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The diversity, structure and regulation of β-lactamases

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Abstract. β-Lactamase production is responsible for the appearance of a large number of pathogenic bacterial strains exhibiting a high degree of resistance to β-lactam antibiotics. A large number of enzymes have been described with very diverse primary structures and catalytic profiles. Nevertheless, all known three-dimensional structures of active-site serine β-lactamases exhibit a high degree of similarity with apparently equivalent chemical functionalities in the same strategic positions. These groups might not, however, play identical roles in the various classes of enzymes. Structural data have also been recently obtained for the zinc metallo-β-lactamases, but the detailed catalytic mechanisms might also differ widely, depending on the enzyme studied. Similarly, the induction of the synthesis of β-lactamases is now better understood, but many questions remain to be answered.

Key words. β-lactamases; penicillins; cephalosporins; regulation; classification.

The diversity of β-lactamases

Bacterial resistance was detected concomitantly with the discovery of antibiotics. The use of a novel antibiotic, generally introduced for high antibacterial activity, is often followed within a few years by the emergence of unsuspected 'novel' resistant bacterial species such as the recently reported *Corynebacterium urealyticum*, *Stenotrophomonas maltophilia*, *Alcaligenes xylosoxydans* and *Bordetella bronchiseptica*. However, the main issue is acquired resistance which can be related to various genetic tricks. In particular, β-lactamases are enzymes which inactivate β-lactam antibiotics via either the utilization of zinc ions or more frequently the serine ester mechanism and constitute the major cause of resistance in both Gram-positive and Gram-negative bacteria. About 200 enzymes have been identified. They determine well-characterized resistance phenotypes, particularly those resulting from the synthesis of cephalosporinases, penicillinases, broad-spectrum or extended-spectrum β-lactamases, or carbapenemases. The classification proposed by Ambler distinguishes four classes of β-lactamases – A, B, C and D – according to their primary structures.

These enzymes exhibit very diverse amino acid sequences and can be characterized according to their kinetic properties, and possibly the phenotype they determine in bacteria. Table 1 illustrates this diversity among the four classes as subdivided into functional clusters or groups by the Bush-Jacoby-Medeiros scheme [1].

Class C or group 1 includes chromosomal β-lactamases called cephalosporinases, but some 'novel' plasmid-mediated members of this group (FOX-1, MOX-1, for example) have amino acid sequences rather dissimilar (<60% identity) from those of the chromosomal AmpC β-lactamases of *Citrobacter freundii*, *Enterobacter cloacae* or *Morganella morganii*, in contrast to others such as CMY-1, LAT-1, BIL-1 and ACT-1 (>90% identity). But the sequences of class C enzymes highlight the presence of the usual conserved motifs (see below), and the enzymes exhibit similar kinetic parameters. Their

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Table 1. Classification of β-lactamases according to the Bush-Jacoby-Medeiros scheme.

Enzyme class	Group (No.)	Activity*			Examples of β-lactamases		Types of β-lactamases (bla)
		CLA	TGC	IMP	chromosomal	plasmid-mediated	
A	2a (20)	+	−	−	*Bacillus*	*S. aureus*, *E. faecium*	penicillinases of Gram +
	2b (16)	+	−	−	*K. pneumoniae*	TEM-1-2, SHV-1,	broad-spectrum bla
	2be (38)	+	+	−		SHV-2-12, TEM-3-46	extended-spectrum bla (ESB)
	2br (9)	−	−	−		TEM-30-40	inhibitor resistant TEM (IRT)
	2c (15)	+	−	−		PSE-1 (CARB-4)	carbenicillinases
	2e (19))	+	+	−	*P. vulgaris*, *P. penneri*		cefuroximases
	2f (3)	+	+	+	*S. marcescens*, *E. cloacae*		carbapenemases (Zn −)
B	3 (15)	−	+	+	*S. maltophilia*	IMP-1	carbapenemases (Zn +)
C	1 (53)	−	+	−	*C. freundii*, *E. cloacae* *P. aeruginosa*	BIL-1, LAT-1, CMY-2, MIR-1, ACT-1, CMY-1, FOX-1, MOX-1	cephalosporinases
D	2d (18)	±	−	−		OXA-1–OXA-10 OXA-11–OXA-16	oxacillinases extended-spectrum oxacillinases (ESB)

