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 usual 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.

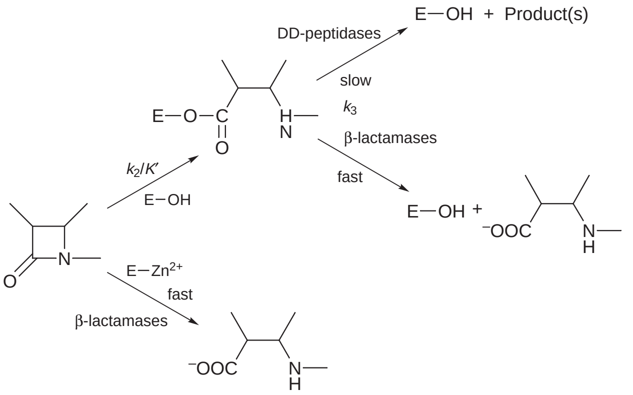


Fig. 1 Interaction between β-lactam antibiotics and active site serine penicillin-recognizing enzymes. E-Zn^{2+} represents a metallo β-lactamase and E-OH an active-site serine DD-peptidase (PBP) or β-lactamase. With the DD-peptidases, k_{+3} is very small and the enzyme is inactivated as the acyl-enzyme. With β-lactamases, k_{+3} can be very large, and in consequence the antibiotic is rapidly hydrolysed. Note that the $k_{\mathrm{cat}}/K_{\mathrm{m}}$ parameter [$k_{\mathrm{cat}}/K_{\mathrm{m}} = k_2/K'$ where $K' = (k_{-1} + k_2)/k_{+1}$, see eqn. 1] is independent of the deacylation rate (k_3) and reflects the acylation rate.

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 permeability[16] and, more recently, efflux mechanisms[9] 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]

Benzylpenicillin, 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'[20] and leading to the present diversity of β-lactam structures (Fig. 2), recently and exhaustively reviewed by Burton *et al.*[21] Presently, more than 200 different β-lactamases have presently been described, exhibiting a wide range of primary structures and catalytic properties.[22] 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.[23–26]

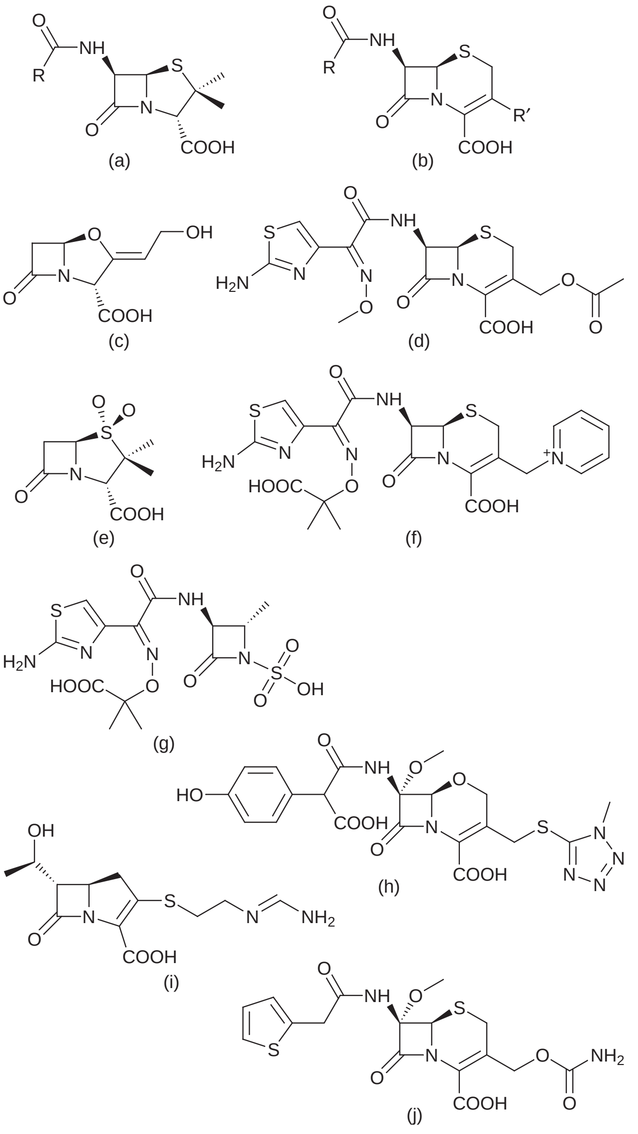


Fig. 2 Structure of some β-lactam antibiotics. (a) Penams (*e.g.* benzylpenicillin, ampicillin, amoxycillin), (b) cephems (cephalosporins), (c) clavulanic acid (oxapenam), (d) cefotaxime (oximino cephalosporin), (e) sulbactam (penam sulfone), (f) ceftazidime (oximino cephalosporin), (g) aztreonam (monobactam), (h) moxalactam (oxacephamycin), (i) imipenem (carbapenem), (j) cefoxitin (cephamycin).

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,[27] it seems that the naturally occurring cation is always Zn^{2+}.[28] 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.[29–30] 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):[8,10,31]

$$E + C \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EC \xrightarrow{k_2} EC^* \xrightarrow{k_3} E + P(s) \qquad (1)$$

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.[32]

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,[33–35] 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 three-dimensional structures, including the class D β-lactamases and the large number of PBPs whose primary structures have been deduced from the corresponding gene sequences[26,36–39] 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.[40] The structures of five class A enzymes (Fig. 3) have been obtained by X-ray crystallography[34,41–44] and the function of many residues has been probed by site-directed mutagenesis.[45] The catalytic properties and primary structures of class A β-lactamases differ considerably, making them a highly diverse class.[10]

These medium-sized proteins (M_r about 29 000) show a wide distribution of pI values, ranging from ~ 3.5 to ~ 10.[22] Of their 260–280 residues, it appears today that only *nine* residues are strictly conserved.[40] 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 *

	Element 1	Element 2	Element 3
Class A	70 Ser*-Xaa-Xaa-Lys	130 Ser-Asp-Asn Ser-Asp-Ser Ser-Asp-Gly	234 Lys-Thr-Gly Lys-Ser-Gly Arg-Thr-Gly Arg-Ser-Gly
Class C	64 Ser*-Xaa-Ser-Lys	150 Tyr-Ala-Asn Tyr-Ser-Asn	314 Lys-Thr-Gly
Class D	70 Ser*-Xaa-Xaa-Lys	144 Tyr-Gly-Asn	214 Lys-Thr-Gly
S. R61 DD-peptidase	62 Ser*−Val-Thr-Lys	159 Tyr-Ser-Asn	298 His-Thr-Gly
Other known PBPs	Ser*-Xaa-Xaa-Lys	Ser-Xaa-Asn Ser-Xaa-Cys Tyr-Gly-Asn	Lys-Thr-Gly Lys-Ser-Gly

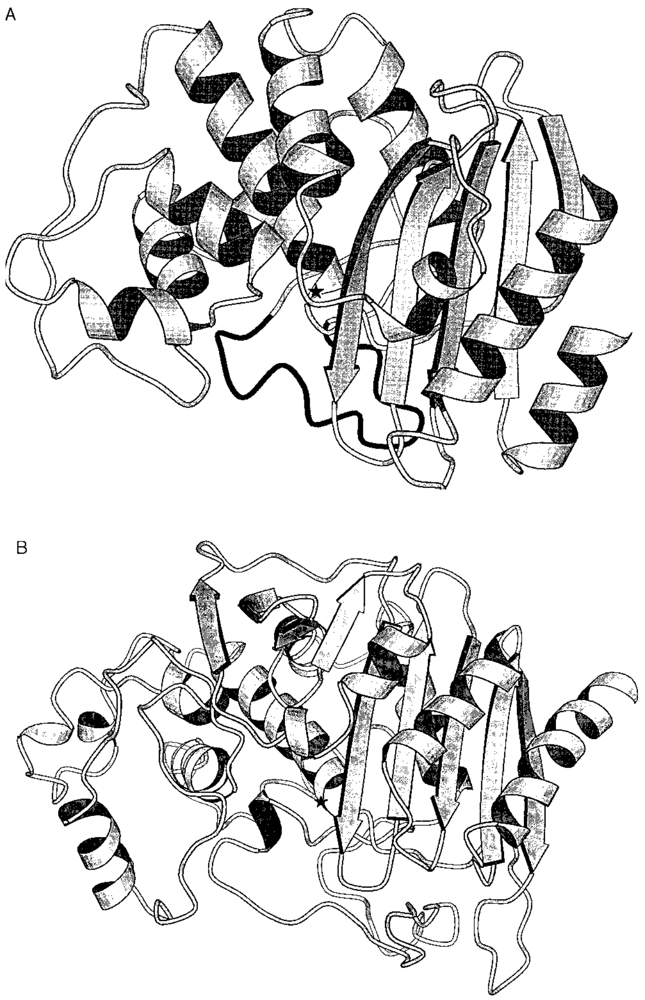


Fig. 3 Comparison of the tertiary structures of the class A TEM-1 β-lactamase (A) and the *E. cloacae* P99 class C β-lactamase (B). The α-carbon of the active site serine is marked by a star and in the class A enzyme, the Ω-loop is in black. These diagrams were generated using MOLSCRIPT.[225] [PDB entry codes: 1XPB (TEM-1) and 2BLT (P99)].

These nine strictly conserved residues remain the most distinct feature of class A β-lactamases and thus the molecular structure classification first proposed by Ambler[23] when only four sequences were known, remains totally valid. An alternative classification scheme for β-lactamases based on functional characteristics has been proposed by Bush and co-workers,[22] who tried to correlate molecular structure with catalytic profiles and sensitivity to inactivators. Three major groups of enzymes were defined: group 1 ‘cephalosporinases’ 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 classification[22] 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 (2800–84 000 mM^{-1} s^{-1}) and class C (1200–75 000 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 2be, 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

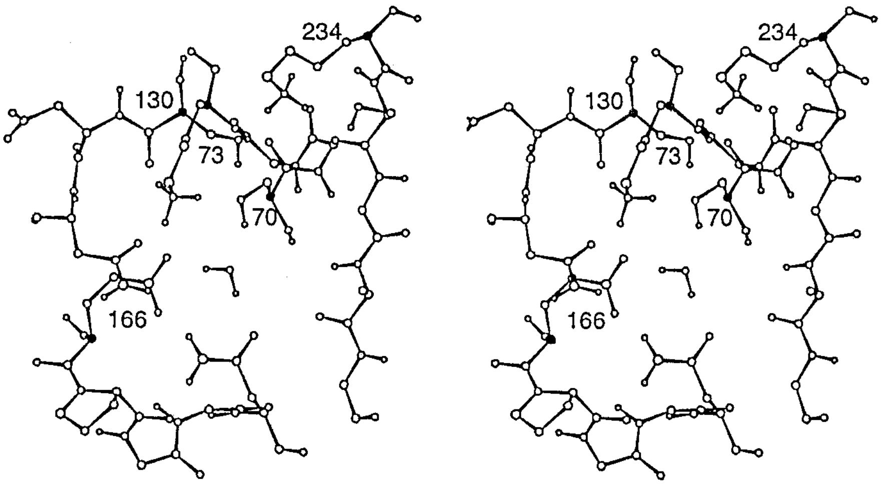


Fig. 4 Simplified stereo view of the active site of the class A TEM β-lactamase. The important catalytic residues Ser-70, Lys-73, Ser-130, Glu-166 and Lys-234 are numbered, and the conserved water molecule W1 is shown. Besides those of the backbone NH groups, the protons on the following groups are shown: the hydroxyls of Ser-70, Ser-130 and Ser-235, the alkylammoniums of Lys-73 and Lys-234, and the amides of Asn-132 and Asn-170.

