acts as an activator of the $\text{ampC}$ gene but, in the uninduced cell, it behaves as a repressor after binding the most abundant intracellular precursor of the peptidoglycan biosynthetic pathway, the UDP-muramyl-pentapeptide. Under these conditions, a low, basal level of $\beta$-lactamase is produced. In the presence of penicillin, larger amounts of the degradation products re-enter the cell. In addition, and for reasons which are still mysterious, the concentration of UDP-muramyl-pentapeptide is simultaneously decreased. Under these conditions, the anhydromuramyl-tripeptide displaces the precursor from AmpR, thus restoring its activating properties.\textsuperscript{196} In $\text{ampD}^{-}$ mutants,\textsuperscript{196,198} the same degradation product understandably accumulates to much higher levels, resulting in a similar, but even more drastic reactivation of AmpR and thus of the transcription of $\text{ampC}$. This model also nicely explains the effects of mutations which inactivate the AmpG protein and thus preclude the re-entry of the degradation product, resulting in low, constitutive production of enzyme.

This system is found in all enterobacteria (and the related \textit{Pseudomonas sp.})\textsuperscript{110} with the exception of \textit{E. coli}, where the $\text{ampR}$ gene is absent and the $\text{ampC}$ gene is transcribed with a poor efficiency under all conditions, despite the presence of the $\text{ampD}$ gene on the chromosome.\textsuperscript{24}

At the present time, the only inducible plasmid-encoded class C enzyme has been found in \textit{Morganella morganii},\textsuperscript{115} where the plasmid contains both the $\text{ampR}$ and $\text{ampC}$ genes in an arrangement similar to that found in the chromosomes of enterobacteria.

In \textit{Proteus vulgaris}, the synthesis of a class A $\beta$-lactamase is similarly controlled and transformation by plasmids carrying the \textit{Citrobacter freundii} $\text{ampG}$ and $\text{ampD}$ (but not $\text{ampR}$) genes can restore the original inducible phenotype which has been lost following mutations in the corresponding, original chromosomal genes.\textsuperscript{201}

6.2 Induction in \textit{Streptomyces cacaoi}
$\text{S. cacaoi}$ produces two inducible $\beta$-lactamases, BlaL and BlaU.\textsuperscript{202,203} Four open reading frames are found near and upstream of the $\text{blaL}$ gene, of which two, $\text{blaA}$ and $\text{blaB}$ are both necessary for induction.\textsuperscript{204} BlaA is a repressor/activator of the LysR family which binds in the intercistrionic region between $\text{blaA}$ and $\text{blaL}$.\textsuperscript{202} BlaB contains the SXXK and KTG conserved elements characteristic of PBPs but is located on the inner face of the cytoplasmic membrane.\textsuperscript{205} Several site-directed mutants of BlaB have been obtained: they all impair the induction process, but the protein remains membrane-bound in all cases. Nothing is known about the primary induction signal.

6.3 Induction in \textit{Bacillus licheniformis}
Three genes $\text{blaI}$, $\text{blaR1}$ and $\text{blaR2}$ are involved in the regulation of the structural gene $\text{blaP}$ which encodes a class A $\beta$-lactamase.\textsuperscript{206} BlaI acts as a typical repressor, $\text{blaR1}$ encodes a transmembrane protein whose extracellular C-terminal domain covalently binds $\beta$-lactams and detects the presence of these compounds in the external medium. The message is then transmitted into the cytoplasm via the four transmembrane helices of BlaR1.\textsuperscript{207} The role of BlaR2 and how the message reaches BlaI is still mysterious, although recent results suggest an increased proteolysis of BlaI under inducing conditions.\textsuperscript{208} Mutations in the $\text{blaR2}$ gene can result in constitutive production of the $\beta$-lactamase. The $\text{balR1}$, $\text{ball}$ and $\text{blaF}$ genes are contiguous and the two regulatory genes are oppositely oriented when compared to their structural neighbour. The $\text{blaR2}$ gene is in a completely different locus. It is likely that the expression of the class A $\beta$-lactamase of \textit{B. cereus} is controlled by a similar mechanism. The $\text{blaI}$, $\text{blaR}$ and $\text{blaF}$ genes of \textit{S. aureus} are organized in the same way on the transposon TN$\text{N552}$.\textsuperscript{209} In \textit{Streptococcus epidermidis}, a resistance gene ($\text{mrsA}$ or $\text{meca}$) codes for a $\beta$-lactam-resistant PBP, in a related system.\textsuperscript{210}