*, + inhibitory activity (CLA, clavulanate) or hydrolysis (TGC, third-generation cephalosporins; IMP, imipenem); ±, variable; −, no activity.

expression confers a typical susceptibility pattern including resistance to aminopenicillins, first-generation cephalosporins, cefoxitin, and to the combination of amoxycillin and the β-lactamase inactivator clavulanate.

As shown in figure 1 and table 1, there is substantial diversity within the chromosomal and plasmid-encoded β-lactamases of class A resulting in various phenotypes (1) such as resistance to penicillins only (2b), to penicillins and some cephalosporins (2b, 2c), to cephalosporins and cefuroxime (2e), to third generation cephalosporins (2be), to β-lactamase inactivators (2br) or to imipenem (2f).

Some β-lactamases, for example SHV-1 and SHV-5, TEM-1 and TEM-12, OXA-10 and OXA-11, are very similar (identity >97%). These apparent minor modifications of structure, which demonstrate molecular evolution, have large effects on kinetic parameters and resistance expression [2, 3]. Thus these 'similar' β-lactamases are classified into two separate groups because of the major differences in β-lactam hydrolysis. TEM-1 belongs to the group of broad-spectrum types (2b). TEM-12, although differing from TEM-1 by only one amino acid substitution, belongs to the group of extended-spectrum β-lactamases (2be) which mediate multiresistance to β-lactams, including first-, second- and third-generation cephalosporins. The latter were reputed to be highly stable to enzymatic hydrolysis when first introduced. The diversity of expression in terms of bacterial resistance was recently illustrated by the discovery of TEM-46 (with a novel substitution) which confers in vitro resistance to third-generation cephalosporins but not to the first-generation molecules. Other examples of the molecular evolution of TEM types involve substitutions in other regions of the protein, resulting in a decrease of affinity for β-lactams, including β-lactamase inhibitors (group 2br).

β-Lactamases provide the most widespread mechanism of bacterial resistance [3]. In the recent past and in response to the clinical use of new drugs, 'novel' β-lactamases have emerged carrying mutations close to the binding cavity of well-established β-lactamases. The diversity of these enzymes has been more recently illustrated by the recruitment of new β-lactamase genes (e.g. FOX-1, MOX-1) without any known ancestor. The determination of DNA sequences of β-lactamases naturally produced by some enterobacteria or other bacterial clusters may supply clues to the origin of these mobilized genes.

Structures and mechanisms

The Zn^{++} enzymes

For a long time, the only known representatives of this class of enzymes were produced by the nearly innocuous *Bacillus cereus*, but the situation changed [4] with the appearance of metallo-β-lactamase-producing pathogens such as *Bacteroides*, *Aeromonas* and *Chryseobacterium* (*Flavobacterium*) and of the plasmid-encoded Imp1 enzyme in clinical isolates of *Pseudomonas aeruginosa*, *Serratia marcescens* and *Klebsiella pneumoniae*. The most worrying property of these homologous enzymes, whose M_r values are in the 22,000–30,000 range, was their capacity to hydrolyse