Table 2 Comparison of the kinetic parameters $k_{cat}(s^{-1})$ and k_{cat}/K_m (M^{-1} s^{-1}) for the hydrolysis of some characteristic β-lactams by various class A β-lactamases

	Group[a]		PG[b]	AMPI	CARB	OXA	CR	CT	CTX	CAZ	AZT	IMI
B. licheniformis[c]	2a	k_{cat}	2200	1500	400	10	630	48	6.7	15	ND	ND
		k_{cat}/K_m	29 000	11 000	8100	1200	5000	2500	33	9	ND	ND
S. albus G[c,d]	2a	k_{cat}	2800	3900	> 1000	270	200	260	> 1	< 0.001	> 0.5	0.04
		k_{cat}/K_m	2800	6100	100	630	620	370	1	< 0.001	0.4	0.32
S. aureus PC1[e]	2a	k_{cat}	130	240	190	> 7	0.074	0.006	ND	ND	ND	ND
		k_{cat}/K_m	40 000	20 000	70	1.3	100	8	ND	ND	ND	ND
TEM-1[e,f,g]	2b	k_{cat}	1600	1050	150	60	1500	170	9	0.3	1	0.04
		k_{cat}/K_m	84 000	33 000	9000	20 000	2200	650	1.5	0.07	0.7	1.5
S. fonticola[h]	2be	k_{cat}	770	ND	100	105	2530	7150	920	30	38	(−)
		k_{cat}/K_m	33 500	ND	2500	2500	14 500	40 900	11 500	60	930	(−)
TEM-7[f]	2be	k_{cat}	40	18	ND	9.5	26	20	1.5	9	4	ND
		k_{cat}/K_m	13 000	1100	ND	9500	300	250	15	9	3	ND
A. R39[c,d]	2d	k_{cat}	370	1900	220	940	440	200	280	> 13	20	< 0.0001
		k_{cat}/K_m	7500	16 000	870	5900	11 600	3700	400	13	76	9
P. vulgaris[i]	2e	k_{cat}	3.8	4.7	ND	0.8	85	37	78	1.9	3.3	0.004
		k_{cat}/K_m	640	450	ND	710	1100	1700	470	10	160	1000
IMI-1[j]	2f	k_{cat}	36	190	ND	ND	2000	120	3.4	0.0068	51	89
		k_{cat}/K_m	560	240	ND	ND	1900	920	18	0.0024	550	520

[a] Bush–Jacoby–Medeiros classification.[22] [b] PG, benzylpenicillin; AMPI, ampicillin; CARB, carbenicillin; OXA, oxacillin; CR, cephaloridine; CT, cephalothin; CTX, cefotaxime; CAZ, ceftazidime; AZT, aztreonam; IMI, imipenem. The data are from[c] Matagne *et al.*[40] [d] Matagne *et al.*[47] [e] Matagne and Frère, unpublished data; [f] Raquet *et al.*[48]; [g] Zafaralla and Mobashery[49]; [h] Péduzzi *et al.*[50]; [i] Datz and Frère, unpublished; [j] Rasmussen *et al.*[51] (—), no activity detected. ND, not determined.

to 7000 s^{-1}). Despite these high turn-over numbers, Waley and collaborators have succeeded in measuring the microscopic rate constants of some class A enzymes with various penicillin substrates.[10,32,52] Remarkably, the rate constants for acylation and deacylation, measured for the hydrolysis of benzylpenicillin by four different class A enzymes (*B. cereus* I, *B. licheniformis*, TEM-1 and *S. aureus* PC1), are similar. This, together with the high values for both k_{cat}/K_m and k_{cat}, suggested that these β-lactamases are 'fully efficient enzymes'.[10,32]

2.2 Fighting the β-lactamases

Two fundamentally different strategies have been devised in the fight against β-lactamases.[7,53] 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-lactamase-sensitive compounds. Thus, clavulanic acid has been widely used (Augmentin) to increase the efficiency of amoxycillin 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 ß-lactam combinations (second strategy).[5, 54]

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,[47] 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 variants[48] 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.* ceftazidime and cefotaxime; 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 identity[61] and which are very broad spectrum β-lactamases, hydrolysing efficiently both classical penams and cephems 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 though 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]

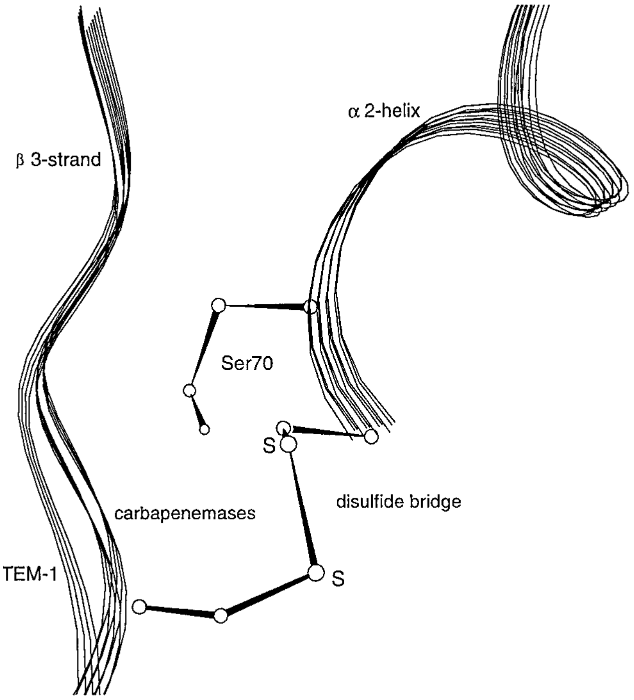


Fig. 5 Schematic drawing showing the modification of the β-3 strand in the class A carbapenem-hydrolysing enzymes (adapted from Raquet *et al.*[62]). The TEM-1 structure is used as a reference.

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 guanidinium 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 enzymes, in which two distinct residues have been proposed as potential general bases (Fig. 6). In one hypothesis,[59,75–77] this role is played by the conserved Glu-166. Its importance in the catalytic process was revealed by both crystallographic[71,78] and mutagenesis[75,79] studies. The detailed study of the E166D mutant of the *B. cereus* 569/H β-lactamase I[75] indicated that both the k_2 and k_3 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 deacylation 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 data[76] have suggested that a conserved water molecule (W1), which forms a bridge between the Ser-70 hydroxy group and the Glu-166 carboxylate (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 Ω-loop.[80]

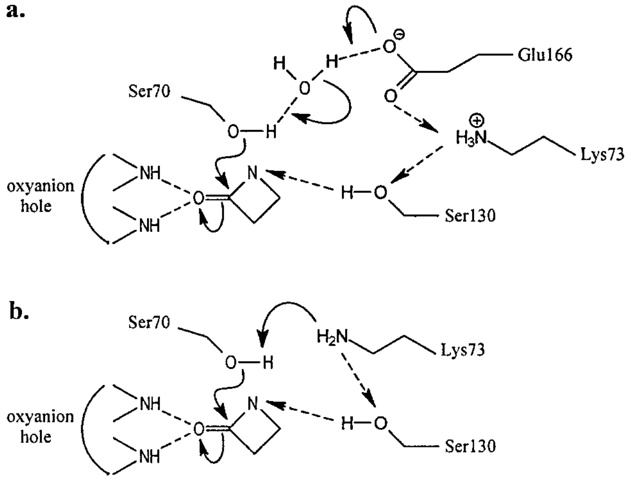


Fig. 6 Putative mechanisms for the formation of the tetrahedral intermediate in the acylation of class A β-lactamases. The role of general base is fulfilled by Glu-166 (a) or Lys-73 (b). In both cases, the breakdown of the tetrahedral intermediate involves the transfer of a proton from the general base to the β-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 activated O_γ of Ser-70 can then attack the β-lactam carbonyl carbon on the well exposed α-face of the molecule and the proton would be delivered back to the leaving nitrogen atom of the β-lactam *via* a network of hydrogen bonds involving the ε-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 β-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 and ensure back-delivery of the abstracted proton to the Ser-70 O_γ atom, ultimately leading to free enzyme regeneration.[76] Interestingly, the phosphonate structure[81] mentioned above indicates a major reorientation of the Lys-73 side chain, which is displaced by 0.3 Å (Nγ-H···Oε-H distance) from the Glu-166 side chain. A similar displacement (~ 0.6 Å) has been predicted in the tetrahedral intermediate formed with the *S. albus* G β-lactamase and benzylpenicillin,[76] which can be easily explained if Glu-166 is protonated at this stage.

The second hypothesis[70] 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 (by 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 carboxylate group of Glu-166 is much closer to the ε-amino group of Lys-73 than is any positively charged side-chain.[44] Moreover, NMR titration of the ^{13}C-labelled lysine residues in the TEM-1 β-lactamase[82] indicated a 'normal' pK_a value ($\geqslant 10$) for the Lys-73 side chain, making it a very unlikely candidate for proton abstraction in catalysis. These experimental findings have recently been further strenghtened by a calculation of the pK_a values (> 10) for Lys-73 in two class A enzymes (TEM-1 and *B. licheniformis*).[83]

Several kinetic studies performed with Glu-166 mutants of various class A β-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 ~ 100 fold decrease of the value of k_2 for benzylpenicillin hydrolysis, while its E166D counterpart causes a ~ 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 studies[47,48,85–87] of β-lactam compounds bearing a methoxy (cefoxitin, moxalactam and temocillin; Fig. 2h,j) or a hydroxyethyl (imipenem; Fig. 2i) substituent side chain on the α-face of the β-lactam ring, or also an oximino moiety on the C-7 side chain of cephalosporins (cefotaxime, ceftazidime 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 β-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 β-lactamases. A more detailed discussion of the many available data can be found in reference.[40]

3 Class C β-lactamases

The isolation and characterization of stable acylated-serine enzyme complexes by biochemical techniques[88–90] and recently the direct observation of the acyl-enzyme intermediate with the help of electrospray mass spectrometry[91] have firmly established class C β-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 1 β-lactamases of the Bush–Jacoby–Medeiros classification.[22]

Class C β-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.[95]

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 β-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 β-lactamases which exhibit an extended spectrum of activity against aztreonam, cefotaxime, ceftazidime, ceftriaxone and other oximino β-lactams.[117]

3.2 Kinetic properties

Class C β-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_{cat} and K_m, which means in practice that they are readily saturated to form acyl-enzymes which hydrolyse relatively slowly. Although the k_{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.

The generally low k_{cat} and K_m values (Table 3) of class C enzymes can in most cases be attributed to small k_{+3} values (*i.e.* rate limiting deacylation step).[90] This was confirmed by the determination of individual rate-constants through rapid kinetics experiments[118] and by the detection of stable acyl-enzyme complexes formed with poor substrates such as aztreonam,[68] oxacillin[88] and carbenicillin[91] which exhibit k_{cat} values of 10^{-3} s^{-1} or less.[119] This particularity makes the class C enzymes less 'efficient' than their class A counterparts, which have developed a different and more effective deacylation mechanism.[32]

Generally speaking, class C β-lactamases catalyze the hydrolysis of linear acyclic substrates (depsipeptides and thiolesters but not peptides, see below) more efficiently than do class A enzymes.[120–122] This fact emphasizes the adaptability and reactivity of the active-site cavity of these enzymes, which can accommodate either linear acyclic or bicyclic non-planar structures with a (thiol)ester or peptidic reactive bond.[123]

3.3 Inhibitors and inactivators

Class C β-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_{+2}/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 β-lactamases because of low affinity, poor acylation rate and non-negligible turnover.

