REVIEW ARTICLE

Catalytic properties of class A β-lactamases: efficiency and diversity

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β-Lactamases are the main cause of bacterial resistance to penicillins, cephalosporins and related β-lactam compounds. These enzymes inactivate the antibiotics by hydrolysing the amide bond of the β-lactam ring. Class A β-lactamases are the most widespread enzymes and are responsible for numerous failures in the treatment of infectious diseases. The introduction of new β-lactam compounds, which are meant to be 'β-lactamase-stable' or β-lactamase inhibitors, is thus continuously challenged either by point mutations in the ubiquitous TEM and SHV plasmid-borne β-lactamase genes or by the acquisition of new genes coding for β-lactamases with different catalytic properties. On the basis of the X-ray crystallography structures of several class A β-lactamases, including that of the clinically relevant TEM-1 enzyme, it has become possible to analyse how particular structural changes in the enzyme structures might modify their catalytic properties. However, despite the many available kinetic, structural and mutagenesis data, the factors explaining the diversity of the specificity profiles of class A β-lactamases and their amazing catalytic efficiency have not been thoroughly elucidated. The detailed understanding of these phenomena constitutes the cornerstone for the design of future generations of antibiotics.

1. INTRODUCTION

The introduction of benzylpenicillin in clinical trials about 50 years ago clearly represents one of the major breakthroughs in modern chemotherapy. The success of penicillins and related compounds (β-lactam antibiotics or β-lactams), which are currently the most widely used antibacterial agents, rests on both their high efficiency and their specificity [1]. However, as soon as β-lactams were introduced as chemotherapeutic weapons, this efficiency was challenged by the emergence of resistant pathogenic strains. In consequence, new molecules have been progressively introduced, with structures increasingly different from those of the original drugs. Thus the β-lactam antibiotics form an ever-expanding family of compounds, whose only common feature is the four-membered β-lactam ring (Figure 1).

The β-lactam antibiotics interfere in a specific way with the biosynthesis of the peptidoglycan [1–3]. This major constituent of the bacterial cell wall forms a three-dimensional network which completely surrounds the bacterium and protects it from its own osmotic pressure [4]. The peptidoglycan is also an essential element in maintaining the shape and rigidity of the cell wall, in both Gram-positive and Gram-negative bacteria. This macromolecule is unique to the bacterial world, which explains the high specificity of antibiotics interfering with its biosynthesis.

2. TARGET ENZYMES: THE BACTERIAL DD-PEPTIDASES

The physiological targets of β-lactam compounds are membrane DD-peptidases [often called penicillin-binding proteins (PBPs)], which are responsible for the synthesis and remodelling of the peptidoglycan [1,2,5]. These lethal targets in bacteria are active-site serine enzymes which perform their catalytic cycle according to an acylation/deacylation mechanism, involving transient acyl-enzyme adducts [4,6]. Similarly, β-lactam antibiotics acylate the active-site serine residue of the DD-peptidases, forming rather stable covalent non-catalytic acyl-enzymes. This results in the formation of non-functional peptidoglycan and, eventually, in cell death [4]. This mechanism is schematically represented by a three-step model [1]:

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

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 Figure 2).

The two complexes EC and EC* are enzymically inactive and the antibiotic efficiency rests on both a high k_2/K' value (generally due to a high k_2 value) and a very low k_3 value, which results in acyl-enzyme (EC*) accumulation at the steady-state. The covalent complex EC* results from the nucleophilic attack of the carbonyl carbon atom of the β-lactam ring by the hydroxyl group of the side-chain of the essential active-site serine. This model applies to all DD-peptidases [1] and the same serine residue is also critical for the interaction of these enzymes with their physiological substrates (D-alanyl-D-alanine-terminated peptides; [7–9]). It seems that the enzyme forms a similar acyl-enzyme adduct with its peptide substrates, but the k_3 value is much higher than with penicillins. The β-lactam antibiotics, which are recognized as natural substrates by the PBPs, are thus mechanism-based inactivators or suicide substrates of the DD-peptidases/PBPs.

3. β-LACTAMASES

As mentioned above, resistance phenomena have recently become increasingly widespread and worrying (see [10,11], and related papers in the same issues). Besides DD-peptidases of increased resistance, and the modification of the permeability barrier [3,12,13], the synthesis of one or several β-lactamases represents the most widespread and often the most efficient mechanism

Abbreviations used: PBP, penicillin-binding protein; MIC, minimum inhibitory concentration; ES, extended spectrum; IRT, inhibitor-resistant TEM β-lactamase.

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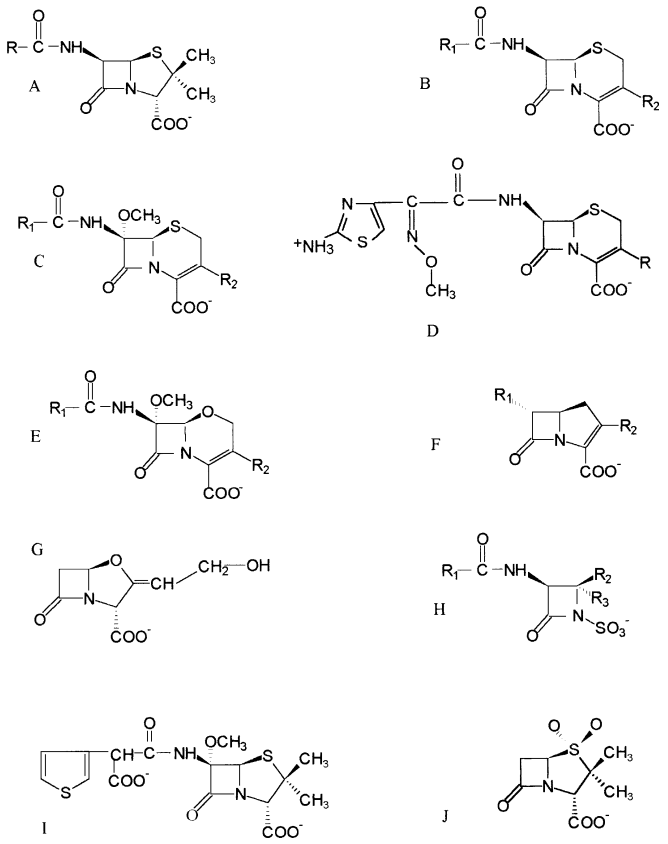

Figure 1 Structure of some β-lactam antibiotics

(**A**) Penams (e.g. benzylpenicillin, ampicillin, amoxycillin), (**B**) cephems (cephalosporins), (**C**) cephamycins (e.g. cefoxitin), (**D**) cefotaxime (oximino cephalosporin; R = CH_2–O–CO–CH_3), (**E**) oxacephamycins (e.g. moxalactam), (**F**) carbapenems (e.g. imipenem), (**G**) clavulanate (oxapenam), (**H**) monobactams (e.g. aztreonam), (**I**) temocillin (6-α-methoxy penam) and (**J**) sulbactam (penam sulphone).

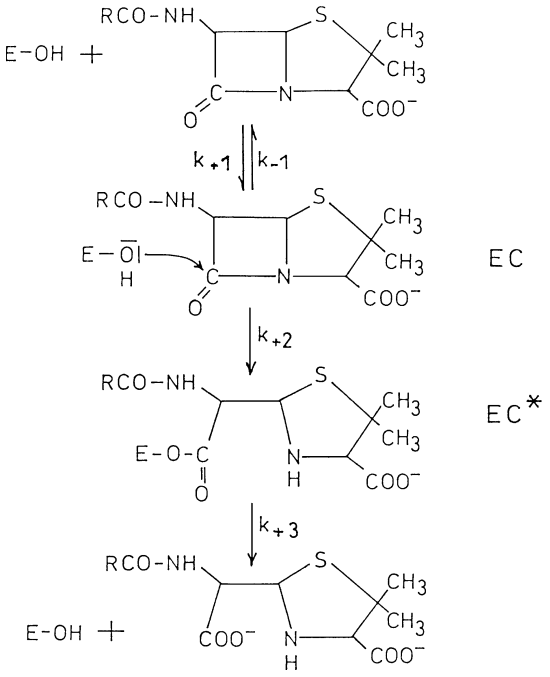

Figure 2 General catalytic pathway of active-site serine β-lactamases

In general, both acylation (k_2) and hydrolysis of the acyl-enzyme (k_3) are rapid, resulting in high turnover numbers [$k_{cat} = k_2 \times k_3/(k_2 + k_3)$] and specificity constants [$k_{cat}/K_m = k_2/K'$ where $K' = (k_{-1} + k_2)/k_{+1}$)]. Note that the k_{cat}/K_m parameter (also sometimes misleadingly referred to as the 'catalytic efficiency') corresponds to the apparent second-order rate constant for acyl-enzyme formation (k_2/K'), and thus is independent of the deacylation rate. Hence the genuine catalytic efficiency of the enzyme rests on high values of both k_{cat}/K_m and k_{cat}.

devised by bacteria to escape the lethal action of β-lactam antibiotics [10,14–16].

β-Lactamases catalyse very efficiently the irreversible hydrolysis of the amide bond of the β-lactam ring, thus yielding biologically inactive product(s) ([14,17]; Figure 2). Today, it clearly appears that the therapeutic future of the β-lactam antibiotics is seriously challenged and that a solution to the problem of bacterial resistance due to β-lactamase production is urgently needed. This demanding task is further complicated by the 'genetic fluidity' of microbial populations, which allows a widespread and frightening dissemination of resistance genes [10,16,18,19], and by the occurrence of single-point mutations in these β-lactamase-coding genes, resulting in the production of an ever-expanding number of enzymes with new substrate profiles [20–23]. β-Lactamases, which can be chromosome or plasmid encoded and produced in a constitutive or inducible manner, are secreted into the periplasmic space of Gram-negative strains or into the outer medium by their Gram-positive counterparts. Membrane-associated enzymes have also been reported, but only in a few instances (*Bacillus licheniformis* and *Bacillus cereus* [24]; *Bacteroides vulgatus* [25]).

The main division of β-lactamases is based on the chemistry of their catalytic mechanisms, and distinguishes serine and zinc enzymes [17]. Most β-lactamases display an active-site serine residue and function by the three-step mechanism described above for the DD-peptidases, involving the transient formation of an acyl-enzyme (model 1 and Figure 2) in which the hydroxyl group of the essential serine residue is esterified by the carbonyl group of the antibiotic moiety [13,17,26]. On the basis of their amino acid sequences, these active-site serine β-lactamases are divided into three molecular classes A, C and D [27–30]. A minority of enzymes are metalloproteins whose activity relies on the presence of one or two Zn^{2+} ion(s) in their active site and appears to involve only non-covalent intermediates [17,31]. For historical reasons, they are known as class B β-lactamases. All these Zn^{2+}-dependent enzymes significantly hydrolyse carbapenems (Figure 1F; section 9.2) and, in some cases, the classical inactivators of the more prevalent class A β-lactamases. Although they represent a new clinical problem [32,33], Zn^{2+}-enzymes are still vastly outnumbered by the active-site serine β-lactamases which represent a more immediate threat.

4. ACTIVE SITE SERINE β-LACTAMASES

Interestingly, according to model 1 (and Figure 2), the β-lactam compounds appear to form a similar adduct when reacting with their physiological targets (the DD-peptidases) and with active-site serine β-lactamases. The difference between these two types of enzymes is purely quantitative. Indeed, with the former, the k_3 values are very low (hardly ever above 0.001 s^{-1}; [1]) and the acyl-enzymes are very stable ($t_{1/2} > 10$ min), whereas with the latter very high k_3 values are observed [up to $\sim 10\,000$ s^{-1} for the interaction between the *B. licheniformis* and the *Streptomyces albus* G β-lactamases and benzylpenicillin at 30 °C (A. Matagne, S. G. Waley and J.-M. Frère, unpublished work). Also the rate

of acylation (k_{cat}/K_m, see Figure 2) is generally larger with β-lactamases, sometimes close to the diffusion-limited values of 10^7–10^8 $M^{-1} \cdot s^{-1}$. These purely quantitative differences result in sharp qualitative differences, as β-lactamases inactivate penicillins, whereas penicillins inactivate PBPs [1].