6.4 \textit{Aeromonas jandei}
This Gram-negative bacterium (formerly known as \textit{Aeromonas sobria} AER14) expresses three inducible $\beta$-lactamases, belonging respectively to classes $B$ (AsbM1), $C$ (AsbA1) and $D$ (OXA-12 or AsbB1).\textsuperscript{160} That their expression shares a common regulatory pathway is suggested by the fact that mutant strains have been isolated which simultaneously and constitutively produce the three enzymes. Other genetic results indicate that a two-component sensor-regulator system of the \textit{cre} family might be responsible for this phenomenon.

7 Linear (depsipeptide) substrates of $\beta$-lactamases
In a seminal paper,\textsuperscript{211} Pratt and Govardhan demonstrated, for the first time, that linear depsipeptide analogues of the peptide substrates of DD-peptidases could be hydrolysed by class $A$ $\beta$-lactamases, belonging respectively to classes $B$ (AsbB1).\textsuperscript{160} That their expression shares a common regulatory pathway is suggested by the fact that mutant strains have been isolated which simultaneously and constitutively produce the three enzymes. Other genetic results indicate that a two-component sensor-regulator system of the \textit{cre} family might be responsible for this phenomenon.


13
ester 7 and the class C P99 enzyme, but data for the analogous thiol ester are not available. In class A and for the same substrate, significant differences are observed depending upon the enzyme tested, reflecting the highly diverse catalytic properties of these enzymes with their usual β-lactam substrates. Class B and class D enzymes appear to be less active but, with the former, inhibition by the thiol-containing product seriously complicates the analysis of the interactions with the thiol esters.

A surprising observation is the loss of stereospecificity for D-type residues: a lactate or thiolactate Rα residue is not a better leaving group than the non-chiral glycollate or thioglycollate but, more surprisingly, at the level of the Xaa residue, the L isomer of ester 6 was somewhat hydrolysed by the P99 enzyme and the L isomer of thiol ester 6 was even a better substrate of the same enzyme than its D counterpart. Similar phenomena have been observed with some DD-peptidases, but mainly at the level of the leaving group.121

Finally, the transacylation properties of the P99 enzyme have been studied in great detail by Pratt and co-workers214–216 and these studies revealed a complex reaction pathway involving multiple binding sites for the acyl donor substrate, a situation not unlike that described for the Streptomyces R61 DD-peptidase. A very inefficient catalysis of transacylation reactions by some class A β-lactamases has also been reported.217

8 Non β-lactam inhibitors

8.1 Boronic acids

Compounds of general structure R-B(OH)2 were first found to inhibit class C enzymes reversibly126 with Kᵢ values in the micromolar range. Further studies revealed a two-step mechanism, the slower step probably being associated with an enzyme conformation change and the formation of an O–B bond by the active-serine γ–O atom.124 For the class C enzymes, simple R groups such as C₆H₅- or (m) H₂N-C₆H₄-yielded efficient inhibitors, but for the B. cereus I class A enzyme, a more complex side-chain (C₆H₅–CH₂–CO–NH–CH₂–) was necessary to obtain a Kᵢ value of about 15 μM.124 The same compound was also an efficient inhibitor of the P99 enzyme. More recently, (1R)-1-acetamido-2-(3-carboxyphenyl)ethane boronic acid was also found to be a highly effective inhibitor (Kᵢ = 110 nM) of the TEM-1 enzyme.219