Another important difference is observed with cephamycins (*e.g.* cefoxitin, Fig. 2j) which contain a 7α-methoxy substituent. Generally class A enzymes do not interact efficiently with this compound, because the α-methoxy group displaces the catalytically important W1 water molecule in the active-site cavity.[85] 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 β-lactamase from

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

		PG[a]	AMPI	CARB	OXA	CR	CT	CTX	CAZ	AZT	IMI	COX	CLA
E. cloacae P99	k_{cat}	15	0.7	0.003	0.006	700	200	0.015	0.012	0.0002	0.003	0.06	0.2
	k_{cat}/K_m	23000	1800	260	7000	10 000	20 000	1500	2.5	260	60	2500	0.04
C. freundii	k_{cat}	31	6.5	0.002	0.005	700	210	0.017	ND	0.0002	0.016	0.32	ND
OS60	k_{cat}/K_m	75 000	30 000	7000	12 000	20 000	16 000	3400	ND	180	140	1300	ND
E. coli K12	k_{cat}	45	4.2	0.004	ND	130	300	0.17	ND	0.00016	0.01	0.2	0.15
	k_{cat}/K_m	10 000	1200	230	ND	760	7000	100	ND	135	11.2	300	0.017
S. marcescens	k_{cat}	75	0.46	ND	ND	1100	1100	1.7	ND	0.0007	0.001	0.014	1
	k_{cat}/K_m	44 000	46 000	ND	ND	4000	16 000	140	ND	12	27	40	0.2
P. aeruginosa	k_{cat}	76	4.4	0.005	004	110	430	0.15	ND	0.0023	0.03	0.12	ND
18SH	k_{cat}/K_m	45 000	9000	240	1600	5000	17 000	750	ND	58	88	2400	ND

[a] PG, benzylpenicillin; AMPI, ampicillin; CARB, carbenicillin; OXA, oxacillin; CR, cephaloridine; CT, cephalothin; 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.

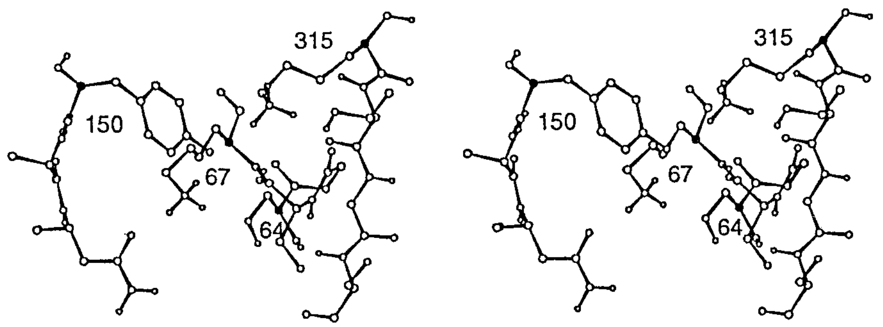


Fig. 7 Simplified stereo view of the active site of the class C *E. cloacae* P99 β-lactamase. The important catalytic residues Ser-64, Lys-67, Tyr-150 and Lys-315 are numbered. Besides those of the backbone NH groups, the protons on the following groups are shown: the hydroxy groups of Ser-64 and Thr-316, the alkylammoniums of Lys-67 and Lys-315, and the amide of Asn-152.

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 β-lactamases.[130,131] They react through the phosphonylation of the active-site serine to form a tetrahedral intermediate analog.[132] Inactivation of class C enzymes is relatively rapid ($\sim 10^3$ M^{-1} s^{-1}) whereas reactivation is very slow ($\sim 10^{-6}$ s^{-1}).[130] The crystal structure of a phosphonate derivative of the *Enterobacter cloacae* P99 β-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 at a pH close to neutrality, the optimal pH (k_{cat}/K_m) of the P99 β-lactamase being about 7.5.[135]

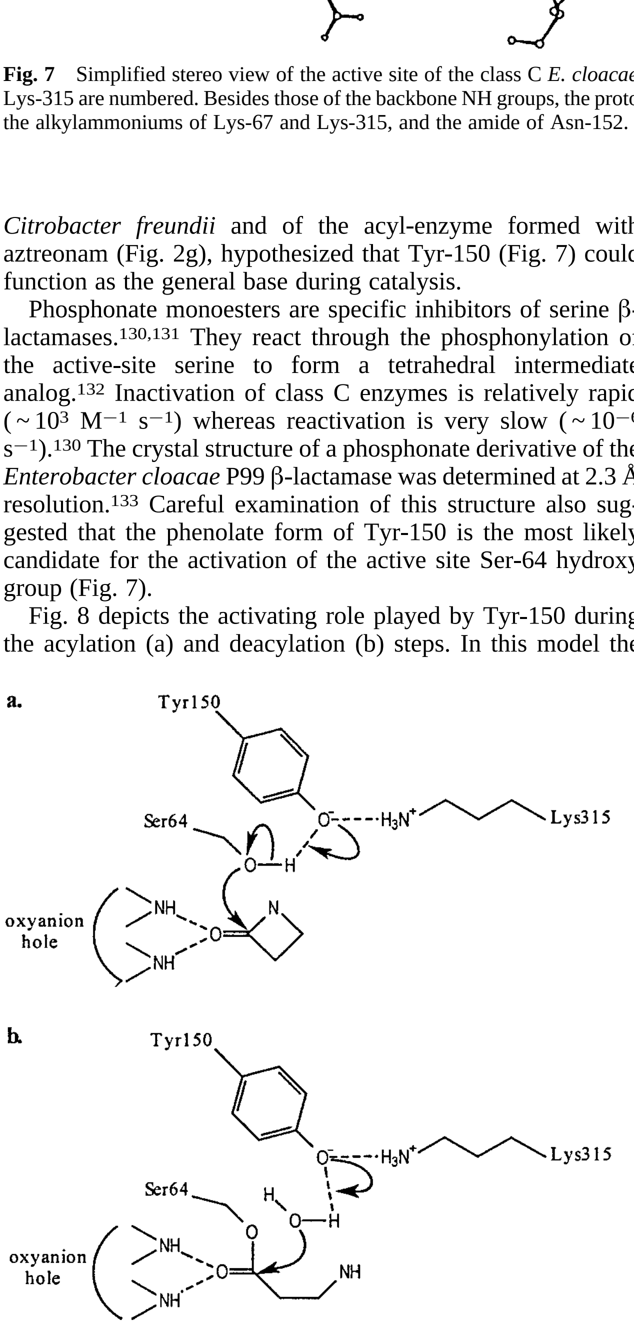


Fig. 8 Putative mechanism of class C β-lactamases. a: acylation reaction: the Tyr-150 phenolate anion acts as a general base for activating Ser-64. b: deacylation: the same residue activates a water molecule.

This hypothesis is also supported by results obtained from kinetic analysis of a number of site-directed mutants involving most of the postulated key residues of the catalytic cavity.[136] Conversely to what is observed with class A enzymes,[137] replacement of Lys-315 by His[138] failed to produce an active enzyme. 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 Oγ, 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 β-lactam is expected to bind.[133] The positive charges carried by the lysine side-chains and that due to the dipole of the α-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 β-lactamases. In conclusion, as in class A, it appears unlikely that the unprotonated amino group of Lys-67 acts as a general base.

3.5 Evolutionary considerations

These observations, added to the facts that class C β-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 β-lactamases, evolutionarily closer to DD-peptidases than to other serine β-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[141] 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 β-lactamase-producing organisms has been due to cephalosporins rather than penicillins.

4 Class D β-lactamases

Class D β-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*.[144] Their existence has been known for a long time,[145] but their identification as a new distinct molecular class came with the determination of the first primary structures in the late 1980s.[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.[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 'oxacillinases'. A comparative study of three members of this class showed that this behaviour was their most remarkable common feature.[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. [145]

Class D β-lactamases exhibit 'burst' or biphasic kinetics with many substrates. An extensive study[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-sensoring BLAR protein,[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/Asp residue and a Tyr-Gly-Asn motif at locations equivalent to the Glu-166 and Tyr-150 of class A and C β-lactamases, respectively.[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, cefpirome, ceftriaxone, moxalactam and aztreonam was recently isolated and shown to arise from OXA-2 through a single amino-acid replacement.[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).[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^{2+}-requiring enzymes were found in *Stenotrophomonas maltophilia* (*Xanthomonas maltophilia*)[154] and in an increasing number of nosocomial strains such as *Pseudomonas aeruginosa*,[30] *Serratia marcescens*,[29] *Klebsiella pneumoniae*,[155] *Bacteroides fragilis*,[156,157] *Aeromonas hydrophila*[158] and *Chryseobacterium meningosepticum*.[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.[61,158,160,161] Other organisms, *i.e. B. cereus, B. fragilis* and *S. maltophilia*, produce a chromosomal metallo-β-lactamase together with a serine β-lactamase.[28] Thus, *B. cereus* secretes a class A β-lactamase (BcI) together with the metallo BcII enzyme.[27] Furthermore, the production of the different enzymes can be induced by the presence of β-lactams in the media.[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.[153,162] With the latter, this was found to be independent of the nature of the C-3 substituent. However, esterification of the carboxylic 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.[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 carbapenems, cephamycins and third-generation cephalosporins (Table 4). Monobactams are the sole β-lactam antibiotics to be poorly recognized. By contrast, the Zn^{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.[162–164]

The β-lactamases produced by *B. fragilis* cfiA (CfiA),[166] *S. maltophilia* ULA511 (L1)[162-164] and *S. marcescens* TN9011 (IMP-1)[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^{-1} s^{-1}). They are the most active enzymes against compounds of the cephamycin (Fig. 2j) and oxacephamycin (Fig. 2h) families ($k_{cat}/K_m = 10^6$ M^{-1} s^{-1} for L1 *vs.* 100 M^{-1} s^{-1} for BcII). Tazobactam poorly inactivates these enzymes at very high molar ratios (> 10 000).[171]

The presence of chelating agents, such as EDTA, EGTA, dipicolinic acid and *o*-1,10-phenanthroline not surprisingly inhibits the metallo-β-lactamases.[153,172] The interactions between the enzymes and the chelating agent lead to the formation of ternary enzyme–metal ion–chelatant complexes, which slowly decay into apoenzyme and chelatant-metal complexes. In the case of the *A. hydrophila* AE036 β-lactamase, EDTA is the most effective inactivating agent with a second order rate constant of 10000 M^{-1} s^{-1}.[172] The formation of the ternary complexes increases the difficulty of obtaining good preparations of apoenzymes. Preparation of the *A. hydrophila* and *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 *B. cereus* 569H BcII,[173] *B. fragilis* CfiA,[157] *S. maltophilia*,[174] *A. hydrophila* AE036[158] and *S. marcescens* TN9011[29] indicates a rather low degree of isology (Fig. 9). In this comparison, it should be kept in mind that the *S. marcescens*, *P. aeruginosa* (N. Laraki, M.

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 β-lactams by various class B β-lactamases

		PG[a]	AMPI	CARB	OXA	CR	CTX	COX	AZT	IMI
B. cereus	k_{cat}	680	1100	16	325	25	60	0.2	(—)	> 100
(BcII)[b,c,d]	k_{cat}/K_m	450	720	36	190	19	670	0.095	(—)	120
B. fragilis	k_{cat}	190	190	190	ND	42	98	10	(—)	200
(CfiA)[b]	k_{cat}/K_m	4800	1600	800	ND	7000	3600	90	(—)	740
C. menigosepticum	k_{cat}	280	ND	ND	ND	14	39	6	(—)	350
(BlaB)[e]	k_{cat}/K_m	6850	ND	ND	ND	480	220	250	(—)	950
S. marcescens	k_{cat}	320	950	ND	ND	53	1.3	16	(—)	46
(IMP-1)[f]	k_{cat}/K_m	620	4800	20	ND	2400	350	2000	(—)	1200
A. hydrophila	k_{cat}	I	I	I	0.75	0.12	0.07	I	(—)	1400
(CphA)[b,c,d,g]	k_{cat}/K_m	I	I	I	30	0.63	2.7	I	(—)	7600
X. maltophilia	k_{cat}	280	175	280	285	28	66	1.4	(—)	65
(ULA511)[b,c,d]	k_{cat}/K_m	1400	4400	180	11 000	93	2600	550	(—)	730

[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] Rossolini *et al.*[159]; [f] Watanabe *et al.* [30]; [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] β-lactamases are identical. For the same reason, we only take account of the *B. fragilis* CfiA and *A. hydrophila* CphA sequences, and discard those of *B. fragilis* CcrA, CcrA3 and CcrA4,[61,156,157] *A. veronii* ImiS[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.