5. X-RAY CRYSTALLOGRAPHIC DATA

The similarity between β-lactamases and DD-peptidases is not restricted to 'mechanistic' properties. Indeed, X-ray diffraction studies have highlighted striking structural analogies between the *Streptomyces* R61 (*S.* R61) DD-peptidase and several class A and class C β-lactamases [6,34–37]. These enzymes are all medium-sized monomeric proteins (M_r values of about 29000 and 39000 for class A and class C β-lactamases respectively, and 37500 for the *S.* R61 DD-peptidase), which are made up of two structural domains (an all-α and an α/β domain) with the active site situated in a groove between the two domains (Figure 3). Compared with the class A β-lactamases, the class C enzymes and the *S.* R61 PBP have additional loops and secondary structures on the surface of the all-α domain, at a distance from the active site. The active serine is situated at the N-terminus of the rather long, hydrophobic first helix of the all-α domain (α-2 helix). Interestingly, the first three-dimensional crystal structure of a high-molecular-mass PBP [6] has recently been reported at 3.5 Å resolution [39]. This large molecule (M_r value $\sim$ 80000)

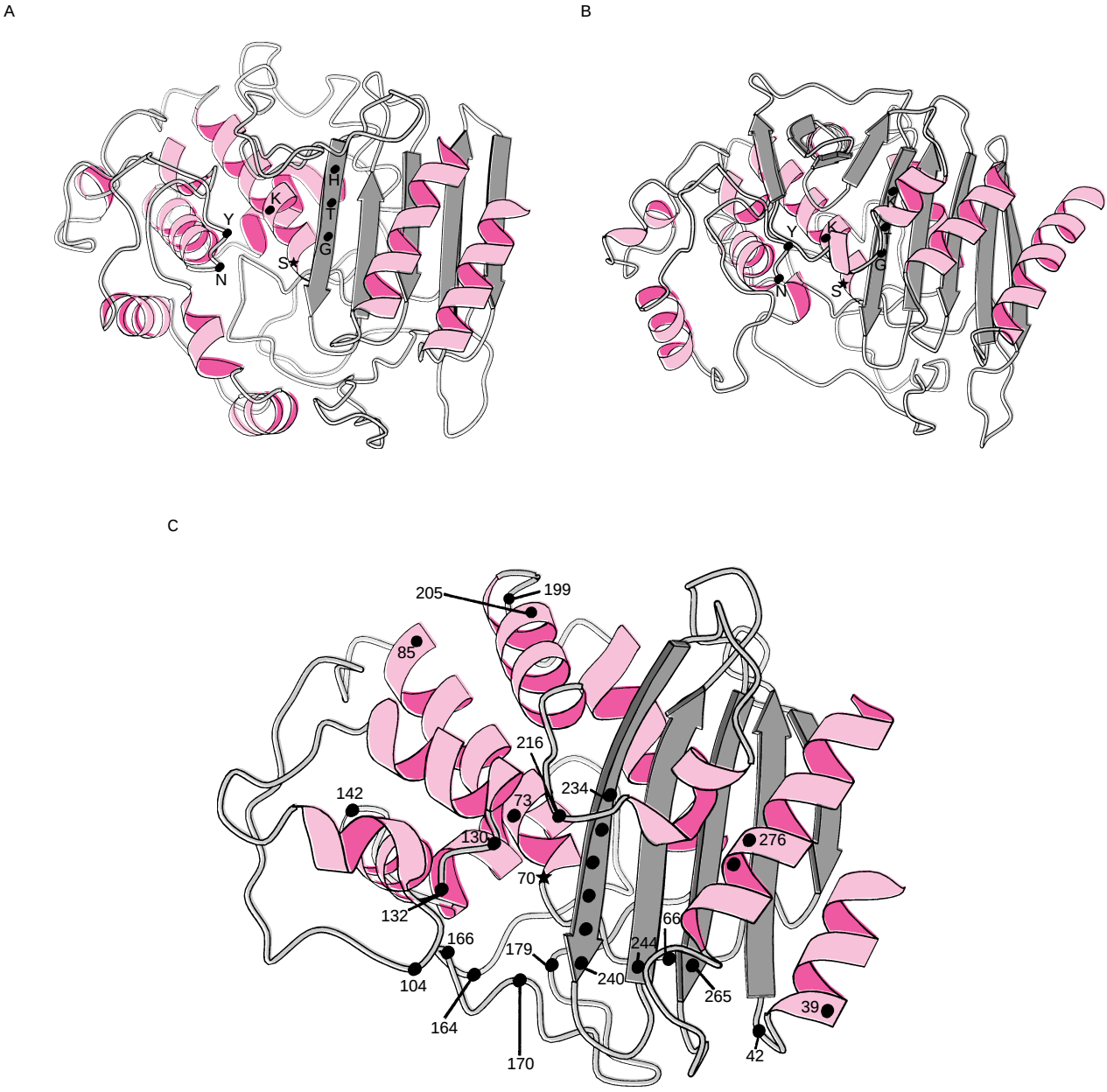

Figure 3 Comparison of the tertiary structures of the *S.* R61 DD-peptidase (A), the *E. cloacae* class C β-lactamase (B) and the class A TEM β-lactamase (C)

The α-carbon of the active-site serine is marked by a star and those of the residues discussed in the text by a dot. These diagrams were generated using MOLSCRIPT [38].

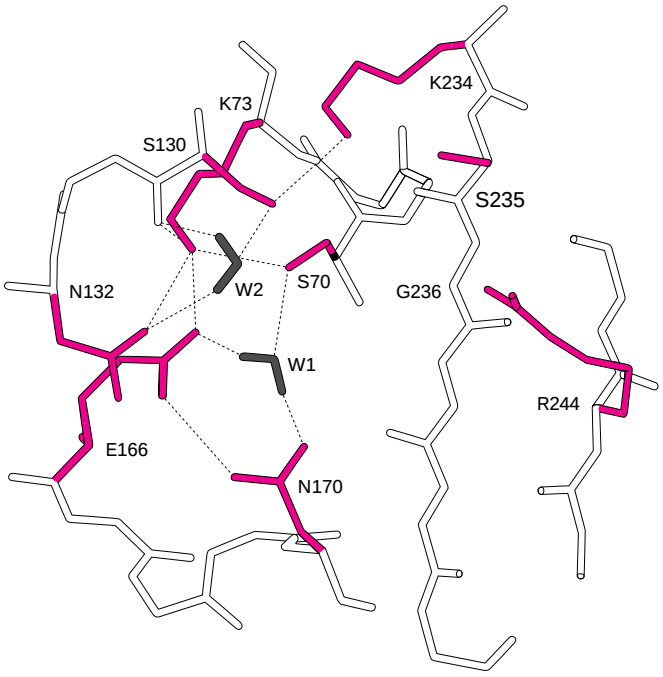

Figure 4 Residues and hydrogen bond network (dotted lines) in the active site of the TEM β-lactamase

The side chains of residues thought to take part in the catalytic process are coloured red and identified. The two conserved water molecules which are hypothesized to take part in the catalytic process are also shown (W1 and W2).

has three domains, the central one being responsible for the transpeptidase activity and for penicillin binding. This transpeptidase domain also shows marked similarities to the active-site serine β-lactamases. These new structural data supply further evidence supporting the existence of a close relationship between these two families of penicillin-recognizing enzymes [2,34,35,37,40] and support the hypothesis of a common ancestor protein for DD-peptidases and β-lactamases [41]. This superfamily of active-site serine penicillin-recognizing enzymes [42] would thus be the perfect illustration of a divergent evolution from a common precursor, analogous to the case of pancreatic serine proteases (trypsin, chymotrypsin and elastase), of carboxypeptidases A and B, and of hen egg white and bacteriophage T4 lysozymes [43,44].

In the immediate vicinity of the active-site serine residue of DD-peptidases and β-lactamases (Figure 3), several conserved elements have been identified which appear to be directly or indirectly involved in the substrate recognition and catalytic processes. Careful comparisons of the primary and tertiary structures have allowed identification of the same structural and functional elements in all active-site serine penicillin-recognizing enzymes [2,30,42,45].

The first element contains the active serine and, one helix-turn downstream, a lysine residue whose side-chain also points into the active site (Ser-Xaa-Xaa-Lys sequence). The proximity of the serine and lysine side-chains, which are hydrogen-bonded (Figure 4), suggested the likely involvement of the lysine side-chain amino group in the catalytic process [46–48].

The second element is situated on a short loop in the all-α domain, where it forms one side of the catalytic cavity. It consists of Tyr-Xaa-Asn (β-lactamases of classes C and D, some PBPs) or Ser-Xaa-Asn (β-lactamases of class A, most PBPs) sequences. The side-chains of the first and third residues point into the active-site cleft, while that of the second residue lies in the protein core. The first residue (Ser or Tyr) is hydroxylated, and the third is nearly always an asparagine. In class A β-lactamases, the Ser^{130}-Asp^{131}-Asn^{132} motif (also called the SDN loop) is nearly invariant, the only exceptions being the $Asn^{132} \rightarrow Ser$ and the $Asn^{132} \rightarrow Gly$ substitutions in the *B. cereus* III and *Mycobacterium tuberculosis* β-lactamases respectively (Table 1).

The third element is on the innermost strand of the β-sheet (α/β domain) and forms the opposite wall of the catalytic cavity. It is generally a Lys-Thr-Gly sequence, but Lys is replaced by His or Arg in a few exceptional cases and Thr by Ser in several class A β-lactamases (position 235 in Figure 3C). A positive side-chain, followed by one bearing a hydroxyl group, appears to be universally conserved. The third member of this so-called KTG triad is devoid of side-chains and is strictly conserved in the superfamily of active-site serine penicillin-recognizing enzymes [42]. Actually any side-chain at this position would severely impair the approach of the substrate. The side chain of the Lys residue forms a hydrogen-bond with the hydroxyl group of the Ser/Tyr residue of the second element (Figure 4).

A fourth element, containing a negatively charged residue, has been tentatively identified in all enzymes, but it seems to play a catalytic role only in class A β-lactamases, where it is situated on a 16–19 residues loop (Arg^{161}-Asn^{179} in TEM-1 [49]) usually referred to as the Ω-loop. In most cases, this loop contains the Glu^{166}-Xaa-Glu-Leu-Asn^{170} sequence where the two residues Glu-166 and Asn-170 seem essential in positioning the conserved water molecule W1 very close to the active serine [50].

X-ray data, where available, indicate that most of these conserved residues in the superfamily of penicillin-recognizing enzymes are intimately involved in a dense, structurally conserved hydrogen-bonding network within the active-site cavity (Figure 4).

Of all these enzymes, class A β-lactamases are the most numerous and the most widely studied. A very high number of enzymes have been reported and more than 45 sequences determined (not considering the numerous variants in the TEM and SHV families; see section 7). The three-dimensional structures of six class A enzymes are known, most of them at high resolution {*B. cereus* I [35]; *S. albus* G [50,51]; *Staphylococcus aureus* PC1 [46,52]; *B. licheniformis* 749/C [47,53]; TEM-1 [48,49,54,55]; NMC-A (P. Swaren, J.-M. Frère and J. P. Samama, unpublished work)}, and the function of numerous residues has been probed by site-directed mutagenesis. Nevertheless, their catalytic mechanism remains controversial and it proves particularly hazardous to establish correlations between the sequence data and the highly variable substrate profiles [56]. This review focuses on the properties of class A β-lactamases. Many studies have been performed involving the modification of the conserved residues described above, and has been extensively reviewed by us [57]. Here we discuss the most recent findings arising from the analysis of both newly discovered enzymes and newly designed mutants. Throughout this discussion, the consensus numbering scheme proposed by Ambler et al. [58] will be used to facilitate comparisons between these homologous proteins (ABL scheme).