8.2 Phosphonic acid derivatives

Pratt and co-workers130,131,220 have described a series of rather efficient transient inactivators of class C enzymes which have the general structure R–CO–NH–CH₂–(PO₂)₂–X where X is a good leaving group (Table 7). They appear to form a transition-state analogue, where X is replaced by the active-serine Oγ atom as shown by X-ray crystallography.133 The covalent adduct is then very slowly hydrolysed. The compound with X = d-lactate is not an inactivator, an observation which suggests that the quality of the leaving group is much more

Table 6

<table>
<thead>
<tr>
<th>Compound</th>
<th>Class A&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Class C&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Class D</th>
<th>Class B</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ester 1</td>
<td>90</td>
<td>310</td>
<td>ND</td>
<td>ND</td>
<td>120</td>
</tr>
<tr>
<td>Ester 2</td>
<td>very low (&lt; 1)</td>
<td>140</td>
<td>ND</td>
<td>ND</td>
<td>120</td>
</tr>
<tr>
<td>Ester 3</td>
<td>0.1–0.9</td>
<td>2500</td>
<td>ND</td>
<td>5</td>
<td>123</td>
</tr>
<tr>
<td>Ester 4</td>
<td>120–290</td>
<td>4600</td>
<td>ND</td>
<td>ND</td>
<td>120</td>
</tr>
<tr>
<td>Ester 5</td>
<td>ND</td>
<td>2200</td>
<td>ND</td>
<td>ND</td>
<td>120</td>
</tr>
<tr>
<td>Ester 6</td>
<td>50–300</td>
<td>20 000</td>
<td>&lt; 5</td>
<td>ND</td>
<td>121</td>
</tr>
<tr>
<td>Ester 7</td>
<td>300–13 000</td>
<td>54 000</td>
<td>ND</td>
<td>ND</td>
<td>212</td>
</tr>
<tr>
<td>Aziridine</td>
<td>120–1900</td>
<td>39 000</td>
<td>ND</td>
<td>4000</td>
<td>123</td>
</tr>
<tr>
<td>Thiolester 1</td>
<td>4500–16 000</td>
<td>2400</td>
<td>200</td>
<td>ND</td>
<td>121</td>
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<tr>
<td>Thiolester 2</td>
<td>6000–11 000</td>
<td>3600</td>
<td>265</td>
<td>ND</td>
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<tr>
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<td>6700</td>
<td>ND</td>
<td>150</td>
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<td>ND</td>
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<td>Thiolester 5&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>330</td>
<td>&lt; 5</td>
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<td>121</td>
</tr>
<tr>
<td>Thiolester 6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&lt; 10</td>
<td>1200</td>
<td>&lt; 5</td>
<td>ND</td>
<td>121</td>
</tr>
</tbody>
</table>

<sup>a</sup> The enzymes tested were those from B. licheniformis, S. albus, S. aureus PC1, B. cereus I and the TEM plasmid.  
<sup>b</sup> P99 is an E. cloacae strain. Very similar results were obtained with the E. coli K12 enzyme.  
<sup>c</sup> A very low activity (10 M⁻¹ s⁻¹) was recorded with the L-isomer.  
<sup>d</sup> Much lower values were obtained with the diacetyl-L-Lys R side chain, which is more similar to that of good DD-peptidase peptide substrates. ND, not determined.

Fig. 11 Structures of some linear depsipeptide substrates of β-lactamases. The general structure is R-CO-Xaa-R'. Asymmetric carbons in R' are always D.
important than the similarity of its structure to that of the depsipeptides described above.