	1				50
BcII	SQKVEKTVIK	NETGTISISQ	LNKNVWVHTE	LGSFNGEA.V	PSNGLVLNTS
Blab	....	QENPDVKIEK	LKDNLYVYTT	YNTFNGTK.Y	AANAVYLVTD
Cfia	.....AQKSV	KISDDISITQ	LSDKVYTYVS	LAEIEGWGMV	PSNGMIVINN
IMP-1	.........A	ESLPDLKIEK	LDEGVYVHTS	FEEVNGWGVV	PKHGLVVLVN
CphA	....	........AG	MSLTQVSGPV	YVVEDNYY.V	QENSMVYFGA
L1	...AEVPLPQ	LRAYTVDASW	LQPMAPLQIA	DHTWQIGT.E	DLTALLVQTP
	51			* *	* 100
BcII	KGLVLVDSSW	DDKLTKELIE	MVEKKFQK.R	VTDVIIT**H**A**H**	A**D**RIGGIKTL
Blab	KGVVVIDCPW	GEDKFKSFTD	EIYKKHGK.K	VIMNIAT**H**S**H**	D**D**RAGGLEYF
Cfia	HQAALLDTPI	NDAQTEMLVN	WVTDSLHA.K	VTTFIPN**H**W**H**	G**D**CIGGLGYL
IMP-1	AEAYLIDTPF	TAKDTEKLVT	WFVERGY..K	IKGSISS**H**F**H**	S**D**STGGIEWL
CphA	KGVTVVGATW	TPDTARELHK	LIKRVSRK.P	VLEVINT**N**Y**H**	T**D**RAGGNAYW
L1	DGAVLLDGGM	PQMASHLLDN	MKARGVTPRD	LRLILLS**H**A**H**	A**D**HAGPVAEL
	101				150
BcII	KERG.IKAHS	TALTAELAKK	N.........	.......GYE	EPLGDLQTVT
Blab	GKIG.AKTYS	TKMTDSILAK	E.........	.......NKP	RAQYTFDNNK
Cfia	QRKG.VQSYA	NQMTIDLAKE	K.........	.......GLP	VPEHGFTDSL
IMP-1	NSRS.IPTYA	SELTNELLKK	D.........	.......GKV	QATNSFS.GV
CphA	KSIG.AKVVS	TRQTRDLMKS	DWAEIVAFTR	KGLPEYPDLP	LVLPNVVHDG
L1	KRRTGAKVAA	NAESAVLLAR	G....GSDDL	HFGDGITYPP	ANADRIVMDG
	151	*			* 200
BcII	NLKFGNMKVE	TFYPGKG**H**TE	DNIVVWLPQY	......NILV	GG**C**LVKSTSA
Blab	SFKVGKSEFQ	VYYPGKG**H**TA	DNVVVWFPKE	......KVLV	GG**C**IIKSADS
Cfia	TVSLDGMPLQ	CYYLGGG**H**AT	DNIVVWLPTE	......NILF	GG**C**MLKDNQA
IMP-1	NYWLVKNKIE	VFYPGPG**H**TP	DNVVVWLPER	......KILF	GG**C**FIKPY..
CphA	DFTLQEGKVR	AFYAGPA**H**TP	DGIFVYFPDE	......QVLY	GN**C**ILK....
L1	EVITVGGIVF	TAHFMAG**H**TP	GSTAWTWTDT	RNGKPVRIAY	AD**S**LSAPG.Y
	201			*	250
BcII	KDLGNVAD.A	YVNEWSTSIE	VNLKRYRNIN	AVVPG**H**GE.V	GDKGLLLHTL
Blab	KDLGYIGE.A	YVNDWTQSVH	NIQQKFSGAQ	YVVAG**H**DD.W	KDQRSIQHTL
Cfia	TSIGNISD.A	DVTAWPKTLD	KVKAKFPSAR	YVVPG**H**GD.Y	GGTELIEHTK
IMP-1	.GLGNLGD.A	NIEAWPKSAK	LLKSKYGKAK	LVVPS**H**SE.V	GDASLLKLTL
CphA	EKLGNLSF.A	DVKAYPQTLE	RLKAMKLPIK	TVIGG**H**DSPL	HGPELIDHYE
L1	QLQGNPRYPH	LIEDYRRSFA	TVRAL..PCD	VLLTP**H**PG.A	SNWDYAAGAR
	251			281	
BcII	DLLK......	....	....	.	
Blab	DLINEYQQKQ	KASN......	....	.	
Cfia	QIVNQYIEST	SKP.......	....	.	
IMP-1	EQAVKGLNES	KKPSKPSN..	....	.	
CphA	ALIKAAPQS.	....	....	.	
L1	AGAKALTCKA	YADAAEQKFD	GQLAKETAGA	R	

Fig. 9 Sequence alignment of class B β-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*; CfiA: *B. fragilis*; IMP-1: *S. marcescens* TN9011; CphA: *A. hydrophila* AE036; L1: *S. maltophilia* ULA511.

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. mennigoscepticum*[159] and *A. hydrophila*[158] 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 found in at least four other nosocomial microorganisms, *i.e. P. aeruginosa, P. putida, Alcaligenes xylosidans* and *K. pneumoniae*.[177] In *P. aeruginosa* 101/1477, *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 presence of increasing concentrations of imipenem results in the appearance of a small number of highly resistant strains in which the β-lactamase gene is activated. In the case of the *B. fragilis* cfiA enzyme, activation of the transcription of the *cfiA* gene is due to the insertion of a 1.3 kb sequence (*IS1186*) immediately upstream of the coding gene. Upon insertion, transcription of *cfiA* is driven by a promoter identified at the downstream end of the IS element.[179,180]

5.4 Three-dimensional structures and mechanisms

The three-dimensional structures of the metallo-β-lactamases of *B. cereus* 569H[181] and *B. fragilis* cfiA[182,183] 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 Zn^{2+}-ligands are disposed in a distorted tetrahedral shape. A hydrogen-bonded network involving the Zn^{2+} 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.

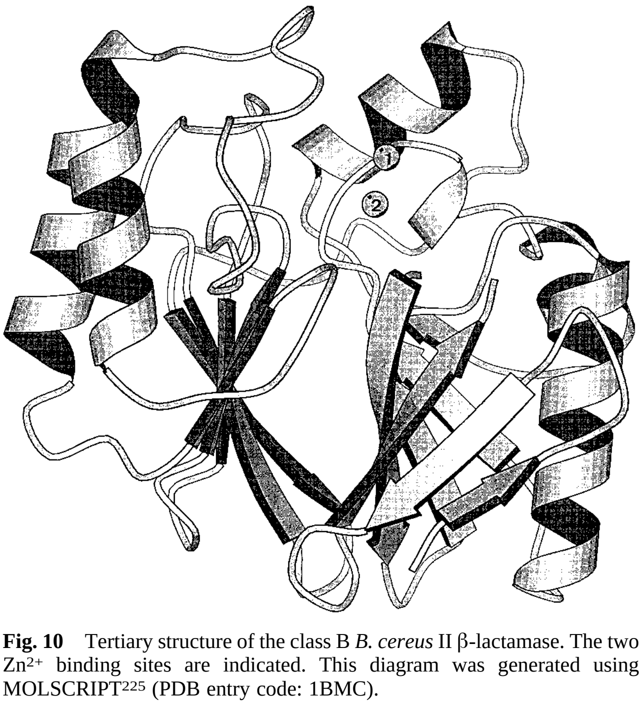


Fig. 10 Tertiary structure of the class B *B. cereus* II β-lactamase. The two Zn^{2+} binding sites are indicated. This diagram was generated using MOLSCRIPT[225] (PDB entry code: 1BMC).

As observed in other zinc hydrolases, the metal ion can play a dual role in catalysis. Firstly, the Zn^{2+}-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 pK_a value from 14 to 7, as shown for carbonic anhydrase.[185] At physiological pH, a non-negligible proportion of the water molecules would be present as hydroxide ions. Secondly, the Zn^{2+} 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 above. Asp-92, 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-Zn^{2+} 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 bipyramid 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[182] 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,[182] 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* 569H (BcII) and *B. fragilis* CfiA β-lactamases contain two metal ion binding sites. In the case of CfiA,[186] both zincs appear to be tightly bound. The enzyme can bind either two Zn^{2+} or two Co^{2+} ions per mole. Electron paramagnetic resonance studies of the Co(II) 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(II) 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.[186]

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^{2+}-β-lactamase II and of nitrocefin by the Zn^{2+}-β-lactamase II at sub-zero temperature indicated the prevalence of a branched mechanism with two non-covalent intermediates ES^1 and ES^2.[187] Spectroscopic studies of these complexes suggested that the metal is pentacoordinated in ES^1 and tetracoordinated in ES^2. These studies also suggested that the cysteine residue (Cys-193) is directly interacting with the metal during the catalytic cycle.[188]

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.[172]

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.[189] 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.[190] These compounds behave as suicide-substrates. The hydrolysis of the thiolester 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.[191,192] Thus, trifluoromethyl ketones derived from L-alanine, D-alanine or D-phenyalanine 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.[193]

6 Genetic support and induction

Chromosome and plasmid-encoded proteins are found in all four classes of β-lactamases.[22] 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-

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

	Constitutive	Inducible
Class A		
plasmid	TEM, SHV	*S. aureus* PC1 (6.3)
chromosome	*K. oxytoca, K. pneumoniae,* *S. albus* G	*B. licheniformis* (6.3) *S. cacaoi* (6.2) *P. vulgaris* (6.1)
Class C		
plasmid	BIL, CMY, FOX	*M. morganii* (6.1)
chromosome	AmpD mutants	All other Enterobacteria (6.1)
	E. coli	*Pseudomonas* sp. (6.1) *A. jandaei* AsbA1 (6.4)
Class D		
plasmid	All OXA but OXA-12	
chromosome		*A. jandaei* AsbB1 (OXA-12) (6.4)
Class B		
plasmid	*K. pneumoniae, B. fragilis* cfiA, *S. marcescens, . . .* (see text)	
chromosome	*B. fragilis, B. cereus* 569/H, *B. cereus* 5/B/6, *A. hydrophila*	*S. maltophilia,* *B. cereus* 569, *A. jandaei* AsbM1 (6.4)

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.[195]

Presently, four distinct induction mechanisms have been identified.