6. CATALYTIC MECHANISM

By analogy with the widely studied mechanism of serine proteases [44,59], which also hydrolyse amide bonds and involve an acyl-enzyme intermediate, the active site of β-lactamases would be expected to contain a specific amino acid side-chain which increases the nucleophilicity of the hydroxyl of Ser-70 by acting

Table 1 Conserved residues in all class A β-lactamases

Residues involved in the catalytic mechanism and/or in substrate binding are shown in bold type. Columns corresponding to strictly conserved residues are shaded in grey.

	Amino acid at position													
β-Lactamase	37	45	66	**70**	**73**	81	107	**130**	131	**132**	134	136	144	156
ABL consensus*	Glu	Gly	Phe	**Ser**	**Lys**	Leu	Pro	**Ser**	Asp	**Asn/ (Ser)**	Ala	Asn	Gly	Gly
M. tuberculosis										Gly				
N. lactamadurans	Gln													
P. vulgaris														Asn
L. enzymogenes													Asn	
Per-1/Per-2†	Val											Asp		
B. uniformis (cblA)	Leu											Asp		Ser
B. fragilis (cepA)	Ile		Tyr			Ala						Asp		
B. vulgatus (cfxA)‡	Val		Tyr			Cys							Asn	Pro
Sme-1/NmcA/IMI-1														

	Amino acid at position												
β-Lactamase	157	164	**166**	169	179	180	199	207	233	**234**	**235**	236	References
ABL consensus*	Asp	Arg	**Glu**	Leu	Asp	Thr	Leu	Leu	Asp	**Lys/ (Arg)**	**Thr/ (Ser)**	Gly	[58,140,158,165,176–178]
M. tuberculosis		Ala											[179]
N. lactamadurans													[158]
P. vulgaris													[158,159,180]
L. enzymogenes													[158]
Per-1/Per-2†	Ile/Val	Ala		Met	Asn	Trp			His				[151,161]
B. uniformis	Ile	Glu		Met	Asn	Trp	Phe		His				[160]
B. fragilis	Ile	His		Met	Asn	Trp	Phe	Ile	His				[160]
B. vulgatus (cfxA)‡	Arg	Tyr		Met	Asn	Tyr	Ile	Ile	His				[160,161]
Sme-1/NmcA/IMI-1								Tyr					[169,171]

* *K. pneumoniae* LEN-1; SHV-1; TEM-1; *P. aeruginosa* PSE-3; PSE-4; *R. capsulata*; *A.* R39; *B. cereus* 569/H type I; *B. cereus* 5/B type I; *B. cereus* 569/H type III; *B. licheniformis*; *S. badius*; *S. cacaoi* blaU; *S. cacaoi* Ulg; *K. oxytoca* E23004; *S. aureus PC1*; *S. aureofaciens*; *S. albus* G; *S. lavendulae*; *S. fradiae*; *H. influenzae* ROB-1; CARB-4; *S. cellulosae*; *C. diversus*; *Y. enterocolitica*; *B. mycoides*; *E. faecalis*; *K. oxytoca* D488; *E. coli* MEN-1 (CTX-M-1); *P. mirabilis* GN 79; *M. fortuitum*; *E. coli* Toho-1; CTX-M-2; *S. fonticola*. Among these 34 sequences, a Ser is found at position 132 only in *B. cereus* III. Three carbenicillin-hydrolysing enzymes (PSE-4 [181], CARB-4 [182] and *P. mirabilis* GN 79 [183]) display an Arg at position 234 (note that a similar K234R substitution also occurs in PSE-1 [184] and CARB-3 [185]). A hydroxylated residue, either Ser (in 13 sequences) or Thr (in 21 sequences), is invariably found at position 235.

† Per-1 and Per-2 display 86.4% isology. An Ile residue and a Val residue are found in position 157 in Per-1 and Per-2 respectively.

‡ Due to a different alignment, Nordmann and Naas [161] found an Ile and a Pro at positions 156 and 157 respectively.

as a general base catalyst. In most serine proteases, this function has long been known to be fulfilled by the imidazole group of a histidine residue. In contrast, the mechanism of proton abstraction and donation during hydrolysis of β-lactam compounds by the active-site serine β-lactamases is still the subject of considerable discussion.

The most controversial situation prevails for class A enzymes, for which two distinct residues have been proposed for the role of general base. In one hypothesis [50,60–62], this role is played by the conserved Glu-166. Crystallographic data have highlighted the presence of a tightly bound water molecule, forming a bridge between the Ser-70 hydroxyl group and the Glu-166 carboxylate side chain. This water molecule (W1) is found in the same position in all the high-resolution class A structures [47,49, 50,52]. Although site-directed mutagenesis experiments suggested that Glu-166 might play the role of general base in the acylation reaction [60], it seems too far away from the Ser-70 OH group to do so directly. Molecular modelling studies [50] have suggested that the conserved water molecule might act as a relay molecule in the transfer of the proton between the Ser-70 and Glu-166 side chains. Alternatively, the flexibility of the Ω-loop might allow the distance between the two residues to be shortened during the acylation process [63]. In both cases, the activated γ-O of Ser-70 can then attack the β-lactam carbonyl carbon on the well-exposed α-face of the molecule, and the abstracted proton is back-donated to the nitrogen atom of the β-lactam through the hydrogen-bonding sub-network (Figure 4) involving a second water molecule (W2), the ϵ-amino groups of Lys-73 and Lys-234 and the hydroxyl group of Ser-130, which acts as the last proton donor [50,61] (note the different proposal by Mobashery and co-workers [64,65], who suggested that Ser-130 lowers the energy barrier for hydrolysis of substrates by merely hydrogen-bonding to the nitrogen atom of the β-lactam ring in the course of Ser-70 acylation). Hydrolysis of this serine-ester-linked penicilloyl-enzyme would subsequently occur according to a symmetrical mechanism, where Glu-166 would activate the W1 water molecule to attack the carbonyl carbon of the acyl-enzyme and ensure back-delivery of the abstracted proton to the Ser-70 γ-O atom, ultimately leading to enzyme regeneration [50]. The role of Glu-166 (proton abstractor) in the deacylation step, originally suggested by Herzberg and Moult [46] on the basis of the refined structure of the *S. aureus* PC1 β-lactamase, has been widely accepted.

During both acylation and deacylation, elusive tetrahedral transition-state intermediates are formed in which the β-lactam carbonyl oxygen is negatively charged. This charge is stabilized

Table 2 Comparison of the k_{cat}/K_m values ($mM^{-1} \cdot s^{-1}$) for the interaction of some characteristic β-lactams with carbapenem-hydrolysing β-lactamases and various enzymes of class A

Values in parentheses are relative to those for benzylpenicillin arbitrarily fixed at 100. The data are from [1] Mariotte-Boyer et al. [175]; [2] P. Swarén, J.-M. Frère and J. P. Samama, unpublished work; [3] Raquet et al. [132]; [4] Rasmussen et al. [171]; [5] Raquet et al. [99]; [6] Fisher et al. [26]; [7] Miyashita et al. [173]; [8] Matagne et al. [56,72,83]. (*) poor inactivator with $K_i \sim 2$ mM [186]. ND, not determined.

	NmcA[1,2]	Sme-1[3]	IMI-1[4]	TEM-1[3,5–7]	*S. albus* G[8]	*A.* R39[8]
Benzylpenicillin	9300 (100)	1400 (100)	560 (100)	84 000 (100)	2800 (100)	7500 (100)
Ampicillin	ND	730 (52)	240 (44)	33 000 (39)	6100 (220)	16 000 (210)
Ticarcillin	530 (6)	130 (9)	ND	ND	125 (4.5)	800 (11)
Penicillanate	ND	ND	ND	66 (0.08)	61 (2.2)	590 (8)
6-α-(Hydroxymethyl)-penicillanate	100 (1)	ND	ND	1.2 (0.0015)	ND	ND
Cephaloridine	ND	1530 (110)	1900 (340)	2200 (2.6)	620 (22)	11 600 (150)
Cephalothin	15 200 (160)	1620 (120)	920 (160)	650 (0.8)	370 (13)	3700 (49)
Nitrocefin	ND	6150 (440)	3500 (630)	17 000 (20)	2400 (86)	8500 (73)
Cefotaxime	300 (3)	ND	18 (3)	1.5 (0.002)	1 (0.04)	400 (5)
Ceftazidime	52 (0.6)	ND	0.024 (0.004)	0.07 (8×10^{-5})	< 0.001	13 (0.2)
Cefoxitin	62 (0.7)	2.2 (0.2)	6.7 (1.2)	0.006 (7×10^{-6})	$< 3 \times 10^{-5}$	0.002 (2×10^{-5})
Moxalactam	29 (0.3)	ND	ND	(*)	$< 3 \times 10^{-5}$	0.0003 (5×10^{-6})
Aztreonam	5600 (60)	ND	550 (98)	0.7 (8×10^{-4})	0.4 (0.01)	76 (1)
Imipenem	11 300 (120)	1620 (120)	520 (93)	1.5–2.5 (~ 0.002)	0.32 (0.01)	9 (0.12)
Meropenem	2750 (30)	ND	380 (69)	ND	ND	ND

by hydrogen-bonding interactions with the backbone NH groups of Ser-70 and residue 237, which thus form an 'oxyanion hole' reminiscent of that found in serine proteases [46,48,50,66,67].

The second hypothesis [48,68,69] mainly rests on the structural properties of the Glu-166 → Asn mutant of the TEM-1 β-lactamase [48], which forms long-lived acyl-enzymes [70]. The authors argued that Glu-166 is expendable for acylation and thus proposed an unsymmetrical mechanism, with two different general bases, Lys-73 and Glu-166, participating in acylation and deacylation respectively. Thus the ϵ-amino group of a lysine side-chain would act as the proton abstractor to increase the nucleophilicity of the active-site serine, a mechanism which has now been proposed for several proteases that contain an essential lysine, but no essential histidine [59]. In this mechanism, the enzyme must provide a favourable local environment to enable the lysine to remain deprotonated at neutral pH. The strongly reduced pK_a value ($\sim$ 5–6 pH units) for the alkylammonium group of Lys-73 in class A β-lactamases would result both from hydrogen-bonds with Ser-70, Asn-132 and the backbone carbonyl of Ser-130 (Figure 4), and from a very positive electric field created by the α-2 helix dipole, the alkylammonium group of Lys-234 and, possibly, some other basic residues, such as Arg-244 and Arg-164, located near the enzyme active site [48].

However, in the wild-type enzyme, the carboxylate group of Glu-166 is much closer to the ϵ-amino group of Lys-73 than is any positively charged side-chain [49], and thus the active-site negative charge of Glu-166 would be expected to hinder the catalytic efficiency of the acylation process. Hence, removal of this proximal negatively charged residue should actually increase the rate of the acylation process. However, in all class A β-lactamase mutants where Glu-166 was replaced by a neutral residue (Ala, Cys, Tyr or Asn), the acylation step was found to be significantly impeded.

Several Glu-166 mutants were prepared with various class A enzymes, and whenever detailed kinetic studies were performed, both acylation and deacylation rates appeared to be decreased by the mutation, deacylation being sometimes more affected than acylation (for a detailed review of these data, see [57]). Moreover, as reported for the *B. cereus* 569/H β-lactamase I [60], the K73R mutation causes a $\sim$ 100-fold decrease in the value of k_2 for benzylpenicillin hydrolysis, while its Glu-166 → Asp counterpart causes a $\sim$ 2000-fold decrease in 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. Most recently, careful re-examination of the catalytic properties of the Glu-166 → Asn mutant of the TEM-1 β-lactamase [71] unambiguously indicated that, if the mutation truly causes conversion of the penicillin-hydrolysing TEM-1 β-lactamase into a PBP (due to an extremely large decrease in the k_3 value, i.e. $\sim 10^9$-fold for benzylpenicillin), the k_2/K' value, which characterizes the efficiency of acylation, was also significantly impaired ($\sim 10^3$-fold for benzylpenicillin). All these kinetic data seem to weaken the hypothesis of an unsymmetrical mechanism [48] and are most consistent with the mechanism proposed by Lamotte-Brasseur et al. [50], where both acylation and deacylation involve Glu-166 acting as a general base via a conserved water molecule.