These compounds exhibit only a very poor activity against class A enzymes (Table 7), but complexes could nevertheless be studied by X-ray crystallography, showing the same types of adducts as those formed with the P99 enzyme.223,281

### 8.3 Phosphonamidates

Among the various phosphonamidates studied as β-lactamase inactivators,221,222 the best results were obtained with C₆H₅–CH₂–O–CO–NH–CH₂–, where R was t-phenylalanine, β-alanine or β-phenyl-β-alanine, exhibiting second order acylation rate constants of 230, 150 and 250 M⁻¹s⁻¹, respectively, with the P99 enzyme.223 The same compounds were about 100-fold less efficient as inactivators of some class A enzymes.222

### 9 Conclusions

The production of one or several β-lactamases by pathogenic bacteria represents the most widespread resistance mechanism to antibiotics of the penicillin family. The appearance of penicillin-resistant PBPs has, at the present time and with very few exceptions, remained limited to bacterial species which appear to be unable either to produce a β-lactamase, to modify a preexisting enzyme in a way that enlarges its specificity profile or to acquire a β-lactamase gene from the vast pool of plasmids and transposons available in other genera or even in related species. This observation can be explained by the fact that any modification of a PBP which might decrease its affinity for β-lactams must preserve its transpeptidase activity. The probability of such an exquisite rearrangement of the PBP active site understandably remains quite low, although it should be kept in mind that, even among the PBPs generally considered as ‘sensitive’ the affinity for penicillins can vary by several orders of magnitude.

This review highlights the spectacular diversity in the primary structures of class A β-lactamases which parallels similarly astounding variations in their specificity profiles. A total number of 9 strictly conserved residues is only marginally increased by a few conserved chemical functionalities in other positions. Surprisingly, modifications of some of these residues by site-directed mutagenesis (Ser 130,224 the hydroxy group at position 235) have failed to result in the expected impairment of enzymatic activity, at least for some substrates. Usually, cephalosporins are much more sensitive to these alterations than penicillins, as also observed with class C enzymes. These results might point to the former compounds as the ‘natural’ selective agents,142 before man started to interfere with the process by massively utilizing β-lactams in the hope of controlling bacteria-mediated infectious diseases. By contrast, the crystal structures which have presently been determined exhibit a very high degree of similarity, with identical or chemically equivalent residues in the key positions. The diversity of the specificity profiles explains the relatively rapid development of resistance phenomena when new compounds are introduced in the chemotherapeutic arsenal.

Although less spectacular, the same trends are observed with class C and class D enzymes. The class B metallo-β-lactamases presently remain less prevalent, but the very broad activity spectrum of the plasmid-borne members of this class could possibly be further extended by a similar mutation/selection mechanism, a rather frightening perspective.

Three very different pathways have emerged in the selection of resistant bacteria. Strains producing entirely new enzymes have been isolated and, alternatively, point mutations in pre-existing and well studied proteins have dramatically increased their activity against β-lactams first described as ‘β-lactamase stable’. Plasmid and transposon-borne genes have rapidly spread among pathogens, some of them producing more than one β-lactamase, resulting in serious clinical problems which have been worsened by the third mechanism: the deregulation of the induction pathway which allows some opportunistic Enterobacteriaceae and related Pseudomonaceae to synthesise such enormous amounts of their chromosomal class C β-lactamase that they become resistant even to very poor substrates of the enzyme.

The other strategy devised to circumvent the β-lactamase problem was the coadministration of a β-lactamase inactivator together with a classical, β-lactamase sensitive antibiotic. The success of these composite drugs such as Augmentin is however endangered by the recent appearance of inactivator-resistant mutants and by the presence, in the pool of existing β-lactamases, of enzymes which exhibit a low intrinsic sensitivity to clavulanic acid, sulbactam or tazobactam. Class C and some naturally occurring class A enzymes belong to this latter category. The fact that mycobacterial enzymes might belong to the latter group underlines the urgent need for new compounds which would efficiently inhibit or inactivate a large number of β-lactamases. In this search for the ideal inhibitor, it will be worth remembering that such a compound must not only exhibit a high affinity for the enzymes, but also escape hydrolysis if a branched pathway is prevalent. Moreover and not surprisingly, the metallo-β-lactamases not only escape the action of the mechanism-based inactivators of their active-site serine counterparts but also hydrolyse some of them and might thus protect the latter in mixed infections or when a strain produces both a metallo- and an active-site serine enzyme.