6.1 The AmpG-AmpD-AmpR-AmpC system

With the exception of *E. coli*, naturally occurring chromosome-encoded class C β-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.[92,94]

This acute clinical problem has fuelled active interest in the induction of class C β-lactamases which has been extensively studied[194] and it has been found that a simple disrupting mutation in the *amp*D gene could result in the constitutive, overproducing phenotype.[195]

Rather than detecting the presence of the β-lactam in the medium, the induction mechanism of class C β-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 PBPs), β-lactams modify this balance and trigger the induction mechanism.[196]

The *ampC* gene encodes the β-lactamase.[24] It is under the control of the product of the nearby *ampR* gene, a DNA-binding protein of the LysR family which also represses its own expression.[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.[198] The AmpD enzyme, an intracellular amidase, then liberates the tripeptide which is reintroduced in the biosynthetic pathway.[199] The free AmpR protein acts as an activator of the *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 β-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.[196] In *ampD*$^-$ mutants,[196,200] 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 *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 *Pseudomonas* sp.)[100] with the exception of *E. coli*, where the *ampR* gene is absent and the *ampC* gene is transcribed with a poor efficiency under all conditions, despite the presence of the *ampD* gene on the chromosome.[24]

At the present time, the only inducible plasmid-encoded class C enzyme has been found in *Morganella morganeii*,[115] where the plasmid contains both the *ampR* and *ampC* genes in an arrangement similar to that found in the chromosomes of enterobacteria.

In *Proteus vulgaris*, the synthesis of a class A β-lactamase is similarly controlled and transformation by plasmids carrying the *Citrobacter freundii ampG* and *ampD* (but not *ampR*) genes can restore the original inducible phenotype which has been lost following mutations in the corresponding, original chromosomal genes.[201]

6.2 Induction in *Streptomyces cacaoi*

S. cacaoi produces two inducible β-lactamases, BlaL and BlaU.[202,203] Four open reading frames are found near and upstream of the *blaL* gene, of which two, *blaA* and *blaB* are both necessary for induction.[204] BlaA is a repressor/activator of the LysR family which binds in the intercistronic region between *blaA* and *blaL*.[202] BlaB contains the SXXK and KTG conserved elements characteristic of PBPs but is located on the inner face of the cytoplasmic membrane.[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 *Bacillus licheniformis*

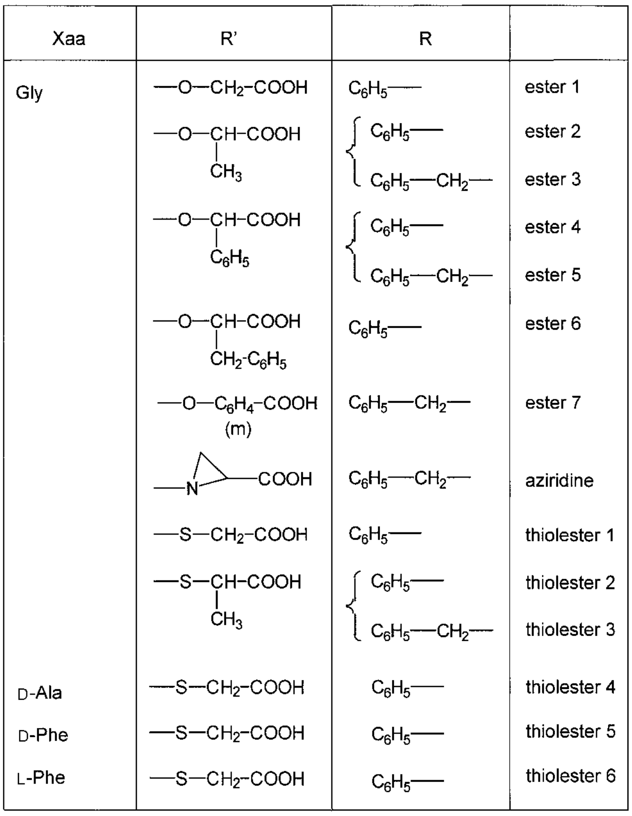
Three genes *blaI, blaR1* and *blaR2* are involved in the regulation of the structural gene *blaP* which encodes a class A β-lactamase.[206] BlaI acts as a typical repressor. *blaR1* encodes a transmembrane protein whose extracellular C-terminal domain covalently binds β-lactams and detects the presence of these compounds in the external medium. The message is then transmitted into the cytoplasm *via* the four transmembrane helices of BlaR1.[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.[208] Mutations in the *blaR2* gene can result in constitutive production of the β-lactamase. The *balR1, balI* and *blaP* genes are contiguous and the two regulatory genes are oppositely oriented when compared to their structural neighbour. The *blaR2* gene is in a completely different locus. It is likely that the expression of the class A β-lactamase of *B. cereus* is controlled by a similar mechanism. The *blaI, blaR* and *blaP* genes of *S. aureus* are organized in the same way on the transposon TN552.[209] In *Streptococcus epidermidis*, a resistance gene (*mrsA* or *mecA*) codes for a β-lactam-resistant PBP, in a related system.[210]

6.4 *Aeromonas jandei*

This Gram-negative bacterium (formerly known as *Aeromonas sobria* AER14) expresses three inducible β-lactamases, belonging respectively to classes B (AsbM1), C (AsbA1) and D (OXA-12 or AsbB1).[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 *cre* family might be responsible for this phenomenon.

7 Linear (depsipeptide) substrates of β-lactamases

In a seminal paper,[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, class B and class C β-lactamases. Moreover, the latter also catalysed acyl transfer reactions when supplied with adequate acceptors, such as D-amino-acids. A variety of ester, thiol ester and aziridine derivatives of general structure R-CO-Xaa-R′ were later studied, and the structures and properties of the most characteristic compounds are summarized in Fig. 11 and Table 6. In fact, β-lactamases of all four classes exhibit activity on at least some of the tested compounds, although this activity remains significantly lower than with their best β-lactam substrates. The highest activities are usually recorded with class C β-lactamases, although class A enzymes can be more active against thiol esters 1, 2 and 3. As could be expected on the basis of their respective chemical reactivities, thiol esters 1, 2 and 3 were better substrates than their ester counterparts. Nonetheless, the highest k_{cat}/K_m value is found for the interaction between



Xaa	R′	R	
Gly	$-O-CH_2-COOH$	C_6H_5-	ester 1
	$-O-CH(CH_3)-COOH$	C_6H_5-	ester 2
		$C_6H_5-CH_2-$	ester 3
	$-O-CH(C_6H_5)-COOH$	C_6H_5-	ester 4
		$C_6H_5-CH_2-$	ester 5
	$-O-CH(CH_2-C_6H_5)-COOH$	C_6H_5-	ester 6
	$-O-C_6H_4-COOH$ (m)	$C_6H_5-CH_2-$	ester 7
	$-N-COOH$	$C_6H_5-CH_2-$	aziridine
	$-S-CH_2-COOH$	C_6H_5-	thiolester 1
	$-S-CH(CH_3)-COOH$	C_6H_5-	thiolester 2
		$C_6H_5-CH_2-$	thiolester 3
D-Ala	$-S-CH_2-COOH$	C_6H_5-	thiolester 4
D-Phe	$-S-CH_2-COOH$	C_6H_5-	thiolester 5
L-Phe	$-S-CH_2-COOH$	C_6H_5-	thiolester 6

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.

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.[121] 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 R′ 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-workers[214–216] 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.[217] 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 reversibly[126] with K_i 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 C_6H_5- or (m) $H_2N-C_6H_4$-yielded efficient inhibitors, but for the *B. cereus* I class A enzyme, a more complex side-chain ($C_6H_5-CH_2-CO-NH-CH_2-$) was necessary to obtain a K_i value of about 15 μM.[124] The same compound was also an efficient inhibitor of the P99 enzyme.[218] More recently, (1*R*)-1-acetamido-2-(3-carboxyphenyl)ethane boronic acid was also found to be a highly effective inhibitor (K_i = 110 nM) of the TEM-1 enzyme.[219]

8.2 Phosphonic acid derivatives

Pratt and co-workers[130,131,220] have described a series of rather efficient transient inactivators of class C enzymes which have the general structure

$$R-CO-NH-CH_2-(PO_2^-)-X$$

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 Second order rate constants k_{cat}/K_m(M^{-1} s^{-1}) for the acylation of various β-lactamases by the depsipeptides (R-CO-Xaa-R′) described in Fig. 11

	Enzymes				
Compound	Class A[a]	Class C P99[b]	Class D OXA-2	Class B *B. cereus* II	References
Ester 1	90	310	ND	ND	120
Ester 2	very low (< 1)	140	ND	ND	120
Ester 3	0.1–0.9	2500	ND	5	123
Ester 4	120–290	4600	ND	ND	120
Ester 5	ND	2200	ND	ND	212
Ester 6	50–300	20 000[c]	< 5	ND	121
Ester 7	300–13 000	54 000	ND	ND	212
Aziridine	120–1900	39 000	ND	4000	123
Thiolester 1	4500–16 000	2400	200	ND	121
Thiolester 2	6000–11 000	3600	265	ND	121
Thiolester 3[d]	340–540	6700	ND	150	213
Thiolester 4	< 10–100	240	16	ND	121
Thiolester 5	< 10	330	< 5	ND	121
Thiolester 6	< 10	1200	< 5	ND	121

[a] The enzymes tested were those from *B. licheniformis, S. albus, S. aureus* PC1, *B. cereus* I and the TEM plasmid. [b] P99 is an *E. cloacae* strain. Very similar results were obtained with the *E. coli* K12 enzyme. [c] A very low activity (10 M^{-1} s^{-1}) was recorded with the L-isomer. [d] 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.

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–CH_2–(PO_2^-)–X

		Enzymes				
R	X	*E. cloacae* P99	TEM	*S. aureus* PC1	*B. cereus* I	References
C_6H_5–CH_2–	–O–C_6H_4–NO_2 (*p*)	56 000	0.9	4	0.02	131, 220
	–O–C_6H_4–NO_2 (*o*)	55 000	1.3	0.8	0.01	220
	–O–C_6H_4–NO_2 (*m*)	23 000	0.04	0.11	<0.01	220
	–O–C_6H_4–COOH (*m*)	1100	(—)	(—)	(—)	130
	–O–CH(CH_3)–COOH[a]	(—)	(—)	(—)	(—)	130
C_6H_5–CH_2–O–	–O–C_6H_4–NO_2 (*p*)	33 000	2.7	2.5	0.3	130
	–S–C_6H_5	26 000	2	0.07	0.3	130
	–F	26 000	2.1	0.34	0.3	130

[a] D-Stereochemistry. (—): no detectable inactivation.

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.[72,81]

8.3 Phosphonamidates

Among the various phosphonamidates studied as β-lactamase inactivators,[221–223] the best results were obtained with C_6H_5–CH_2–O–CO–NH–(PO_2^-)–R, where R was L-phenylalanine, β-alanine or β-phenyl-β-alanine, exhibiting second order acylation rate constants of 230, 150 and 250 $M^{-1}s^{-1}$, 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[141,142]) 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 preexisting 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 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 cross-talk mechanism which allows a response of the control of the biosynthetic pathways to modifications in the environment as detected by the balance between the synthesis and the degradation of the cell wall peptidoglycan.[196]

A final surprising observation is also worth mentioning: despite strong selective pressure, the β-lactamase of *Staphylococcus aureus* has failed to adapt and evolve into an enzyme which can hydrolyse methicillin and cephalosporins. Similarly, these bacteria did not acquire genes encoding broader-spectrum enzymes. When compared to the remarkable adaptability of the TEM and SHV enzymes and the facile spreading of the corresponding genes, this remains a haunting question, although it can be argued that, when the β-lactamase is secreted into the growth medium, a potential mutant would protect the whole population and the producing cell would be diluted out as soon as the selective pressure is removed. This did not however impair the initial rapid emergence of the β-lactam resistant strains of the same *Staphylococci* as soon as the parent benzylpenicillin was first introduced in our chemotherapeutic arsenal. Thus, many questions remain unanswered and it can be predicted that the study of β-lactamases will remain an exciting field for generations of biochemists.