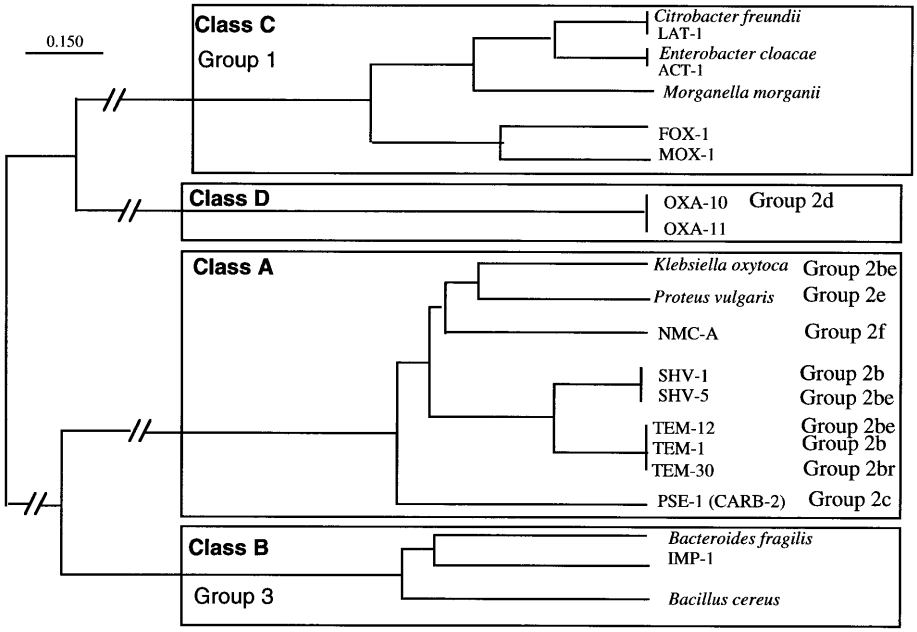

Figure 1. Relationships between β-lactamases on the basis of amino acid sequences and functional properties.

carbapenems, a group of β-lactams which generally escape the activity of the active-serine β-lactamases. The structure of the *B. cereus* enzyme was first determined at pH 5.5 [5] and found, as expected, to contain one Zn^{++} ion per molecule liganded by three histidine imidazole side chains and one water molecule. However, at pH 7.5, both the *B. cereus* and *Bacteroides fragilis* enzyme molecules bind two metal ions in neighbouring sites [6]. In the former case, the binding of the second ion is accompanied by a significant increase in activity. In the latter, the two binding constants are very high, and no mono-Zn derivative has been obtained. By contrast, the *Aeromonas hydrophila* enzyme is fully active with only one Zn ion and is nearly completely inhibited by additional binding of the same ion [7]. Although mechanisms similar to that of metalloproteases can be proposed, the catalytic pathway of these enzymes, which is unlikely to involve covalent intermediates, is still far from being completely understood. It is interesting to note that the general architectures of the *B. cereus* and *B. fragilis* enzymes are devoid of significant similarities to those of other proteins of known three-dimensional (3D) structure.

A more distant relative of the group is produced by *Stenotrophomonas maltophilia*. It is a tetramer (M_r = 90,000) which does not exhibit allosteric properties but cannot be dissociated into active subunits. Each subunit contains two Zn ions.

No clinically useful inhibitor of the Zn-β-lactamases is presently known.

The serine enzymes

The elucidation of the crystal structures of several class A and class C β-lactamases has revealed striking similarities to the penicillin-sensitive DD-peptidases and penicillin binding proteins (PBPs). These studies have underlined the presence, in all these proteins, of three structural elements involved in the formation of the active site and which can be detected in all other PBPs and serine β-lactamases by sequence alignment (for reviews, see refs 8 and 9). The active serine is located near the N-terminus of a long helix and followed, on the next helix turn, by a lysine residue whose long side chain also points into the active site (SXXK element). The second element, on a loop to the left of the active site (fig. 2), contains an SXN or YXN triad. The Tyr hydroxyl group in the *Streptomyces* R61 DD-peptidase nicely superimposes on that of the Ser in class A β-lactamases. A triad exhibiting a basic residue (Lys, Arg or His) followed by Thr or Ser and always terminated by a Gly forms the other side of the active site. The side chain of the basic residue points into the active site, forming a hydrogen bond with the first residue of the second element, and the strict conservation of the Gly residue in the third position is explained by the fact that any side chain would seriously hinder the approach of the substrates.

In class A β-lactamases, a fourth element forms the basis of the cavity and contains the essential Glu 166 residue. A similar loop is found in class C enzymes, but the side-chain of its sole acidic residue is directed away from the active serine [9].