This latter hypothesis is further strengthened by kinetic and modelling studies of β-lactam compounds bearing a methoxy side-chain on the α-face of the β-lactam ring [72]. These molecules (cefoxitin, moxalactam and temocillin; Figure 1) acylate the active serine of most class A enzymes with an exceedingly poor efficiency (Table 2), and whenever acylation occurs, deacylation proves to be even slower, both several orders of magnitude below the rates observed with good substrates. Nevertheless, these molecules easily fit into the active site [72], in a way very similar to that observed with the best substrates, but the bulky methoxy substituent displaces the water molecule W1, thus making both acylation and deacylation very unlikely, as expected from the symmetrical mechanism involving Glu-166. Conversely, the three substrates considered in this study do not perturb the hydrogen-bond between Ser-70 and Lys-73 and thus they should readily acylate the enzyme if the second hypothesis were valid. Additionally, and quite notably, the acylation rates by these very bad substrates were found to be significantly facilitated in the Glu-166 → Asn mutant of TEM-1 [71]. These data suggested that the acylation of the mutant might rely on an alternative

mechanism, where the Lys-73 side chain would effectively replace Glu-166 as the general base, as suggested by Strynadka et al. [48] for the wild-type β-lactamase. In the mutant, removal of the negative charge might explain the large decrease in the Lys-73 pK_a value when compared with the wild-type enzyme. This alternative mechanism may explain why in many Glu-166 → Xaa mutants, the acylation step was found to be less affected than the deacylation step.

Finally, the pH dependence of the chemical modification of ϵ-amino groups and the NMR titration of the ^{13}C-labelled lysine residues in the TEM-1 β-lactamase [73] clearly indicated that the pK_a value ($\geqslant 10$) of the Lys-73 side-chain is not significantly decreased in the wild-type enzyme, making it a very unlikely candidate for proton abstraction in catalysis. Note also that a crystallographic study of the Lys-73 → His mutant of the *S. aureus* PC1 β-lactamase, whose catalytic properties are severely impaired, was recently reported [74]. The X-ray data indicated that the imidazole ring is positively charged and that the only significant deviation in side-chain conformation is that of the catalytic serine residue, which adopts an alternative conformation incompatible with nucleophilic attack on substrates. These observations suggest that the positively charged alkylammonium group of Lys-73 helps in orientating the Ser-70 hydroxyl group for efficient catalysis.

Thus, although it can still be argued that a substantially lowered pK_a value prevails for Lys-73 when the substrate is bound to the active site [67,74,75] (see also the mechanism proposed by Swarén et al. [76] who argue for a substantial upward shift in the pK_a of the unprotonated Lys-73 upon substrate binding), most presently available data are inconsistent with the second hypothesis (see [77,78] for further comments), and seem to indicate that Glu-166 is the genuine general base catalyst in both formation and hydrolysis of the acyl-enzyme intermediate.

7. EXTENDED-SPECTRUM β-LACTAMASES OF THE TEM AND SHV FAMILIES

In the struggle against β-lactamase-producing pathogenic bacteria, one strategy has been to search for, and use, β-lactam compounds that are resistant to the hydrolytic action of these enzymes, while retaining antibacterial activity [12,79]. Several families of so-called 'β-lactamase-stable' antibiotics are now available. Third-generation cephalosporins (e.g. cefotaxime and ceftazidime), which differ from first- and second-generation cephalosporins in their chain substituents at C-3 and C-7 (R_1 and R_2 in Figure 1B), and hence in their increased spectrum of activity and potency [80,81], represent one such group, besides cephamycins (cefoxitin), oxacephamycins (moxalactam), 6-α-methyoxypenams (temocillin), monobactams (aztreonam) and carbapenems (imipenem) (Figure 1). Their stability is neither absolute nor general [56,82–84] and most of them behave as sluggish substrates of the chromosome-encoded class C and plasmid-borne SHV-1 and TEM-1 enzymes, but nevertheless display fair to high efficiency against strains producing these β-lactamases. In the past 15 years, the extensive and sometimes abusive clinical utilization of these drugs has been responsible for the appearance of an increasing number of resistant strains [3]. In most cases, this could be attributed to the production of new extended-spectrum (ES) β-lactamases [20–22,85]. These plasmid-mediated enzymes confer resistance to 'β-lactamase-stable' compounds such as cefotaxime, ceftazidime and aztreonam, all characterized by β-acyl side-chains containing an oximino group (Figure 1). The large majority of these new enzymes belong to the SHV and TEM families [86], the parent enzymes, TEM-1/2 (see below) and SHV-1, sharing very similar primary structures [87] and biochemical properties. To date, at least 29 TEM- and 11 SHV-related enzymes exhibiting increased hydrolytic activities against the oximino β-lactams have been described [23,88–90].

Table 3 Extended-spectrum β-lactamases of the SHV family

The data are compiled from previously published reports [21,92,107,187], where all references can be found. Note that residues 238 and 240 are adjacent in both the SHV and TEM sequences, an insertion occurring at this level in some homologous class A enzymes.

	Amino acid at position					
	35	179	205	238	240	No. of mutations*
SHV-1	Leu	Asp	Arg	Gly	Glu	—
SHV-2				Ser		1
SHV-2a	Gly			Ser		2
SHV-3			Leu	Ser		2
SHV-4			Leu	Ser	Lys	3
SHV-5†				Ser	Lys	2
SHV-7‡				Ser	Lys	3
SHV-8		Asn				1
SHV-11	Gln					1
SHV-12	Gln			Ser		2

* Number of point mutations when compared with the parental type SHV-1.

† The same mutations occur in SHV-9. However, one deletion (Gly-45) and three substitutions (Arg-140 → Ala, Asn-192 → Lys and Val-193 → Leu) differentiate SHV-9 from SHV-5, which are nevertheless functionally indistinguishable [156].

‡ An additional mutation occurs at position 43, where the highly conserved Arg is replaced by Ser [187]. The role of that new substitution might be structural, although this is not clear [110].

Each variant enzyme has from one to four amino acid substitutions when compared with the parental enzymes (Tables 3 and 4), and those mutations responsible for the new activity profiles are restricted to Glu-104 (TEM), Arg-164 (TEM), Asp-179 (SHV), Arg-205 (SHV), Ala-237 (TEM), Gly-238 and Glu-240 (in both TEM and SHV), with the recent addition of Val-42 in the TEM-42 variant [91] and by Leu-35 in several SHV derivatives [92]. In the TEM family, all the variants arise from point mutations in the gene encoding either TEM-1 or TEM-2. The Gln-39 → Lys (TEM-2) and Thr-265 → Met (TEM-13) mutations, at positions quite distant from the active site (Figure 3C), do not result in significant differences in the catalytic properties [21,93–95], and thus TEM-1, TEM-2 and TEM-13 correspond to parental phenotypes.

Not surprisingly, most residues involved in the extended-spectrum properties of these enzymes were found to be located in close proximity to the active-site cavity (Figure 3C). None of the modified side-chains appears to be involved in the catalytic mechanism of class A β-lactamases, but these mutations, which extend the substrate profile of the variants, often concomitantly lower their catalytic efficiencies against the classical, good substrates of the parent enzymes [96–99]. Three other substitutions, i.e. Leu-35 → Gln and Arg-205 → Leu in the SHV family and Ala-42 → Val in the TEM family (Tables 3 and 4), observed further away from the active site, appear to be not negligible, but of lesser importance [86,91,92], and their real significance remains to be firmly established.

An attempt was first made to explain the specificity of the variants on the basis of the three-dimensional structures of related enzymes (see, e.g., [98,100]). These studies (reviewed in [57]) drew attention to both the effect of individual mutations (particularly as positions 164 and 238) and the spectacular synergistic effect obtained by combining two or three mutations.

Table 4 Extended-spectrum β-lactamases of the TEM family

The data are compiled from previously published reports [21,86,91,95,188–194], where all references can be found.

	Amino acid at position								
	39	42	104	164	237	238	240	265	No. of mutations*
TEM-1	Gln	Ala	Glu	Arg	Ala	Gly	Glu	Thr	—
TEM-2	Lys								—
TEM-3	Lys		Lys			Ser			2
TEM-4			Lys			Ser		Met	3
TEM-5				Ser	Thr		Lys		3
TEM-6†			Lys	His					2
TEM-7	Lys			Ser					1
TEM-8	Lys		Lys	Ser		Ser			3
TEM-9			Lys	Ser				Met	3
TEM-10				Ser			Lys		2
TEM-11	Lys			His		?			1–2
TEM-12				Ser					1
TEM-13	Lys							Met	1
TEM-14	Lys		Lys			Ser		Met	3
TEM-15‡			Lys			Ser			2
TEM-16	Lys		Lys	His					2
TEM-17			Lys						1
TEM-18	Lys		Lys						1
TEM-19						Ser			1
TEM-24	Lys		Lys	Ser	Thr		Lys		4
TEM-25						Ser		Met	2
TEM-26			Lys	Ser					2
TEM-27				His			Lys	Met	3
TEM-28				His			Lys		2
TEM-29				His					1
TEM-42	Lys	Val				Ser	Lys	Met	4
TEM-46			Lys	Ser			Lys		3

* Number of point mutations when compared with the parental type TEM-1/2. Note that silent mutations can also result in additional differences between the various genes (see e.g. [163,190,195,196]).

† The same mutations as for the recently reported TEM-43 enzyme [197], which displays yet an additional Met-182 → Thr mutation (see Table 5).

‡ The same mutations in TEM-21, which bears an additional His-153 → Arg mutation [191].

More recently, the determination of the X-ray structure of the TEM-1 β-lactamase allowed a more rational examination of the role of the residues at positions 104, 164, 238 and 240 in the TEM family [99]. A detailed kinetic study of six TEM mutants obtained by site-directed mutagenesis was performed and their properties were analysed using molecular modelling, since no data on the structures of the variants are presently available. The key roles of mutations at positions 164 (Arg-164 → Ser/His) and 238 (Gly-238 → Ser) could be rationalized on the basis of structural changes (sections 7.1 and 7.2). Most interestingly, docking the optimized structures of substrates with bulky oximino moieties on their C-7 side-chain (cefotaxime and ceftazidime) into the active site of the wild-type TEM-1 enzyme led to short contacts between the oxime substituents and the side-chain of Asn-170, which in turn resulted in a displacement of the carboxylate side-chain of Glu-166 [99]. Moreover, with ceftazidime, the position of the catalytic water molecule W1 was also modified, an observation which is consistent with the even poorer activity of the enzyme against this substrate. A similar displacement of the catalytically important water molecule W1 was also deduced from molecular-modelling analysis of the interaction of the *S. albus* G β-lactamase with various β-lactamase-stable antibiotics, including cefotaxime, ceftazidime and aztreonam [72,83] and, more recently, from a similar study of the mechanism of hydrolysis of cefepime, a novel oximino cephalosporin, by TEM-1 [101]. These results confirm yet again, the intimate implication of Glu-166, Asn-170 and the conserved water molecule W1 in the catalytic machinery of class A β-lactamases (see also section 9.2).