The β-lactam ring is present in many naturally occurring compounds and it is thus not surprising to find enzymes which can catalyse its hydrolysis. Conversely, peptidoglycan synthesis, a specific bacterial pathway, remains a choice target for potential antibiotics, and hitting the active site of the DD-peptidases, which is oriented towards the outer medium, still appears to constitute a particularly obvious and efficient goal. In this respect, the synthesis of entirely original, non-β-lactam inactivators of these enzymes should represent a highly attractive alternative to the present strategies.

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11 References

1 J. Davies, *Science*, 1994, **264**, 375.
2 W. Witte, *Science*, 1998, **279**, 996.
3 J. M. Frère and B. Joris, *CRC. Crit. Rev. Biochem.*, 1985, **11**, 299.
4 J. M. Ghuysen, *Ann. Rev. Microbiol.*, 1991, **45**, 37.
5 J. Coyette, M. Nguyen-Distèche, J. Lamotte-Brasseur, B. Joris, E. Fonzé and J. M. Frère, *Adv. Comp. Environ. Physiol.*, 1994, **20**, 233.
6 M. Jamin, J. M. Wilkin and J. M. Frère, *Essays Biochem.*, 1995, **29**, 1.
7 J. R. Knowles, *Acc. Chem. Res.*, 1985, **18**, 97.
8 J. M. Frère, B. Joris, B. Granier, A. Matagne, F. Jacob and C. Bourguignon-Bellefroid, *Res. Microbiol.*, 1991, **142**, 705.
9 H. Nikaido, *J. Bacteriol.*, 1996, **178**, 5853.
10 S. G. Waley, in *The chemistry of β-lactams*, ed. M. I. Page, 1992, pp. 198–228, A. & P. Blackie, London.
11 G. A. Jacoby, *Trends Microbiol.*, 1994, **2**, 357.
12 J. M. Frère, *Mol. Microbiol.*, 1995, **16**, 385.
13 J. M. Frère, M. Nguyen-Distèche, J. Coyette and B. Joris, in *The chemistry of β-lactams*, ed. M. I. Page, 1992, pp. 148–197, A. & P. Blackie, London.
14 C. G. Dowson, T. J. Coffey and B. G. Spratt, *Trends Microbiol.*, 1994, **2**, 361.
15 B. Spratt, *Science*, 1994, **264**, 388.
16 H. Nikaido, *Science*, 1994, **264**, 382.
17 S. G. Waley, *Microbiol. Sci.*, 1987, **4**, 143.
18 H. Nikaido and S. Normark, *Mol. Microbiol.*, 1987, **1**, 29.
19 J. M. Frère, *Biochem. Pharmacol.*, 1989, **38**, 1415.
20 R. B. Sykes and K. Bush, in *The Chemistry and Biology of β-lactam antibiotics*, vol. 3, ed. R. Morin and M. Gorman, 1982, pp. 155–207, Academic Press, New York.
21 G. Burton, N. F. Osborne, M. J. Pearson and R. Southgate, in *Burger's Medicinal Chemistry and Drug Discovery* (Fifth Edition), ed. M. E. Wolff, 1998, vol. 4: Therapeutic Agents, pp. 277-363, John Wiley & Sons, Inc.
22 K. Bush, G. A. Jacoby and A. A. Medeiros, *Antimicrob. Agents Chemother.*, 1995, **39**, 1211.
23 R. P. Ambler, *Philos. Trans. R. Soc. Lond.*, 1980, **B289**, 321.
24 B. Jaurin and T. Grundström, *Proc. Natl. Acad. Sci. USA*, 1981, **78**, 4897.
25 P. Huovinen, S. Huovinen and G. A. Jacoby, *Antimicrob. Agents Chemother.*, 1988, **32**, 134.
26 B. Joris, P. Ledent, O. Dideberg, E. Fonzé, J. Lamotte-Brasseur, J. A. Kelly, J. M. Ghuysen and J. M. Frère, *Antimicrob. Agents Chemother.*, 1991, **35**, 2294.
27 R. B. Davies and E. P. Abraham, *Biochem J.*, 1974, **143**, 129.
28 D. J. Payne, *J. Med. Microbiol.*, 1993, 93.
29 E. Osano, Y. Arakawa, R. Wacharotayankun, M. Ohta, T. Horii, H. Ito, F. Yoshima and N. Kato, *Antimicrob. Agents Chemother.*, 1994, **38**, 71.
30 M. Watanabe, S. Yiobe, M. Inoue and S. Mitsuhashi, *Antimicrob. Agents Chemother.*, 1991, **35**, 147.
31 J. Fisher, J. G. Belasco, S. Khosla and J. R. Knowles, *Biochemistry*, 1980, **19**, 2895.
32 H. Christensen, M. T. Martin and S. G. Waley, *Biochem. J*, 1990, **266**, 853.
33 J. A. Kelly, O. Dideberg, P. Charlier, J. P. Wery, M. Libert, P. Moews, J. Knox, C. Duez, C. Fraipont, B. Joris, J. Dusart, J. M. Frère and J. M. Ghuysen, *Science*, 1986, **231**, 1429.
34 B. Samraoui, B. Sutton, R. Todd, P. Artymiuk, S. G. Waley and D. Phillips, *Nature*, 1986, **320**, 378.
35 J. R. Knox, P. C. Moews and J. M. Frère, *Chem. Biol.*, 1996, **3**, 937.
36 B. Joris, J. M. Ghuysen, G. Dive, A. Renard, O. Dideberg, P. Charlier, J. M. Frère, J. Kelly, J. Boyington, P. Moews and J. Knox, *Biochem. J*, 1988, **250**, 313.
37 F. Sanschagrin, F. Couture and R. C. Levesque, *Antimicrob. Agents Chemother.*, 1995, **39**, 887.
38 I. Massova and S. Mobashery, *Antimicrob. Agents Chemother.*, 1998, **42**, 1.
39 C. Goffin and J. M. Ghuysen, *Microbiol. Mol. Biol. Rev.*, 1998, in the press.
40 A. Matagne, J. Lamotte-Brasseur and J. M. Frère, *Biochem. J.*, 1998, **330**, 581.