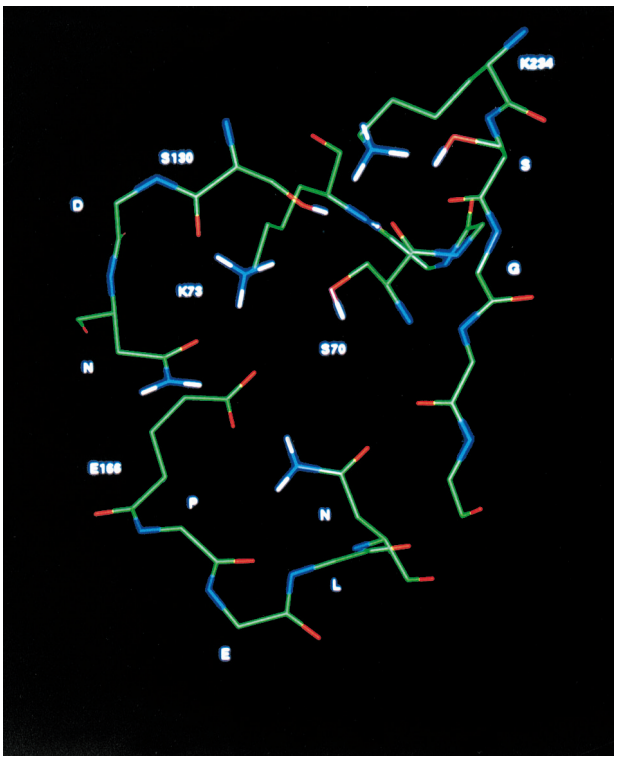

Figure 2. Conserved elements forming the active site of the TEM β-lactamase. The first residue of each element is numbered and only the side chains pointing into the active site are shown. Colour code: green, C; blue, N; red, O; white, H, but only the hydrogen atoms thought to be important in the mechanism are shown. The first element extends from Ser70 to Lys73, the second from Ser130 to Asn132, the third from Lys234 to Gly236 and the fourth from Glu166 to Asn170.

As in the classical serine proteases, a general base is expected to increase the nucleophilicity of the active serine by abstracting its hydroxyl proton. The identity of this general base is still an unsolved problem [8]. In those enzymes containing a Tyr in the second element (class C and class D β-lactamases, and a small number of PBPs), the anionic phenolate form of this residue has been hypothesized to fulfil this function. This would require a significantly decreased pK_a value for the Tyr phenolic group which could be explained by the very positive environment in the active site [10]. In class A β-lactamases, the role of general base has been attributed to the Glu 166 carboxylate or to the deprotonated alkylamino group of the Lys residue in the SXXK tetrad. Again, the latter hypothesis involves a strongly decreased pK_a value for the Lys alkylammonium side chain, which is explained as above, but nuclear magnetic resonance (NMR) titration of the Lys 73 residue in the free TEM β-lactamase has demonstrated a normal pK_a of about 10 for this group [11]. A significant modification of this value on substrate binding remains, however, a distinct possibility.

Numerous site-directed mutagenesis experiments have been devised to probe the role of several conserved residues in β-lactamases and PBPs, but the results have failed to yield a simple and coherent picture. At first sight, one may even conclude that similar side chains, situated in equivalent positions, do not play exactly the same role in the various classes of enzymes, and the replacement of some very conserved residues has not always resulted in the expected drastic impairment of catalytic or penicillin-binding activities [12].

Whatever the detailed mechanism, all active-site serine β-lactamases and PBPs (E) interact with β-lactams (C) according to a three-step pathway involving the transient formation of an acylenzyme EC* [8].

$$E + C \underset{k_{-1}}{\overset{k_{+1}}{\longleftrightarrow}} EC \xrightarrow{k_2} EC^* \xrightarrow[H_2O]{k_3} E + P(s)$$