From a biochemist’s point of view and in addition to the evident medical relevance of the subject, β-lactamases represent ideal tools for the detailed study of enzymatic catalysis and specificity. Indeed, the large number of enzymes and potential substrates offer a ‘garden of delights’ in which the enzymologist can spend hours of fruitful exploration. Both acylation and deacylation rates exhibit a wide range of values and finding

### Table 7: Second-order rate constants (M⁻¹ s⁻¹) for the inactivation of β-lactamases by phosphonic acid derivatives of general structure R–CO–NH–CH₂–(PO₂–)–X

<table>
<thead>
<tr>
<th>R</th>
<th>X</th>
<th>E. cloacae</th>
<th>TEM</th>
<th>S. aureus</th>
<th>B. cereus</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₆H₅–CH₂–</td>
<td>–O–C₆H₅–NO₂ (p)</td>
<td>56 000</td>
<td>0.9</td>
<td>4</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>–O–C₆H₅–NO₂ (o)</td>
<td>55 000</td>
<td>1.3</td>
<td>0.8</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>–O–C₆H₅–NO₂ (m)</td>
<td>23 000</td>
<td>0.04</td>
<td>0.11</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td></td>
<td>–O–C₆H₅–COOH (m)</td>
<td>1100</td>
<td></td>
<td>(—)</td>
<td>(—)</td>
</tr>
<tr>
<td></td>
<td>–O–C(CH₃)₂–COOH (o)</td>
<td>(—)</td>
<td></td>
<td>(—)</td>
<td>(—)</td>
</tr>
<tr>
<td></td>
<td>–O–C₆H₅–NO₂ (p)</td>
<td>33 000</td>
<td>2.7</td>
<td>2.5</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>–S–C₆H₅</td>
<td>26 000</td>
<td>2</td>
<td>0.07</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>–F</td>
<td>26 000</td>
<td>2.1</td>
<td>0.34</td>
<td>0.3</td>
</tr>
</tbody>
</table>

* d-Stereochemistry, (—): no detectable inactivation.

explanations to these variations remains a highly challenging problem. It is however striking that, despite the availability of several high resolution structures, the exact acylation mechanism of class A enzymes remains controversial, a fact that underlines the difficulty of bridging the gap between static images, however beautiful, and the understanding of catalytic phenomena which occur within a millisecond time scale. It is interesting to remember that the TEM β-lactamase can be considered as a ‘perfect’ enzyme for which evolution has resulted in an optimization of the catalytic process for its best substrates. 32

In a somewhat different field, the large number of primary structures yielding similar 3D architectures and the relative facility of obtaining point mutants also make these enzymes excellent models in the analysis of factors affecting the folding of medium-sized proteins.

The origin of β-lactamases remains mysterious. If it can now be safely assumed that they derived from enzymes involved in peptidoglycan biosynthesis, the extensive sequence differences between the 3 classes of active site-serine enzymes and even within class A suggest a very early divergence. How and why were such diverse sequences selected? Do β-lactamases have a still undiscovered physiological function? For β-lactam producing species, such as several members of the Actinomycetales family, the synthesis of these enzymes might constitute a regulation mechanism, a defence against their own potentially suicidal secondary metabolites. But, with the exception of some Bacillus strains, which also produce a metallo-β-lactamase, Gram positive bacteria only synthesize class A enzymes. What is the origin of the class C genes found in many Enterobacteriaceae and whose expression is moreover tightly and exquisitely regulated? This phenomenon might represent a still undiscovered physiological function? For

We are indebted to the late Stephen G. Waley (1922–1993) for his remarkable contribution to our present knowledge of β-lactamases.

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11 References

[References list]