41 O. Dideberg, P. Charlier, J. P. Wery, P. Dehottay, J. Dusart, T. Erpicum, J. M. Frère and J. M. Ghuysen, *Biochem. J.*, 1987, **245**, 911.
42 O. Herzberg, *J. Mol. Biol.*, 1991, **217**, 701.
43 J. R. Knox and P. C. Moews, *J. Mol. Biol.*, 1991, **220**, 435.
44 C. Jelsch, L. Mourey, J. M. Masson and J. P. Samama, *Proteins: Struct. Funct. Genet.*, 1993, **16**, 364.
45 A. Matagne and J. M. Frère, *Biochim. Biophys. Acta*, 1995, **1246**, 109.
46 A. Matagne, A. M. Misselyn-Bauduin, B. Joris, T. Erpicum, B. Granier and J. M. Frère, *Biochem. J.*, 1990, **265**, 131.
47 A. Matagne, J. Lamotte-Brasseur and J. M. Frère, *Eur. J. Biochem.*, 1993, **217**, 61.
48 X. Raquet, J. Lamotte-Brasseur, E. Fonzé, S. Goussard, P. Courvalin and J. M. Frère, *J. Mol. Biol.*, 1994, **244**, 625.
49 G. Zafaralla and S. Mobashery, *J. Am. Chem. Soc.*, 1992, **114**, 1505.
50 J. Péduzzi, S. Farzaneh, A. Reynaud, M. Barthélémy and R. Labia, *Biochim. Biophys. Acta*, 1997, **1341**, 58.
51 B. A. Rasmussen, K. Bush, D. Keeney, Y. Yang, R. Hare, C. O'Gara and A. A. Medeiros, *Antimicrob. Agents Chemother.*, 1996, **40**, 2080.
52 M. T. Martin and S. G. Waley, *Biochem. J.*, 1988, **254**, 923.
53 R. F. Pratt, in *The Chemistry of β-lactams*, edited by M. I. Page, 1992, pp. 229-271, A. & P. Blackie, London. 81.
54 A. A. Medeiros, *Clin. Infect. Dis.*, 1997, **24**(Suppl 1), S19.
55 E. Collatz, R. Labia and L. Gutmann, *Mol. Microbiol.*, 1990, **4**, 1615.
56 W. Sougakoff, S. Goussard, G. Gerbaud and P. Courvalin, *Rev. Infect. Dis.*, **10**, 1988, 879.
57 G. A. Jacoby and A. A. Medeiros, *Antimicrob. Agents Chemother.*, 1991, **35**, 1697.
58 J. A. Sowek, S. B. Singer, S. Ohringer, M. F. Malley, T. J. Dougherty, J. Z. Gougoutas and K. Bush, *Biochemistry*, 1991, **30**, 3179.
59 J. Lamotte-Brasseur, J. R. Knox, J. A. Kelly, P. Charlier, E. Fonzé, O. Dideberg and J. M. Frère, *Biotechnol. Genet. Eng. Rev.*, 1994, **12**, 189.
60 I. Massova and S. Mobashery, *Acc. Chem. Res.*, 1997, **30**, 162.
61 B. A. Rasmussen and K. Bush, *Antimicrob. Agents Chemother.*, 1997, **41**, 223.
62 X. Raquet, J. Lamotte-Brasseur, F. Bouillenne and J. M. Frère, *Proteins: Struct. Funct. Genet.*, 1997, **27**, 47.
63 X. Y. Zhou, F. Bordon, D. Sirot, M. D. Kitzis and L. Gutmann, *Antimicrob. Agents Chemother.*, 1994, **38**, 1085.
64 C. Henquell, C. Chanal, D. Sirot, R. Labia and J. Sirot, *Antimicrob. Agents Chemother.*, 1995, **39**, 427.
65 J. R. Knox, *Antimicrob. Agents Chemother.*, 1995, **39**, 2593.
66 U. Imtiaz, E. Billings, J. R. Knox, E. K. Manavathu, S. A. Lerner and S. Mobashery, *J. Am. Chem. Soc.*, 1993, **115**, 4435.
67 M. Paetzel and R. E. Dalbey, *Trends Biochem. Sci.*, 1997, **22**, 28.
68 C. Oefner, A. Darcy, J. J. Daly, K. Gubernator, R. L. Charnas, I. Heinze, C. Hubschwerlen and F. K. Winkler, *Nature*, 1990, **343**, 284.
69 E. Lobkovsky, P. C. Moews, L. Hansong, Z. Haiching, J. M. Frère and J. R. Knox, *Proc. Natl. Acad. Sci. USA*, 1993, **90**, 11257.
70 N. C. J. Strynadka, H. Adachi, S. E. Jensen, K. Johns, A. Sielecki, C. Betzel, K. Sutoh and M. N. J. James, *Nature*, 1992, **359**, 700.
71 O. Herzberg and J. Moult, *Science*, 1987, **236**, 694.
72 C. C. Chen, J. Rahil, R. F. Pratt and O. Herzberg, *J. Mol. Biol.*, 1993, **234**, 165.
73 J. A. Kelly and A. P. Kuzin, *J. Mol. Biol.*, 1995, **254**, 223.
74 M. Galleni, J. Lamotte-Brasseur, X. Raquet, A. Dubus, D. Monnaie, J. R. Knox and J. M. Frère, *Biochem. Pharmacol.*, 1995, **49**, 1171.
75 R. M. Gibson, H. Christensen and S. G. Waley, *Biochem. J.*, 1990, **272**, 613.
76 J. Lamotte-Brasseur, G. Dive, O. Dideberg, P. Charlier, J. M. Frère and J. M. Ghuysen, *Biochem. J.*, 1991, **279**, 213.
77 J. Lamotte-Brasseur, F. Jacob-Dubuisson, G. Dive, J. M. Frère and J.-M. Ghuysen, *Biochem. J.*, 1992, **282**, 189.
78 O. Herzberg, G. Kapadia, B. Blanco, T. S. Smith and A. Coulson, *Biochemistry*, 1991, **30**, 9503.
79 P. J. Madgwick and S. G. Waley, *Biochem. J.*, 1987, **248**, 657.
80 S. Vijayakumar, G. Ravishanker, R. F. Pratt and D. L. Beveridge, *J. Am. Chem. Soc.*, 1995, **117**, 1722.
81 L. Maveyraud, R. F. Pratt and J. P. Samama, *Biochemistry*, 1998, **37**, 2622.
82 C. Damblon, X. Raquet, L. Lu-Yun, J. Lamotte-Brasseur, E. Fonzé, P. Charlier, G. C. K. Roberts and J. M. Frère, *Proc. Natl. Acad. Sci. USA*, 1996, **93**, 1747.
83 X. Raquet, V. Lounnas, J. Lamotte-Brasseur, J. M. Frère and R. C. Wade, *Biophys. J.*, 1997, **73**, 2416.
84 G. Guillaume, M. Vanhove, J. Lamotte-Brasseur, P. Ledent, M. Jamin, B. Joris and J. M. Frère, *J. Biol. Chem.*, 1997, **272**, 5438.
85 A. Matagne, J. Lamotte-Brasseur, G. Dive, J. R. Knox and J. M. Frère, *Biochem. J.*, 1993, **293**, 607.
86 P. Taibi and S. Mobashery, *J. Am. Chem. Soc.*, 1995, **117**, 7600.
87 P. Taibi-Tronche and S. Mobashery, *J. Am. Chem. Soc.*, 1996, **118**, 7441.
88 V. Knott-Hunziker, S. Petursson, G. S. Jayatilake, S. G. Waley, B. Jaurin and T. Grundström, *Biochem. J.*, 1982, **201**, 621.
89 B. Joris, J. Dusart, J. M. Frère, J. van Beeumen, E. L. Emanuel, S. Petursson, J. Gagnon and S. G. Waley, *Biochem. J.*, 1984, **223**, 271.
90 V. Knott-Hunziker, S. Petursson, S. G. Waley, B. Jaurin and T. Grundström, *Biochem. J.*, 1982, **207**, 315.
91 R. T. Aplin, J. E. Baldwin, C. J. Schofield and S. G. Waley, *FEBS Lett.*, 1990, **277**, 212.
92 D. M. Livermore, *Eur. J. Clin. Microbiol.*, 1987, **6,** 439.
93 F. Lindberg and S. Normark, *Rev. Infect. Dis.*, 1986, **8** (Suppl 3), S292.
94 W. E. Sanders and C. C. Sanders, *Rev. Infect. Dis.*, 1988, **10,** 830.
95 M. N. Swartz, *Proc. Natl. Acad. Sci. USA*, 1994, **91**, 2420
96 F. Lindberg and S. Normark, *Eur. J. Biochem.*, 1986, **156**, 441.
97 M. Galleni, F. Lindberg, S. Normark, S. Cole, N. Honore, B. Joris and J. M. Frère, *Biochem. J.*, 1988, **250**, 753.
98 K. Nomura and T. Yoshida, *FEMS Microbiol. Lett.*, 1990, 295.
99 J. M. Lodge, S. D. Minchin, L. J. Piddock and J. W. Busby, *Biochem. J.*, 1990, **272** , 627.
100 A. Seoane, M. V. Francia and J. M. García Lobo, *Antimicrob. Agents Chemother.*, 1992, **36**, 1049.
101 T. R. Walsh, L. Hall, A. P. MacGowan and P. M. Bennett, *J. Antimicrob. Chemother.*, 1995, **36**, 41.
102 G. Feller, Z. Zekhnini, J. Lamotte-Brasseur and C. Gerday, *Eur. J. Biochem.*, 1997, **244**, 186.
103 G. A. Papanicolaou, A. A. Medeiros and G. A. Jacoby, *Antimicrob. Agents Chemother.*, 1990, **34**, 2200.
104 T. Horii, Y. Arakawa, M. Ohta, T. Sugiyama, R. Wacharotayankun, H. Ito and N. Kato, *Gene*, 1994, **139**, 93.
105 A. P. Fosberry, D. J. Payne, E. J. Lawlor and J. E. Hodgson, *Antimicrob. Agents Chemother.*, 1994, 38, 1182.
106 M. Gonzalez Leiza, J. C. Perez-Diaz, J. Ayala, J. M. Casellas, J. Martinez-Beltran, K. Bush and F. Baquero, *Antimicrob. Agents Chemother.*, 1994, 38, 2150.
107 A. Bauernfeind, S. Wagner, R. Jungwirth, J. Schneider and D. Meyer, *Antimicrob. Agents Chemother.*, 1997, 41, 2041.
108 A. Marchese, G. Arlet, G. C. Schito, P. H. Lagrange and A. Philippon, *Antimicrob. Agents Chemother.*, 1998, **42**, 464.
109 L. S. Tzouvelekis, E. Tzelepi and A. F. Mentis, *Antimicrob. Agents Chemother.*, 1994, **38**, 2207.
110 M. Gazouli, L. S. Tzouvelekis, E. Prinarakis, V. Miriagou and E. Tzelepi, *Antimicrob. Agents Chemother.*, 1996, **40**, 1736.
111 A. Bauernfeind, I. Stemplinger, R. Jungwirth, R. Wilhelm and Y. Chong, *Antimicrob. Agents Chemother.*, 1996, **40**, 1926.
112 A. Bauernfeind, I. Stemplinger, R. Jungwirth and H. Giamarellou, *Antimicrob. Agents Chemother.*, 1996, **40**, 221.
113 O. Gaillot, C. Clement, M. Simonet and A. Philippon, *J. Antimicrob. Chemother.*, 1997, **39**, 85.
114 P. A. Bradford, C. Urban, N. Mariano, S. J. Projan, J. J. Rahal and K. Bush, *Antimicrob. Agents Chemother.*, 1997, **41**, 563.
115 G. Barnaud, G., Arlet, C. Danglot and A. Philippon, *FEMS Microbiol. Lett.*, 1997, **148**, 15.
116 G. A. Jacoby, *Eur. J. Clin. Microbiol. Infect. Dis.*, 1994, **13** (Suppl 1), S2.
117 A. Philippon, G. Arlet and P. H. Lagrange, *Eur. J. Clin. Microbiol. Infect. Dis.*, 1994, **13** (Suppl 1), S17.
118 D. Monnaie, R. Virden and J. M. Frère, *FEBS Lett.*, 1992, **306**, 108.
119 M. Galleni and J. M. Frère, *Biochem. J.*, 1988, **255**, 119.
120 M. Adam, C. Damblon, B. Plaitin, L. Christiaens and J. M. Frère, *Biochem. J.*, 1990, **270**, 525.
121 C. Damblon, G. H. Zhao, M. Jamin, P. Ledent, A. Dubus, M. Vanhove, X. Raquet, L. Christiaens and J. M. Frère, *Biochem. J.*, 1995, **309**, 431.
122 Y. Xu, G. Soto, K. R. Hirsch and R. F. Pratt, *Biochemistry*, 1996, **35**, 3595.
123 B. P. Murphy and R. F. Pratt, *Biochemistry*, 1991, **30**, 3640.
124 I. E. Crompton, B. K. Cuthbert, G. Lowe and S. G. Waley, *Biochem. J.*, 1988, **251**, 453.
125 J. E. Baldwin, T. D. W. Claridge, A. E. Derome, B. D. Smith, M. Twyman and S. G. Waley, *J. Chem. Soc. Chem Commun.*, 1991, 573.
126 S. J. Cartwright and S. G. Waley, *Biochem. J.*, 1984, **221**, 505.
127 A. Matagne, M. F. Ghuysen and J. M. Frère, *Biochem. J.*, 1993, **295**, 705.

128 D. Monnaie and J. M. Frère, *FEBS Lett.*, 1993, **334**, 269.

129 M. Galleni, G. Amicosante and J. M. Frère, *Biochem. J.*, 1988, **255**, 123.

130 R. F. Pratt, *Science*, 1989, **246**, 917.

131 J. Rahil and R. F. Pratt, *Biochem. J.*, 1993, **296**, 389.

132 J. Rahil and R. F. Pratt, *Biochemistry*, 1992, **31**, 5869.

133 E. Lobkovsky, E. M. Billings, P. C. Moews, J. Rahil, R. F. Pratt and J. R. Knox, *Biochemistry*, 1994, **33**, 6762.

134 B. P. Murphy and R. F. Pratt, *Biochem. J.*, 1988, **256**, 669.

135 P. Rahil and R. F. Pratt, *Biochemistry*, 1993, **32**, 10763.

136 A. Dubus, P. Ledent, J. Lamotte-Brasseur and J. M. Frère, *Proteins: Struct. Funct. Genet.*, 1996, **25**, 473.

137 J. Brannigan, A. Matagne, F. Jacob, C. Damblon, B. Joris, D. Klein, B. G. Spratt and J. M. Frère, *Biochem. J.*, 1991, **278**, 673.

138 D. Monnaie, A. Dubus, D. Cooke, J. Marchand-Brynaert, S. Normark and J. M. Frère, *Biochemistry*, 1994, **33**, 5193.

139 D. Monnaie, A. Dubus and J. M. Frère, *Biochem. J.*, 1994, **302**, 1.

140 M. Nukaga, K. Tanimoto, K. Tsukamoto, S. Imajo, M. Ishiguro and T. Sawai, *FEBS Lett.*, 1993, **332**, 93.

141 A. Dubus, J. M. Wilkin, X. Raquet, S. Normark and J. M. Frère, *Biochem. J.*, 1994, **301**, 485.

142 U. Imtiaz, E. K. Manavathu, S. A. Lerner and S. Mobashery, *Antimicrob. Agents Chemother.*, 1993, **37**, 2438.

143 J. M. Wilkin, A. Dubus, B. Joris and J. M. Frère, *Biochem. J.*, 1994, **301**, 477.

144 B. A. Rasmussen, D. A. Keeney, Y. Yang and K. Bush, *Antimicrob. Agents Chemother.*, 1994, **38**, 2078.

145 J. W. Dale and J. T. Smith, *J. Bacteriol.*, 1974, **119** , 351.

146 M. Ouellette, L. Bissonnette and P. H. Roy, *Proc. Natl. Acad. Sci. USA*, 1987, **84**, 7378.

147 J. W. Dale, D. Godwin, D. Mossakowska, P. Stephenson and S. Wall, *FEBS Lett.*, 1985, **191**,39.

148 D. Mossakowska, N. A. Ali and J. W. Dale, *Eur. J. Biochem.*, 1989, **180**, 309.

149 F. Couture, J. Lachapelle and R. C. Levesque, *Mol. Microbiol.*, 1992, **12,** 1693.

150 P. Ledent, X. Raquet, B. Joris, J. Van Beeumen and J. M. Frère, *Biochem. J.*, 1993, **292**, 555.

151 P. Ledent and J. M. Frère, *Biochem. J.*, 1993, **295**, 871.

152 F. Danel, L. M. Hall, D. Gur and D. M. Livermore, *Antimicrob. Agents Chemother.*, 1997, **41**, 785.

153 L. D. Sabbath and P. E. Abraham, *Biochem. J.*, 1966, **98**, 11C.

154 Y. Saino, F. Kobayashi, M. Inoue and S. Mitsuhashi, *Antimicrob. Agents Chemother.*, 1982, **22**, 564.

155 H. Yamaguchi, M. Nukaga and T. Sawaï, *EMBL Data Library*, 1994, **D29636**.

156 B. A. Rasmussen, Y. Gluzman and F. P. Tally, *Antimicrob. Agents Chemother.*, 1990, **34**, 1590.

157 J. S. Thompson and M. H. Malamy, *J. Bacteriol.*, 1990, **172**, 2584.

158 O. Massida, G. M. Rossolini and G. Satta, *J. Bacteriol.*, 1991, **173**, 4611.

159 G. M. Rossolini, N. Franceschini, L. M. Riccio, P. S. Mercuri, M. G. Perilli, M. Galleni, J. M. Frère and G. Amicosante, *Biochem. J.*, 1998, **331**, 145.