7.1. The R164 mutants

In most of these enzymes, Arg-164 forms a strong, buried ionic-bond with the highly conserved Asp-179, which is important for the conformation and the stability of the Ω-loop and hence for the correct positioning of Glu-166 within the active site [102] (note that three additional stabilizing interactions are found in the TEM-1 enzyme, namely between Arg-164 and Glu-171, between Asp-176 and Arg-178 and between Glu-177 and Thr-180, which all contribute to the locking of the Ω-loop [49,103]). In excellent agreement with this, systematic mutagenesis of the active site Ω-loop of the TEM-1 β-lactamase [104–106] failed to produce mutations at positions 164 or 179 (also true for positions 166, 176 and 180), resulting in ampicillin resistance of the host strain. Accordingly, a significant loss of activity of the Arg-164 → Ser and Arg-164 → His mutants derived from TEM-2 (TEM-7 and TEM-11 respectively) towards the best substrates was observed (decreased k_{cat} values [99]), and this negative factor, resulting from the perturbation of the Ω-loop, must actually reflect on the hydrolytic efficiency of all compounds, including cefotaxime and ceftazidime [99]. Nevertheless, the specificity constant (k_{cat}/K_m) of the TEM-7 and TEM-11 mutants against these two substrates was actually increased by their respective mutations at position 164, owing to a larger positive effect, i.e. the disappearance of the short contacts with Asn-170, mainly reflecting on the K' [and hence $K_m = k_3 \times K'/(k_2 + k_3)$] values. In addition, removal of the Arg-164 long side-chain allows more freedom for the oxime carboxylate of ceftazidime, which no longer disturbs the water molecule W1, hence accounting for the specifically high activity against ceftazidime (increased k_{cat} value [99]) of the mutants bearing an Arg-164 → Ser/His mutation. Finally, the important feature in the interaction of those TEM variants harbouring a substitution at position 164 with oximino β-lactams is not the occurrence of a potential hydrogen-bond donor, but rather the disappearance of the salt-bridge between the two highly conserved residues Arg-164 and Asp-179. Recently, Maveyraud et al. [103] proposed that in the Arg-164 → Ser mutant, a significant movement of the Ω-loop would displace the large 85–142 region (Figure 3C) of the enzyme, resulting in better access of cephalosporins with large oximino side-chains to the active-site cavity, and thus explain the specific enhancement of the catalytic efficiency observed for these molecules relative to other good penicillin and cephalosporin substrates.

Notably, although mutations at position 164 have not been reported yet in the SHV family (Table 3), a new member (SHV-8) has been identified which displays a single mutation at position 179 (Asp-179 → Asn; [107]). The effects of this new mutation are most likely analogous to those of the Arg-164 → Ser/His substitutions occurring in the TEM family.

7.2. The Gly-238 → Ser mutant

Consistent with previous suggestions [88,108,109], and contrary to the indirect role suggested for the residue at position 164, Raquet et al. [99] suggested that the Ser side-chain of the Glu-238 → Ser mutants (TEM-19 and the double mutant TEM-3 were studied) points into the active-site cavity and is suitably positioned in order to form a new hydrogen-bond with the oxime oxygen of cefotaxime. However, this was not found to be possible with the bulkier substituent of ceftazidime, which would

create short contacts with the hydroxymethyl group of Ser-238. To avoid these short contacts, the side-chain of ceftazidime must be oriented away from the active site, which allows the return of W1 to its efficient position and hence a better catalytic efficiency. Therefore, with these Ser-238 mutants, the structural changes in the enzyme active site were found to have a similar effect on both k_{cat} and k_{cat}/K_m (increased values, enhanced by the second mutation Glu-104 → Lys in TEM-3) for both cefotaxime and ceftazidime [99], but this improved catalytic efficiency was proposed to originate from very different factors, i.e. on the one hand, a new direct interaction between the enzyme Ser-238 side-chain and cefotaxime and, on the other hand, a different, more favourable, positioning of ceftazidime in the active-site cleft.

In contrast, Knox and co-workers [100,110] suggested that the introduction of a large side-chain (larger than Ala, e.g. the hydroxymethyl group of Ser) at position 238 would displace the β-3 strand and result in a slightly expanded active site which would accommodate cephalosporins with bulky side-chains. This hypothesis has the advantage of partly accounting for the observed increase in activity against cefotaxime of *in vitro* mutant enzymes of the TEM (Gly-238 → Ala, [111]) and SHV (Gly-238 → Val [109]) families, which obviously cannot form a new hydrogen-bond with the oxime moiety of the substrate. However, it was argued [112] that the dense hydrogen-bond network found in the particular area of the active site would impede any movement of the C-terminal edge of the β-3 strand, where residue 238 lies. Rather, modelling of the Gly-238 → Ser substitution in the three-dimensional structure of TEM-1 [99,112] suggested some new interactions between Ser-238 and Asn-170, which might again result in a slight displacement of the Glu-166 side-chain, hence explaining the decreased k_{cat} values observed with the best substrates. Conversely, such subtle conformational changes of that part of the Ω-loop could, 'in some way', be responsible for enhanced catalytic efficiency towards cefotaxime [112] and, more globally, towards compounds with an oxime substituent on C-7 (e.g. ceftazidime).

Similar changes, probably also resulting from steric effects [112], might well be obtained with Ala or Val at position 238, thus explaining the altered specificity of the Gly-238 → Ala and Gly-238 → Val mutants quoted above. Nevertheless, recent screening for *in vitro* mutants with highly (100-fold) increased resistance to ceftazidime [113] confirmed that, although several substitutions at position 238 can increase activity, the best combination of substitutions in that region includes Ser at position 238, Lys or Arg at position 240 and a small amino acid at position 241. One can hypothesize that various side-chains, including the methyl group of Ala at position 238, induce some minor structural changes that allow a better positioning of those substrates bearing large oxime substituents. This positive effect would more than compensate for the negative effect observed with the good substrates, i.e. a decrease in activity, caused by slight perturbations of the Ω-loop and poor positioning of the Glu-166 carboxylate side-chain. The decreased stability of the Gly-238 → Ser mutant [114] might indeed reflect an altered conformation of the active site. Recent experiments [112] suggested that with the Gly-238 → Ser mutant of the TEM-1 enzyme, both the acylation and the deacylation steps might be affected by the change in the active-site conformation. Interestingly, these conclusions are again in good agreement with the proposal [50] of a dual role for Glu-166 in both the acylation and deacylation steps.

Finally an engineered *S. aureus* PC1 β-lactamase (Ala-238 → Ser and Ile-239 deleted) displays enhanced activity towards third-generation cephalosporins [115]. Interestingly, the X-ray structure of the mutant revealed both an altered disposition of the β-strand, resulting in an opening of the active-site cavity, and a significant displacement of Glu-166. Although the *S. aureus* mutant is much less efficient than the TEM enzymes (including TEM-1/2) against cefotaxime and ceftazidime, these observed structural changes are similar to those discussed here for the TEM variants bearing a Gly-238 → Ser mutation.

7.3. The Glu-104 → Lys and Glu-240 → Lys mutations

The prevalence of several mutations in the same enzyme (Tables 3 and 4) has been largely documented and, in that respect, several attempts have been made to unravel the individual contributions of Lys at positions 104 and 240. In both cases, the ammonium group of the long Lys side-chain has been suggested to interact with the carboxylic group on oximino substituents of ceftazidime and aztreonam [110]. Unfortunately, in the absence of three-dimensional structures for these proteins, modelling of the mutants with new Lys side-chains (e.g. TEM-3, TEM-10 and TEM-18) is made difficult by the large number of possible conformations of the long alkyl moiety of this residue [99]. The activity compared with the good substrates of the single Glu-104 → Lys mutant (TEM-18) was found to be unaffected [99], a behaviour very similar to that of the Glu-104 → Lys mutant studied by Sowek et al. [98]. It was shown that the ammonium group of the two Lys side-chains can indeed position similarly in the active site without modifying its structure or inducing short contacts [99]. This agrees with a similar role for the two substitutions. Recent work by Petit et al. [116] has suggested that a lysine at position 104 might alter the precise positioning of residues 130–132 (SDN loop) which are involved in substrate binding and catalysis.

Further details about the role of the individual residues involved in the extended substrate profile of the TEM and SHV variants can be found in the recent and thorough review by Knox [110].

8. INHIBITOR-RESISTANT β-LACTAMASES OF THE TEM FAMILY

The ES β-lactamases derived from TEM-1/2 and SHV-1 described above remain sensitive to β-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam (Figure 1). These compounds, which have generally little antibiotic activity by themselves, behave as mechanism-based inactivators of most class A β-lactamases, and therefore are able to potentiate the action of classical β-lactamase-sensitive compounds by protecting them from enzymic hydrolysis [12,79]. These 'wonder' drugs have been widely used against the β-lactamase-producing bacteria and, for instance, clavulanic acid combined with amoxycillin (Augmentin) represents a powerful clinical strategy to overcome the resistance of bacteria harbouring the TEM β-lactamase variants [117]. However, bacterial susceptibility to such combinations of efficient β-lactam antibiotics and potent β-lactamase inactivators was first reported to be challenged by the overproduction of TEM-1 or SHV-1 β-lactamases [117–121]. More worrying were later reports of novel TEM-type β-lactamases in *Escherichia coli* clinical isolates [122,123], and more recently in other species of Enterobacteriaceae (*K. pneumoniae*, [124]; *Proteus mirabilis*, [125]), which are resistant to these mechanism-based inactivators. The inhibitor-resistant TEM β-lactamases (IRTs) differ from TEM-1/2 by one, two or three amino acids substitutions at new locations (69, 165, 244, 275 and 276; Table 5), which decrease the affinity for β-lactam substrates and alter the inhibitory action of suicide substrates such as clavulanic acid [124–131].

Table 5 Inhibitor-resistant β-lactamases of the TEM family

	Amino acid at position					
	69	165	244	275	276	References
TEM-1	Met	Trp	Arg	Arg	Asn	[198]
IRT-1 (TEM-31)			Cys			[123,124,127,130]
IRT-2 (TEM-30)			Ser			[123–125,127,130,131]
IRT-3 (TEM-32)*	Ile					[126,131,199]
IRT-4 (TEM-35)	Leu				Asp	[128,130,131]
IRT-5 (TEM-33)	Leu					[130,131]
IRT-6 (TEM-34)	Val					[130,131]
IRT-7 (TEM-36)	Val				Asp	[130,131]
IRT-8 (TEM-37)	Ile				Asp	[131]
IRT-9 (TEM-38)	Val			Leu		[131]
IRT-10 (TEM-39)	Leu	Arg			Asp	[131]
IRT-I67 (TEM-40)*	Ile					[131,199]
TEM-41			Thr			[199]
IRT-14 (TEM-45)	Leu			Gln		[200]

* In these two enzymes, the Met-69 → Ile substitution has been found either in combination with an additional mutation at position 182 (Met-69 → Ile + Met-182 → Thr, called IRT-3, [126]) or as a single point mutation (Met-69 → Ile, [131], called IRT-I67 (TEM-40) by Stapleton et al. [199]), but it was clearly established that only the mutation at position 69 (Met-69 → Ile) was involved in resistance to suicide inhibitors [126,137]. However, the presence of a Thr at position 182 might facilitate the required structural change at position 69 [110] and was shown to partially compensate for loss in catalytic activity due to the Met-69 → Ile substitution [137]. Note also that a residue (Thr, Ser or Cys) which can form a hydrogen-bond is found at position 182 in the sequences of most β-lactamases [137].

8.1. Residue 69

A methionine is present at position 69 of the TEM-1 and SHV-1 enzymes. This residue, which is not conserved in class A β-lactamases, is situated just next to the nucleophilic serine, but the two side-chains are not in contact [49]. Residue 69 lies on a short turn preceding the α-2 helix in the all-α domain and is buried at the hydrophobic interface between the two domains, with its side-chain situated behind the β-3 and β-4 strands. It exhibits a side-chain containing at least one C atom in all class A β-lactamases, with the sole exception of *S. cacaoi* Ulg, and it is found to be in a highly constrained structural environment in all known structures [47,49,52,132].

Not very long after several reports of laboratory-derived mutants of the TEM-1 [133,134] and SHV-type OHIO-1 [135] β-lactamases with a mutation at position 69 (Met-69 → Ile, Met-69 → Leu and Met-69 → Val) and resistant to clavulanate, sulbactam and tazobactam, the same mutations have been found in numerous inhibitor-resistant TEM β-lactamases from clinical isolates of *E. coli* (Table 5).