The acylation and deacylation rates are characterized by second order [$k_{+1}k_2/(k_{-1}+k_2)$ or k_2/K'] and first-order (k_3) rate constants, respectively. At high substrate concentrations ([C] > K'), the rate-limiting step can be the formation (k_2) or the hydrolysis (k_3) of the acylenzyme. With PBPs, k_3 is usually extremely low (10^{-3} s^{-1} or less) so that an inactive acylenzyme accumulates, and the sensitivity of the PBP essentially depends on the k_2/K' ratio. 'Resistant' PBPs exhibit very low acylation rates, with k_2/K' values of about 10 M^{-1} s^{-1} or lower and with the enzymes considered as 'sensitive', this value ranges from 200 to more than 100,000 M^{-1} s^{-1}. With β-lactamases, acylation and deacylation are generally rapid, and k_2/K' can reach the diffusion-limited value of 10^8 M^{-1} s^{-1}, while k_3 can be larger than 5000 s^{-1}. 'β-lactamase-resistant' compounds are characterized either by lower acylation rates (third-generation cephalosporins with the TEM-1 or SHV-1 enzymes) or slow deacylation (cephalosporins with the *Staphylococcus aureus* enzyme).

It is not possible to predict the k_2 and k_3 values on the basis of the primary structures of the proteins. In class A and for benzylpenicillin, these two first-order rate constants are similar for the TEM enzyme [13], while k_3 and k_2 are rate-limiting for the *S. aureus* and *Streptomyces albus* G β-lactamases, respectively. In class C, the k_2/k_3 ratio is often high but has not been determined for very good substrates such as cefazolin [14]. Class D enzymes exhibit complex kinetics with many substrates, and their exact catalytic pathway remains a puzzle [15].

As underlined above, the specificity profiles of β-lactamases are extremely diverse, even within the same class. Moreover, this diversity rests on very different structural characteristics. For example, third-generation cephalosporins are well hydrolysed by variants of the

OXA, TEM and SHV β-lactamases, which differ from the original enzymes by a limited number of amino acid substitutions, but also by completely different class A proteins exhibiting very different sequences (40% or less isology with TEM). The recent discovery of class A carbapenemases also illustrates this point. Class C producers have devised another strategy to fight the poor substrates of their β-lactamase: massive overproduction. Although cefotaxime is a very poor substrate of the *Enterobacter cloacae* 908R class C enzyme ($k_{cat} = 10^{-2}$ s^{-1}), the periplasmic concentration of the latter is so high (>0.1 mM) that the minimum inhibitory concentration (MIC) value increases more than 1000-fold when compared with that of an isogenic strain devoid of active enzyme (unpublished results). It is also noteworthy that TEM variants have been isolated which are no longer sensitive to the widely utilized inactivators clavulanic acid and sulbactam (see above). Since the class C enzymes are also insensitive to these compounds, the search for more efficient β-lactamase inactivators should remain a priority for the pharmaceutical industry. In this respect, the spread of plasmid-mediated Amp-C-type enzymes also remains a worrying perspective, as well as the appearance of clinical isolates which produce two or even three different enzymes with complementary specificities.

Regulation of β-lactamase production

Some microorganisms produce β-lactamase in a regulated way: the presence of a β-lactam compound in their environment causes an amplification of the enzyme biosynthesis. By adjusting its metabolic effort to its momentary needs, the bacteria obviously save energy, but this additional step might offer a supplementary target for therapy. These strains could be made sensitive to β-lactams by interfering with the induction mechanism, hence the interest of unravelling the molecular pathway of this regulation.

Regulation in Gram-positive bacteria

***Bacillus* and *Staphylococcus*.** The best-studied regulatory system is that of *B. licheniformis*. In this microorganism, three genes (*blaI*, *blaR1* and *blaR2*) are involved in the regulation of the structural gene *blaP*, which encodes a class A β-lactamase. With the exception of *blaR2*, these genes are grouped on the chromosome, forming a divergeon, which means that the regulatory genes *blaI* and *blaR1* are transcribed divergently from *blaP*. *blaI* exerts a negative control on the transcription of *blaP*. It is a classical repressor. *blaR1* is particularly interesting because it codes for a potential β-lactam-recognizing protein. Its transmembrane structure exposes its penicillin-sensitive domain to the external medium and allows this protein to detect the presence of extracellular antibiotics. The study of the BlaRI topology [16] showed that this receptor is composed of four transmembrane segments, an organization which suggests various possibilities for the molecular mechanism of signal transmission from periplasm to cytoplasm.