160 L. E. Alksne and B. A. Rasmussen, *J. Bacteriol.*, 1997, **179**, 2006.

161 T. R. Walsh, W. A. Neville, M. H. Haran, D. Tolson, D. J. Payne, J. H. Bateson, A. P. Mac Gowan and P. M. Bennett, *Antimicrob. Agents Chemother.*, 1998, **42**, 436.

162 A. Felici, G. Amicosante, A. Oratore, R. Strom, P. Ledent, B. Joris, L. Fanuel and J. M. Frère, *Biochem. J.*, 1993, **291**, 151.

163 A. Felici and G. Amicosante, *Antimicrob. Agents Chemother.*, 1995, **39**, 192.

164 A. Felici, M. G. Perilli, B. Segatore, N. Franceschini, D. Setacci, A. Oratore, S. Stefani, M. Galleni and G. Amicosante, *Antimicrob. Agents Chemother.*, 1995, **39**, 1300.

165 Y. Yonjun and K. Bush, *FEMS Microbiol. Letters*, 1996, **137**, 193.

166 Y. Yang, B. A. Rasmussen and K. Bush, *Antimicrob. Agents Chemother.*, 1992, **36**, 1155-1157.

167 K. Marumo, A. Takeda, Y. Nakurama and K. Nakaya, *Microbiol. Immunol.*, 1995, **30**, 27.

168 Y. Yang, P. Wu and D. M. Livermore, *Antimicrob. Agents Chemother.*, 1990, **34**, 755.

169 R. Bicknell, E. L. Emanuel, J. Gagnon and S. G. Waley, *Biochem. J.*, 1985, **229**, 791.

170 S. C. Bucknell, M. I. Page, J. L. Longridge and S. G. Waley, *J. Chem. Soc., Perkin Trans.*, 1988, **2**, 1815.

171 K. Bush, C. Macalintal, B. A. Rasmussen, V. J.. Lee and Y. Yang, *Antimicrob. Agents Chemother.*, 1993, **37**, 851.

172 M. Hernandez Valladares, A. Felici, G. Weber, H. W. Adolph, M. Zeppezauer, G. M. Rossolini, G. Amicosante, J. M. Frère and M. Galleni, *Biochemistry*, 1997, **36**, 11534.

173 M. Hussain, A. Carlino, M. J. Madonna and D. Lampen, *J. Bacteriol.*, 1985, **164**, 223.

174 T. R. Walsh, L. Hall, S. J. Assinda, W. W. Nichols, S. T. Cartwright, A. P. Mac Gowan and P. M. Bennett, *Biochem. Biophys. Acta*, 1994, **1218**, 199.

175 H. M. Lim, J. J. Pène and R. Shaw, *J. Bacteriol.*, 1988, **170**, 2870.

176 O. Kato, T. Kenda, K. Watanabe and K. Horikoshi, *J. Gen. Microbiol.*, 1985, **131**, 3317.

177 K. Senda, Y. Arakawa, S. Ichiyama, K. Ito, H. Ohsuka, K. Shirokara, N. Kato and M. Ohta, *J. Clin. Microbiol.*, 1985, **34**, 2900.

178 I. Podglajen, J. Breuil, F. Bordon, L. Gutman and E. Collatz, *FEMS Microbiol. Lett.*, 1992, **70**, 21.

179 I. Podglajen, J. Breuil and E. Collatz, *Mol. Microbiol.*, 1994, **12**, 105.

180 R. Ruimy, I. Podglajen, J. Breuil, M. Christen and E. Collatz, *J. Bacteriol.*, 1996, **178**, 1914.

181 A. Carfi, S. Pares, E. Duée, M. Galleni, C. Duez, J. M. Frère and O. Dideberg, *EMBO J.*, 1995, **14**, 4914.

182 N. O. Concha, B. A. Rasmussen, K. Bush and O. Herzberg, *Structure*, 1996, **4**, 823.

183 A. Carfi, E. Duée, R. Paul Soto, M. Galleni, J. M. Frère and O. Dideberg, *Acta Crystallogr.*, 1998, **D54**, 47.

184 A. Carfi, E. Duée, M. Galleni, J. M. Frère and O. Dideberg, *Acta Crystallogr.*, 1998, **D54**, 313.

185 D. W. Christianson, P. R. David and W. N. Lipscomb, *Proc. Natl. Acad. Sci. USA*, 1987, **84**, 1512.

186 M. W. Crowder, Z. Wang, S. L. Franklin, C. P. Zovinka and S. J. Benkovic, *Biochemistry*, 1996, **35**, 12126.

187 R. Bicknell and S. G. Waley, *Biochemistry*, 1985, **24**, 6876.

188 R. Bicknell, A. Schäffer, S. G. Waley and D. S. Auld, *Biochemistry*, 1986, **25**, 7208.

189 L. M. Gilpin, M. Fulster, D. J. Payne, R. Cramp and I. Hocht, *J. Antibiot.*, 1995, **48**, 1081.

190 D. J. Payne, J. H. Bateson, B. C. Gasson, D. Proctor, T. Khushi, T. Farmer, D. Tolson, D. Bell, P. W. Shatt, A. C. Marshall, R. Reid, L, Ghosez, Y. Combret and J. Marchand-Brynaert, *Antimicrob. Agents Chemother.*, 1997, **41**, 135.

191 M. W. Walter, A. Felici, M. Galleni, R. Paul Soto, R. M. Adlington, J. E. Baldwin, J. M. Frère, M. Gololobov and C. J. Schofield, *Bioorg. Med. Chem. Lett.*, 1996, **6**, 2455.

192 M. W. Walter, R. Adlington, J. E. Baldwin and C. J. Schofield, *Tetrahedron*, 1993, **53**, 7275.

193 J. H. Toney, P. M. D. Fitzgerald, N. Grover-Sharma, S. H. Olson, W. J. May, J. G. Sundelof, D. E. Vanderwall, K. A. Cleary, S. K. Grant, J. K. Wu, J. W. Kozarich, D. L. Pompliano and G. G. Hammond, *Chem. Biol.*, 1998, **5**, 185.

194 S. Normark, E. Bartowsky, J. Erickson, C. Jacobs, F. Lindberg, S. Lindquist, K. Weston-Hafer and M. Wikström, in *Bacterial Cell Wall. New Comprehensive Biochemistry*, Vol. 27, J. M. Ghuysen and R. Hakenbeck, eds. (Amsterdam, Elsevier Science B.V.), pp. 485-503.

195 F. Lindberg, S. Lindquist and S. Normark, *J. Bacteriol.*, 1987, **169**, 1923.

196 C. Jacobs, J. M. Frère and S. Normark *Cell*, 1997, **88**, 823.

197 F. Lindberg and S. Normark, *Proc. Natl. Aca. Sci. USA*, 1985, **82**, 4620.

198 C. Jacobs, L. J. Huang, E. Bartowsky, S. Normark and T. Park, *EMBO J.*, 1994, **13**, 4684.

199 C. Jacobs, B. Joris, K., Klarsov, J. Van Beeumen, D. Mengin Lecreux, J. Van Heijenoort, T. Park, S. Normark and J. M. Frère, *Mol. Microbiol.*, 1995, **15**, 553.

200 U. Kopp, S. Wiedemann, S. Lindquist and S. Normark, *Antimicrob. Agents Chemother.*, 1993, **37**, 224

201 M. Datz, B. Joris, E. A. Azab, M. Galleni, J. Van Beeumen, J. M. Frère and H. H. Martin, *Eur. J. Biochem.*, 1994, **226**, 149.

202 J. Magdalena, C. Gérard, B. Joris, M. Forsman and J. Dusart, *Mol. Gen. Genet.*, 1997, **255**, 187.

203 J. Magdalena, M. Forsman, M. Lenzini and J. Dusart, *FEMS Microbiol. Lett.*, 1992, **99**, 101.

204 M. Lenzini, J. Magdalena, C. Fraipont, B. Joris, A. Matagne and J. Dusart, *Mol. Gen. Genet.*, 1992, **235**, 41.

205 J. Magdalena, B. Joris, J. Van Beeumen, R. Brasseur and J. Dusart, *Biochem. J.*, 1995, **311**, 155.

206 T. Kobayashi, Y. F. Zhu, N. Nichols and J. O. Lampen, *J. Bacteriol.*, 1987, **169**, 3873.

207 K. Hardt, B. Joris, S. Lepage, R. Brasseur, J. O. Lampen, J. M. Frère, A. L. Fink and J. M. Ghuysen, *Mol. Microbiol.*, 1997, **23**, 935.

208 P. D. Gregory, P. A. Lewis, S. P. Curnoch and K. G. Dyke, *Mol. Microbiol.*, 1997, **24**, 1025.

209 S. J. Rowland and K. G. Dyke, *Mol. Microbiol.*, 1990, **4**, 961.

210 W. Tesch, C. Ryffel, A. Strässle, F. H. Kayser and B. Berger-Bächi, *Antimicrob. Agents Chemother.*, 1990, **34**, 1703.
211 R. F. Pratt and C. P. Govardhan, *Proc. Natl. Acad. Sci. USA*, 1984, **81**, 1302.
212 C. P. Govardhan and R. F. Pratt, *Biochemistry*, 1987, **26**, 3385.
213 Y. Xu, H. Adachi, M. P. G. Van Der Linden, W. Keck and R. F. Pratt, *Biochem. J.*, 1994, **302**, 851.
214 S. Pazhanisamy, C. P. Govardhan and R. F. Pratt, *Biochemistry*, 1989, **28**, 6863.
215 S. Pazhanisamy and R. F. Pratt, *Biochemistry*, 1989, **28**, 6870.
216 S. Pazhanisamy and R. F. Pratt, *Biochemistry*, 1989, **28**, 6875.
217 M. Jamin, M. Adam, C. Damblon, L. Christiaens and J. M. Frère, *Biochem. J.*, 1991, **280**, 499.
218 K. Curley and R. F. Pratt, *J. Am. Chem. Soc.*, 1997, **119**, 1529.
219 N. C. J. Strynadka, R. Martin, S. E. Jensen, M. Gold and J. B. Jones, *Nat. Struct. Biol.*, 1996, **3**, 688.
220 J. Rahil and R. F. Pratt, *Biochem. J.*, 1991, **275**, 793.
221 A. Laws, M. I. Page and M. J. Slater, *Bioorg. Med. Chem. Lett.*, 1993, **3**, 2317.
222 J. H. Bateson, B. C. Gasson, T. Khushi, J. E. Neale, D. J. Payne, D. A. Tolson and G. Walker, *Bioorg. Med. Chem. Lett.*, 1994, **4**, 1667.
223 D. J. Payne, J. H. Bateson, D. Tolson, B. Gasson, T. Khushi, P. Ledent and J. M. Frère, *Biochem. J.*, 1996, **314**, 457.
224 F. Jacob, B. Joris, S. Lepage, J. Dusart and J.-M. Frère, *Biochem. J.*, 1990, **271**, 399-406.
225 P. Kraulis, *J. Appl. Crystallogr.*, 1991, **24**, 946.

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