These observations have been explained by subtle modifications of the active-site structure [134], and, in particular, a deformation of the oxyanion hole and a possible change in the positioning of the guanidinium group of Arg-244, which is situated on the β-4 strand ([136]; see section 8.3). More recent molecular-modelling studies indicated that the substitution of a Met for an Ile at position 69 (Met-69 → Ile) alters the positions of the essential Glu-166 and Asn-170 side chains, as well as the position of the catalytic water molecule [137]. This very slight displacement (0.3 Å for its oxygen) of W1 has been suggested to be responsible for the decrease in the k_{cat} and K_m values (note that a global decrease in the k_{cat}/K_m values was observed) of the IRT-3 β-lactamase and for resistance to mechanism-based inactivators.

Table 6 Conserved basic residue at homologous positions in class A β-lactamases

The data are adapted from previously reported sequence alignments; all references can be found in Table 1. Residues involved in the catalytic mechanism and/or in substrate binding are shown in bold type.

	Amino acid at position		
Enzymes	220	244	276
Klebsiella pneumoniae LEN-1; SHV-1; TEM-1; *Pseudomonas aeruginosa* PSE-3; PSE-4; *Rhodopseudomonas capsulata*; *A.* R39; *Bacillus cereus* 569/H type I; *Bacillus cereus* 5/B type I; ROB-1; CARB-4; *Bacteroides mycoides*; *Proteus mirabilis* GN 79	Leu	**Arg**	Asn
Bacillus cereus 569/H type III; *Bacillus licheniformis; Streptomyces cacaoi* Ulg; *Staphylococcus aureus* PC1; *Enterobacter faecalis*	Leu	**Arg**	Asp
Streptomyces badius; *Nocardia lactamdurans*	Val	**Arg**	Asp
Streptomyces cacaoi blaU	Leu	**Arg**	Ser
Streptomyces cellulosae	Thr	**Arg**	Thr
Streptomyces albus G	**Arg**	Asn	Asp
Streptomyces lavendulae; Streptomyces fradiae	**Arg**	Asn	Asn
Lysobacter enzymogenes	**Arg**	Thr	Ser
Per-1/Per-2	**Arg**	Thr	Glu
Bacillus uniformis (cblA)	***Lys***	Asp	Asn
Bacillus fragilis (cepA)	**Arg**	Cys	Asn
Bacillus vulgatus (cfxA)	**Arg**	His	Ala
Sme-1/NmcA/IMI-1	**Arg**	Ala	Asp
Mycobacterium tuberculosis	**Arg**	Ala	Glu
Klebsiella oxytoca E23004; *Citrobacter diversus; Klebsiella oxytoca* D488; *Escherichia coli* Toho-1; *Proteus vulgaris; Serratia fonticola*	Ser	Thr	***Lys***
Yersinia enterocolitica	Thr	Thr	**Arg**
Escherichia coli MEN-1 (CTX-M-1); CTX-M-2; *Mycobacterium fortuitum*	Ser	Thr	**Arg**
*Streptomyces aureofaciens**	Thr	Ala	Asn

* In this enzyme, Arg-218 might correspond to that found at positions 220, 244 or 276 in all other class A β-lactamases.

8.2. Tryptophan-165

A Trp residue is found in position 165 of the TEM β-lactamases. It is situated on the Ω-loop, just next to Glu-166, but with its side-chain pointing to the surface of the protein. Of the four Trp residues in the TEM-1 enzyme, only Trp-165 can tolerate amino acid substitutions which maintain ampicillin resistance in *E. coli* [106]. The Trp-165 → Arg substitution was found to make little difference in the catalytic behaviour of the enzyme [138].

A Trp-165 → Arg mutation was found in IRT-10, together with Met-69 → Leu and Asn-276 → Asp. Although it may be expected not to play a major role in the resistance to inactivators, the replacement of Trp-165 by Arg decreases the sensitivity of the enzyme to inhibition by clavulanic acid, possibly due to subtle modifications in substrate positioning [138].

8.3. Arginine-244

An arginine residue is present at position 244 in 22 of the 46 class A sequences considered in this review (Table 6). The α-carbon of this residue is situated on the β-4 strand of the α/β domain (Figure 3C), thus rather remote from the active site. However, its long side-chain points in the direction of the cavity, and the guanidinium group of Arg-244 is actually positioned close to the side of the cavity formed by the adjacent β-3 strand containing the Lys-Thr(Ser)-Gly sequence (Figure 4).

Arg-244 is not conserved in class A enzymes (Table 6), but when it is absent, a basic residue (Arg or Lys) is found either at position 220 [139] or at position 276 [140]. A careful comparison

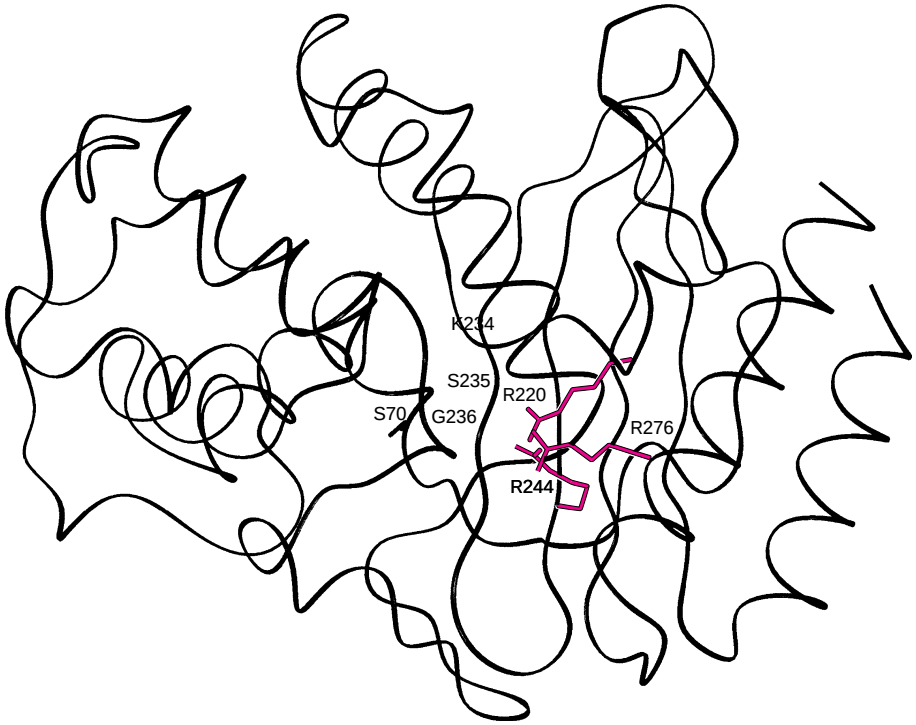

Figure 5 Assumed equivalent positions of the guanidinium groups of arginines 220, 244 and 276

The α-carbon trace and the Arg-244 side-chain are those of the TEM enzyme. The Arg-220 side-chain has been positioned as in the *S. albus* G β-lactamase crystal structure. The Arg-276 side chain is oriented in the same direction as that of Asn-276 in the TEM structure.

of the three-dimensional structures of the *S. albus* G (Arg-220; Asn-244) and of the *B. licheniformis* or TEM-1 (Leu-220; Arg-244) β-lactamases indicated that the guanidinium group of Arg-220 in the former lies in a position equivalent to that of Arg-244 in the latter ([139]; Figure 5). Similarly, Arg at position 276, which is structurally close to position 220 [54], can overlap with those at positions 220 or 244 ([140]; Figure 5). Interestingly, data in Table 6 indicates that a lysine residue can be found in place of an arginine both at positions 220 and 276. Conversely, no 'natural' enzyme has been identified to date with a Lys-244, but the artificial Arg-244 $\rightarrow$ Lys mutant of the TEM-1 β-lactamase was not significantly different from the wild-type enzyme [141], thus suggesting that a lysine residue could well be found at position 244 in presently non-sequenced enzymes.

Site-directed mutagenesis experiments indicated that an arginine (or a lysine) at position 220 or 244 is critical for the catalytic properties of the *S. albus* G [139] and TEM-1 [134,141] β-lactamases respectively, and the same is expected to hold true for Arg-276 in various enzymes [140]. A basic residue at position 220, 244 or 276 might, directly or indirectly, interact with the negatively charged carboxylate group of β-lactam substrates [48,53,64,65,134,139,141] and thus contribute to substrate binding at the ground and/or at the transition state.

Another major feature of Arg-244 is its implication in the mechanism of β-lactamase inactivation by suicide substrates. Indeed, in an effort towards the understanding of the details of the interaction of mechanism-based inactivators with the active-site serine β-lactamases, Mobashery and co-workers [64,65] reported that the Arg-244 $\rightarrow$ Ser mutant of the TEM-1 β-lactamase was resistant to inactivation by clavulanate (both *in vitro* and *in vivo*) and sulbactam. Similar results were also reported by Delaire et al. [134] who observed that replacement of Arg-244 by most other amino acids resulted in increased resistance to inactivation by clavulanate and sulbactam. As suggested for its interaction with the classical substrates, Arg-244 would be involved in effective binding of the inhibitor [64,65]. Additionally, together with the backbone carbonyl group of Val-216, the guanidinium of Arg-244 would serve as an 'electrostatic anchor' for a structurally conserved water molecule which would play a critical role in the inactivation mechanism [64]. This water molecule would be an essential source of proton in the mechanism of β-lactamase inactivation by both clavulanic acid [64] and carbapenem antibiotics [142]. Although Arg-244 is also probably involved in the inactivation chemistry of sulbactam [65], and possibly tazobactam, this is not as well documented as for clavulanate or imipenem.

Not surprisingly, among the naturally occurring TEM mutants recently identified, and which exhibit a decreased sensitivity to clavulanic acid, sulbactam and tazobactam, some were found with a mutation at position 244 (Table 5), thus confirming the alarming findings obtained with the corresponding laboratory mutants.

Importantly, recent site-directed mutagenesis experiments [143,144] and the isolation [145] of a new complex mutant of the TEM-1 enzyme (referred to as CMT-1 or TEM-50) both indicated that the combination of mutations resulting in inhibitor resistance (e.g. Met-69 $\rightarrow$ Leu and Asn-276 $\rightarrow$ Asp in TEM-50) and in ES activity (e.g. Glu-104 $\rightarrow$ Lys and Gly-238 $\rightarrow$ Ser in TEM-50) are largely incompatible. This finding is somewhat reassuring (perhaps temporarily) as it suggests that TEM variants conferring both activity against ES cephalosporins and resistance to β-lactam inactivators cannot be obtained by mere addition of mutations characteristic of each class of mutants (Tables 4 and 5). Thus it appears that it would be more efficient for an *E. coli*

strain to produce two different TEM mutants (an ES and an IRT mutant) simultaneously rather than to produce a single mutant combining mutations of both types [145]. However, this bacterial strategy would be efficient against third-generation cephalosporins and Augmentin (clavulanate + amoxycillin) taken individually, but not against a mixture of a third-generation cephalosporin and clavulanate.

Interestingly, an inhibitor-resistant β-lactamase (SHV-10) derived from an SHV-5 variant (SHV-9, Table 3) was recently reported [146]. In SHV-10, Ser-130 is replaced by Gly. In this case, it was observed that the enzyme partially retains its ability to hydrolyse penicillins, but its activity against cephalosporins, including cefotaxime and ceftazidime, is drastically reduced.

8.4. Residues 275 and 276

An arginine and an asparagine residue are present in the TEM enzymes at positions 275 and 276 respectively. To date, mutations at these two positions have never been observed alone (Table 5), and hence their role in resistance to suicide substrates seems questionable [130,131]. However, crystallographic data indicate that Asn-276, which is situated at the C-terminal extremity of the enzyme in the α/β domain (Figure 3C), restricts the mobility of the Arg-244 side-chain and contributes to maintaining active-site integrity [49,134]. Substitutions at this position might thus be expected to have an effect on the mechanism of resistance to suicide substrates similar to that of mutations at residue 244 [128,131]. Conversely, the role of mutations at position 275 is less clear.