The presence of the third regulatory gene, *blaR2*, rests only on genetic evidence. This locus is not linked to the above-mentioned divergeon. A mutation in *blaR2* results in a high constitutive expression of β-lactamase.

The *blaI*, *blaR* and *blaP* genes of *Staphylococcus aureus* are organized in the same way on the transposon TN552 [17]. In *Streptococcus epidermidis*, a resistance gene (*mrsA* or *mecA*) codes for a β-lactam-resistant PBP, in a related system [18].

***Streptomyces*.** These soil microorganisms very frequently produce class A β-lactamases, most of them expressing the enzyme constitutively; but in *Streptomyces cacaoi*, two regulatory genes, *blaA* and *blaB*, have been identified. They are also located upstream of and diverge from the controlled structural gene, *blaL*. BlaA is a transcriptional activator, belonging to the LysR family, and binds to the intergenic region, *blaA-blaL* [19] at the level of a T-N_{11}-A motif, as described in Goethals et al. [20]. The function of the product of *blaB* remains unknown. Its structure is related to that of the penicillin-recognizing enzymes with their highly conserved motifs and 15 of the 16 secondary structure elements described in these proteins. However, it lacks the features required for a transmembrane disposition like that of *BlaR1*. BlaB does not act as a β-lactamase and does not bind penicillin. Immunotests have shown that it is localized at the internal face of the cytoplasmic membrane. BlaA and BlaB are required not only for induction but also for the basic expression of the enzyme. Inactivation of any of these genes results in a 50-fold decrease of the noninduced production of BlaL. The existence of other genes involved in β-lactamase regulation is not excluded in *S. cacaoi*.

The genome of *S. cacaoi* in fact contains two β-lactamase genes. The second gene, *blaU*, also codes for a class A β-lactamase but is not genetically linked to the *blaB-blaA-blaL* group. In *S. cacaoi*, *blaU* is also inducible by β-lactams, and its promoter region is very similar to that of *blaL*. The control of *blaU* also relies on the *blaA* and *blaB* genes [19].

Regulation in Gram-negative bacteria

In enterobacteria and some related species, a chromosomal gene encodes a class C (*ampC*, enterobacteria and *Pseudomonas* and *Aeromonas* spp.) or class A (*cumA*, *Proteus vulgaris*) β-lactamase. At least four genes are involved in this regulation. *ampR* and *cumR* code for

transcriptional activators of the LysR family. In this case also, regulatory and structural genes are adjacent and are transcribed in a diverging way, and the regulatory proteins, AmpR and CumR, bind to the intergenic region. In contrast to BlaA, AmpR and CumR exert their activating effect only in the presence of the inducer. In the absence of inducer, they act as repressors. Genes unlinked to the preceding ones have been shown to be necessary for induction. *ampG*, which encodes a permease or a permease element, lies on an isolated locus, whereas *ampD* and *ampE* are grouped in an operon. The role of AmpE is not completely understood. Its inactivation is without any effect on induction, but an altered AmpE (deletion of the C-terminal portion) results in a poorly inducible cell. AmpD, an amidase [21], is involved in the recycling of the peptidoglycan degradation products that enter the cytoplasm via AmpG. Under normal conditions, AmpR binds a precursor of the peptidoglycan biosynthesis and represses the transcription of *ampC*. When a β-lactam compound inactivates one or several PBPs, a higher amount of peptidoglycan degradation products enter the cell. One of these degradation products, which is normally recycled through the action of AmpD, accumulates and binds to AmpR, converting it into a transcriptional activator [21]. The same effect is obtained by the inactivation of AmpD. It is not known if the binding of this degradation product implies the displacement of the 'normal' ligand of AmpR.

By contrast, in *Aeromonas jandaei*, the synthesis of three different β-lactamases appears to be under the control of a unique *cre*-type two-component regulon [22].

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