Kinetic, electrospray MS and molecular-modelling studies of the Asn-276 → Asp mutant of TEM-1 [147] indicated that the mutation barely affected the catalytic efficiency of the enzyme (k_{cat}/K_m values reduced to 10–50 % of those of the wild-type) and confirmed the significance of an aspartic acid residue at position 276 in the resistance to inactivation by clavulanic acid. This seems to result from both a substantial loss of affinity for the inactivator (23-fold increase in K_i) and an increase of the partition ratio (2.5-fold increase in k_3/k_4), which represents the ratio of the number of productive turnovers to those leading to irreversible enzyme inactivation. The altered electrostatic interaction between residues at positions 244 and 276 and the possible displacement of the water molecule involved in the inactivation process (hydrogen-bonded to Val-216 and Arg-244, see above) would be responsible for the new catalytic properties of the mutant [147].

The Asn-276 → Gly mutant of the OHIO-1 β-lactamase [148] confirms the contribution of a mutation at position 276 in the resistance to clavulanic acid. However, the affinities for two penam sulphones (sulbactam and tazobactam) were found to increase, a result which contrasts with the data obtained with the Arg-244 → Ser mutant of the TEM-1 β-lactamase [65].

To close this section on IRTs, it is interesting to note that changes at residues 69, 244 and 276 all alter the binding of the substrate to the enzyme. Hence, it is probably not surprising that the natural variants containing changes at these positions (Table 5) are less sensitive to small β-lactams, such as clavulanic acid and sulbactam, which are devoid of an acyl side-chain (Figure 1; tazobactam, which bears a bulky triazole group on C-2, also lacks a substituent on C-6) and would therefore rely primarily on attractive interactions with the oxyanion hole and Arg-244 [110]. In agreement with that proposal, it was observed [139] with the Arg-220 → Leu mutant of the *S. albus* G β-lactamase (where Arg-220 corresponds to Arg-244 in the TEM β-lactamase, see above) that the most affected substrate was 6-aminopenicillanic acid ($k_{cat}/K_m = 2.5 \times 10^3\ M^{-1} \cdot s^{-1}$), which is devoid of acyl side-chain and is a good substrate of the wild-type enzyme ($k_{cat}/K_m = 3.7 \times 10^6\ M^{-1} \cdot s^{-1}$).

9. MORE EXTENDED-SPECTRUM β-LACTAMASES

Although the number of newly identified plasmidic β-lactamases of the TEM and SHV type (not considering the variants with decreased susceptibilities to suicide substrates) has decreased in the past few years [86], several new *bla* genes, coding for ES β-lactamases belonging to the four structural classes A, B, C and D, have emerged [23,149,150]. The *bla* genes for the new class A enzymes, which are located either on a chromosome or on a plasmid (or both in the case of PER-1) in Gram-negative bacteria, are too distant from the TEM- or SHV-type genes to represent direct mutational derivatives. Rather, these genes truly correspond to 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 [10]. These *bla* genes thus constitute 'reservoirs' of new β-lactamases with so far unknown variability, which embody a new generation of ES β-lactamases beyond the TEM and SHV variants [151].

Most of the recently reported enzymes are found in Table 7. Their sequences increase the already large number of reported sequences of class A β-lactamases. Table 1 indicates that addition of these new sequences dramatically reduces the number of residues that are strictly conserved in all the class A enzymes. In Table 7, which was adapted from previously published sequence alignments (for references, see Table 1), we focused the com-

Table 7 Class A extended-spectrum β-lactamases not belonging to the TEM and SHV families

The data are adapted from previously reported sequence alignments (references are included).

	Amino acid at position						
β-Lactamase	104	164	165†	237	238	240	References
ES TEM variants	Lys	Ser/His	Trp	Thr	Ser	Lys	[21]
P. vulgaris	Ala	Arg	Lys	Ser	Gly	Asp*	[140,158]
K. oxytoca	Val	Arg	Thr	Ala	Gly	Asp*	[140,158,164]
L. enzymogenes‡	Ser	Arg	Asn	Ser	Gly	?*	[158,201]
MEN-1/CTX-M-2§	Asn	Arg	Thr	Ser	Gly	Asp*	[140,158,161,176]
E. coli Toho-1	Asn	Arg	Thr	Ser	Gly	Asp*	[140]
S. fonticola	Asn	Arg	Thr	Ser	Gly	Asp*	[165]
C. diversus	Asn	Arg	Lys	Ala	Gly	Asp*	[140,158]
Y. enterocolitica‡	Ser	Arg	Trp	Ser	Gly	Asp*	[140,158,161,164]
B. uniformis (cblA)‡	Thr*	Glu	Thr	Ser	Ser	Gly*	[151,160]
B. fragilis (cepA)‡	Thr*	His	Thr	Thr	Gly	Gly*	[151,160]
B. vulgatus (cfxA)‡	Thr*	Tyr	Thr	Ser	Gly	Gly*	[160,161]
Per-1/Per-2‖	Thr*	Ala	Asn	Thr	Ser*	Gly*	[151,161]
M. fortuitum	Pro	Arg	Trp	Ser	Gly	Asp*	[140,202]
NmcA/IMI-1¶	Phe	Arg	Trp	Ser	Gly*	Ala*	[169,171]
Sme-1	Tyr	Arg	Trp	Ser	Gly*	Ala*	[169,171]

* Due to insertions or deletions in that region of the sequence, the residue corresponding to this particular position in the TEM sequences is uncertain.

† Residue (Trp) at position 165 in the TEM family is not involved in extended-spectrum properties (see the text).

‡ The catalytic properties of these enzymes are unclear. Nevertheless, *L. enzymogenes* was observed to significantly hydrolyse cefotaxime [203], and cfxA was reported to be responsible for cefoxitin resistance in *Bacteroides* species [25]. Conversely, *Y. enterocolitica* is apparently not an ES β-lactamase, as no hydrolysis could be detected after 2 h of incubation with either cefotaxime or cefoxitin [166].

§ MEN-1, also called CTX-M-1, is 84% identical with CTX-M-2 [176].

‖ 86.4% isology [151].

¶ 97% identity [171].

parison on the 23 strictly conserved residues observed by Ambler et al. [58] in their alignment of 20 class A β-lactamases. Table 1 clearly shows that, when the new sequences are included in the alignment (46 of the class A sequences reported to date have been considered here), the number of residues that are strictly conserved decreases to nine, the *Bacteroides* (cblA, cepA and cfxA) and the Per-1/Per-2 enzymes largely contributing to this increased diversity in the sequences. Additionally, important active-site residues at positions 132, 234 and 235 can also be regarded as highly conserved. Indeed, only conservative mutations have been identified at these positions, although a Gly is found at position 132 in the sequence of the *M. tuberculosis* enzyme (Table 1). A similar situation prevails for several residues remote from the active site, but probably playing an important structural role (e.g. residues at position 66 and 199).

Not surprisingly, the essential residues for catalysis [Ser-70, Lys-73, Ser-130, Glu-166 and Lys(Arg)-234; Figure 4] are strictly conserved in all class A β-lactamases. Furthermore, residues at positions 132 (Asn in 44 out of the 46 sequences analysed) and 235 (Ser or Thr) have been shown to be important for enzyme activity, especially towards cephalosporins [61,152–155]. Five residues (Gly-45, Pro-107, Asp-131, Ala-134 and Gly-236) are left, which are most probably conserved for structural reasons. Gly-45 is situated on the β-1 strand in the first hinge-region connecting the two structural domains, i.e. at the interface between these two domains, where no residue other than Gly could be sterically accommodated [49]. Pro-107 forms part of a distorted type I turn [49], and probably makes a critical contribution to the conformation of the polypeptide chain in this region [106]. Asp-131 is located on the short SDN loop between helices α-4 and α-5, which is nearly invariant in class A β-lactamases. Its side-chain is buried in the protein core and is involved in several hydrogen-bonds, probably ensuring the correct positioning of the SDN loop [49,153]. Ala-134 is situated in the hydrophobic core of the α-domain, whereas the strict conservation of Gly-236 in the superfamily of active-site serine penicillin-recognizing enzymes [42] can be attributed to purely steric factors [57].

Interestingly, in their systematic search (by random mutagenesis) for mutants of the TEM-1 β-lactamase conferring wild-type level of ampicillin resistance to *E. coli*, Huang et al. [106] observed that none of these 12 residues tolerated amino acid substitutions, with the sole exception of the residue at position 45, for which a mutant exhibiting an alanine residue was selected. As indicated by this experiment, and although the five residues which do not participate in the catalytic process were found to be strictly conserved in the 46 sequences analysed here (Table 1), it cannot be ruled out that new enzymes with mutations at these apparently important positions will emerge in the future. Indeed, several residues, which not long ago were regarded as being invariant among class A enzymes for important structural reasons (e.g. Glu-37, Gly-144, Arg-164 and Asp-233; Table 1), have been found to be non-conservatively substituted in some of the recently reported ES β-lactamases not belonging to the TEM and SHV families (Table 1). Remarkably, we note that two SHV derivatives (SHV-9 [156] and SHV-10 [146]) with a deletion at position 45 (Gly-45) have been reported recently.

Several attempts have been made to correlate the extended catalytic properties of these enzymes to their structural characteristics (see e.g. [132,157–159]). In Table 7, we compare the residues found to be critical for ES properties in the TEM variants (see section 7) with those occurring at corresponding positions in the sequences of the unrelated ES enzymes discussed here. It should be stressed that this comparison must be considered cautiously. Indeed, in the absence of three-dimensional structures for these β-lactamases (except for NmcA, see section 9.2), it cannot be ruled out that residues found at equivalent, or supposed equivalent, positions in the various sequences aligned according to Ambler et al. [58], might actually occupy substantially different positions in their respective active sites. Moreover, proper alignment of these numerous sequences is made difficult by the presence of insertions or deletions in several enzymes. For instance, the three enzymes from *Bacteroides* species (cb1A, cepA and cfxA) and the PER-1/2 enzymes possess additional amino acid residues at several positions compared with other class A β-lactamases, in particular downstream from the Lys-Thr-Gly triad, where 4–5 additional residues are found [160,161]. Thus the conclusions inferred from studies of the TEM variants, which largely rely on the X-ray structure of the wild type enzyme (TEM-1), do not necessarily apply to enzymes which generally show less than 50% sequence identity with TEM-1.

9.1. Hydroxylated residues at positions 165, 237 and 238

The most striking feature of most of the ES enzymes in Table 7 is the occurrence of a hydroxylated residue (Ser or Thr) at position 237. In class A β-lactamases, the side-chain (if any) of residue 237 (generally Ala or Gly) is on the outer, exposed side of the β-3 strand that forms the right edge of the binding site (Figure 3C). This residue is, however, part of the hydrogen-bond network that stabilizes binding of the substrate in the active site. Indeed, the main-chain NH group of residue 237 interacts with the oxygen of the β-lactam carbonyl, thus contributing to the formation of the oxyanion hole, and a second hydrogen-bond is formed between the main-chain carbonyl group of residue 237 and the amide NH group of the substrate side-chain [46,48,50].

A clear indication of the relevance of Thr at position 237 in the TEM variants came recently from a crystallographic mapping of complexes of cephalothin and cefotaxime with the structurally homologous *S.* R61 DD-peptidase [162]. In this enzyme, cefotaxime, with its branched rigid C-7 acyl side-chain, was found to be unable to form the hydrogen-bond with the backbone CO group at position 237 (301 in the DD-peptidase), which is normally observed with cephalothin. Instead, the 'tilted' cefotaxime binds to the side-chain OH group of Thr, which occupies this position in the DD-peptidase. By analogy, the substitution of Ala-237 by Thr in TEM-5 and TEM-24 was proposed to enhance the binding of β-lactams with oximino substituents and/or to help align the acyl-enzyme intermediate for attack by the hydrolytic water molecule [110,162]. Similarly, the *P. vulgaris* β-lactamase, which contains a Ser residue at position 237, showed significantly decreased activity towards oximino cephalosporins when Ser-237 was replaced by Ala using site-directed mutagenesis ([159]; M. Datz and J.-M. Frère, unpublished work; Table 8). Conversely, the mutation scarcely affected the activity towards the other substrates tested (including aztreonam, Table 8), a slight increase in catalytic efficiency being generally observed, even with first generation cephalosporins (cephaloridine, cephalothin and cefazolin). Further comparison of the kinetic parameter values of the various enzymes in Table 8, some of which are devoid of Ser (or any other hydrogen-bond donor) at position 237, supports the idea [158,163–165] that Ser-237 (or Thr-237) contributes to the ability of ES β-lactamases (e.g. *P. vulgaris*, MEN-1, Toho-1, *S. fonticola* and Per-1) to hydrolyse oximino β-lactams (although this is less clear for aztreonam). However, it also suggests that some other residues are responsible for the high catalytic activity of these enzymes towards these particular compounds and cephalosporins in general. In that respect, it is

Table 8 Comparison of the k_{cat}/K_m values ($mM^{-1} \cdot s^{-1}$) for the hydrolysis of some characteristic β-lactams by various class A ES β-lactamases

The values in parentheses are relative to those for benzylpenicillin arbitrarily fixed at 100. For Per-1, only relative values were reported. The data are from Reynaud et al. [157] (*K. oxytoca* D488), Barthélémy et al. [164] (*K. oxytoca* D488 and MEN-1), M. Datz and J.-M. Frère (unpublished work) (*Proteus vulgaris CumA*), Ishii et al. [140] (Toho-1), Péduzzi et al. [165] (*S. fonticola*), Nordmann et al. [204] (PER-1) and Amicosante et al. [205] (*C. diversus*). ND, not determined. (—) no detectable hydrolytic activity.

Compound	*C. diversus* Ala-237	*K. oxytoca* Ala-237	*P. vulgaris* mutant S237A	*P. vulgaris* Ser-237	MEN-1 Ser-237	Toho-1 Ser-237	*S. fonticola* Ser-237	PER-1 Thr-237
Benzylpenicillin	8300 (100)	33 000 (100)	700 (100)	640 (100)	17 000 (100)	11 000 (100)	33 000 (100)	(100)
Ampicillin	5400 (65)	23 000 (68)	600 (86)	450 (71)	8700 (50)	5700 (51)	ND	ND
Cephaloridine	1250 (15)	5800 (17)	1500 (210)	1100 (170)	ND	1500 (13)	14 000 (43)	(56)
Cephalothin	1300 (16)	5000 (15)	3200 (460)	1700 (270)	21 300 (120)	630 (5)	41 000 (120)	(170)
Cefazolin	8300 (100)	ND	3200 (460)	2700 (430)	ND	ND	ND	ND
Cefuroxime	770 (9)	650 (1.9)	360 (51)	1300 (200)	2000 (12)	ND	27 000 (81)	(93)
Cefotaxime	ND	110 (0.3)	120 (17)	470 (74)	2500 (15)	1900 (17)	11 000 (34)	(150)
Ceftazidime	ND	(—)	1.9 (0.3)	10 (1.6)	20 (0.1)	430 (4)	60 (0.2)	(42)
Aztreonam	(—)	100 (0.3)	150 (21)	160 (25)	ND	91 (0.9)	930 (3)	(0.1)

interesting to note that the Ala-237 → Thr mutation has been found only in two ES β-lactamases of the TEM family (TEM-5 and TEM-24), and, in both cases, it was observed to occur in combination with at least two other mutations important for extended activity (Table 4). It is also noteworthy that no hydrolysis of third-generation cephalosporins could be observed with the β-lactamase of *Y. enterocolitica* [166], which harbours a Ser residue at position 237 and displays a high level of similarity to the *K. oxytoca* (> 70% identity) and MEN-1 (~ 60% identity) enzymes [164]. This observation clearly indicates that a Ser residue at position 237 is not the only requirement for hydrolytic activity against oximino cephalosporins and, again, it draws attention to the subtle differences that exist between the active sites of the various class A enzymes.

Table 8 indicates that *C. diversus* (Ala-237), *K. oxytoca* (Ala-237) and the Ser-237 → Ala mutant of *P. vulgaris* are very efficient enzymes against first-generation cephalosporins ($k_{cat}/K_m > 10^6\ M^{-1} \cdot s^{-1}$), and also show quite good activities ($k_{cat}/K_m > 10^5\ M^{-1} \cdot s^{-1}$) towards second- (cefuroxime) and even third-generation (cefotaxime) cephalosporins. Interestingly, the activity of the *K. oxytoca* enzyme towards cefotaxime has been attributed to Thr-165 [157]. It has been suggested that the hydroxyl group of that residue might interact with the oxime substituant of cefotaxime through the formation of a new hydrogen-bond. Remarkably, it can be seen in Table 7 that a potential hydrogen-bond donor (Thr, Asn or Lys) is found at position 165 in most ES β-lactamases, whereas a hydrophobic residue (Trp, Phe, Tyr, Ile, Val or Leu) is found in most other class A enzymes.

Finally, for the Per-1/2 β-lactamases and the *cfxA* gene product, the strategy is particularly reminiscent of that in the TEM family [99]. Indeed, next to a hydroxylated residue (Ser or Thr) at position 237, residue 238 is a serine in Per-1/2 (also in cb1A, whose catalytic properties remain unknown) and residues 164 (Tyr or Ala) and 179 (Asn) are both neutral, thus precluding the possibility of salt-bridge formation between them.

9.2. Carbapenem-hydrolysing β-lactamases

All the ES β-lactamases described so far, belonging or not to the TEM and SHV families, have been found to hydrolyse imipenem [Figure 1F, with $R_1 = CH(CH_3)OH$ and $R_2 = S{-}(CH_2)_2{-}NH{-}CH{=}NH_2^+$] at very slow rates. Accordingly, most pathogenic bacterial strains producing these enzymes, and hence resistant to ES cephalosporins, are susceptible to imipenem. The high resistance of imipenem and related carbapenem antibiotics (e.g. meropenem) to most β-lactamases, combined with their broad antibacterial activity, make these compounds very efficient antibiotics, often used in the last resort for patients in intensive-care units. However, β-lactamase-mediated resistance to carbapenems has recently been reported. These carbapenem-hydrolysing enzymes ('carbapenemases') belong to two major groups. The first group corresponds to class B metallo-β-lactamases [32,33,167], which require a Zn^{2+} ion for their activity, whereas the second one consists (so far) of three class A β-lactamases from *Enterobacter cloacae* (NmcA and IMI-1) and *Serratia marcescens* (Sme-1), sharing about 70% sequence [168–171].

Kinetic studies performed with the class A carbapenemases (Table 2) showed that they 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.

Molecular modelling analysis performed with TEM-1 provided some clues to the structural determinants of the high stability of imipenem towards class A β-lactamases [132,172]. These studies suggested that the relatively low acylation rate (~ $10^3\ M^{-1} \cdot s^{-1}$) observed with imipenem can be explained by a significantly decreased interaction between the β-lactam carbonyl and the oxyanion hole [132]. Energy-minimized structures for the two acyl-enzyme intermediates (Δ^1- and Δ^2-tautomers) indicated that the hydroxyl group of the C-6-α hydroxyethyl moiety of imipenem makes a strong hydrogen-bond to the side-chain of Asn-132, hence orienting the C-6-α side-chain in a position that displaces the hydrolytic water molecule from its optimal position [172]. This structural factor was proposed to be the basis for the slow turnover ($k_{cat} \ll 1\ s^{-1}$) of imipenem by the TEM-1 enzyme [172] and was judiciously exploited for the design of a new mechanism-based inactivator, 6-α-(hydroxymethyl)penicillanate, of the same enzyme [173]. This molecule, which is a simple structural variant of penicillanic acid (i.e. a relatively poor substrate of TEM-1 and various class A β-lactamases; Table 2), harbours a C-6-α hydroxymethyl side-chain resembling that of imipenem and it behaves, in fact, as a potent inactivator of the TEM-1 β-lactamase, with turnover values very similar to those measured for imipenem ($k_{cat} \approx 0.03$–$0.04\ s^{-1}$ and $K_m \approx 25\ \mu M$; [173]). Interestingly, energy-minimized structures of both the Henri–Michaelis and the acyl-enzyme intermediates for 6-α-(hydroxymethyl)penicillanate in the active site of the TEM-1 β-lactamase indicated a significant displacement of the catalytic

water molecule [173], which was later confirmed by the crystal structure of this acyl-enzyme [103]. As with imipenem, this is likely to explain the retarded rate of hydrolysis of the acyl-enzyme complex [173], but it might also account for the decreased rate of acylation when compared with penicillanic acid (Table 2). A similar displacement of the catalytic water molecule (W1) in the Henri–Michaelis complex was proposed to be responsible for the slow rate of acylation observed with imipenem and the *S. albus* G β-lactamase ([83]; Table 2). Again, this effect on both the acylation and deacylation rates is similar to that observed with cefoxitin and moxalactam (section 7) and with other β-lactamase-stable antibiotics (see above). Consistent with several studies of Glu-166 mutants (section 6), the effect observed here with imipenem and 6-α-(hydroxymethyl)penicillanate seems more critical for deacylation than for acylation, which can still proceed at significant rates (10^2–10^4 $M^{-1} \cdot s^{-1}$; Table 2).

In the class A carbapenemases, the presence of a disulphide bridge between Cys-69 and Cys-238, which creates a new covalent bond between the two domains, significantly modifies the position of the β-3 strand and, consequently, the active-site geometry [55,132]. Compared with TEM-1, the distance between the C-α positions of residues 69 and 238 is reduced by 1.3 Å in NmcA [55]. Docking of imipenem in the three-dimensional models of NmcA and Sme-1 [132] suggested that this unique structural feature might be a major determinant in the characteristic substrate profiles of these β-lactamases. Indeed, in the modelled structures, the carbonyl group of imipenem is found to be closer to the backbone NHs of residues 237 and 70 than in TEM-1, which significantly improves the interaction with the oxyanion hole.

Another major characteristic of these enzymes is the altered position of Asn-132 [due to an insertion after residue 140 (Arg-140a)], which is shifted away from the β-3 strand by 1.2 Å when compared with TEM-1 [55]. This residue has been proposed, both on the basis of crystallographic data [46,53] and of site-directed mutagenesis and molecular-modelling experiments [62,152,153,174], to act as a hydrogen-bond donor to the carbonyl group of the antibiotic side-chain, and to be involved both in the binding of the substrate and in the catalytic mechanism. Molecular modelling and kinetic analysis indicated that, in NmcA, there is no interaction between the oxygen atom of the 6-β-acylamido group of benzylpenicillin and Asn-132, which would be consistent with the 10-fold decrease in k_{cat}/K_m observed for this substrate by NmcA when compared with TEM-1 (P. Swarén, J.-M. Frère and J. P. Samama, unpublished work, Table 2). But most importantly, imipenem was proposed to be better accommodated in the enzyme active site, where its more mobile 6-α-hydroxyethyl substituent, which is at non-binding distance from Asn-132 (P. Swarén, J.-M. Frère and J. P. Samama, unpublished work), probably allows proper positioning of the catalytic water molecule for efficient catalysis. Similarly, NmcA exhibits a fairly good catalytic efficiency towards 6-α-(hydroxymethyl)penicillanate, with a k_{cat}/K_m value about 100-fold higher than that measured with TEM-1 (Table 2).

In addition to their high carbapenemase activity ($k_{cat} \approx$ 10–10^3 s^{-1} for imipenem and meropenem, which is 10^3–10^5-fold higher than for other class A β-lactamases ([132,171,175]; Table 2), another striking feature of NmcA, Sme-1 and IMI-1 is their ability to hydrolyse compounds bearing a methoxy side-chain on the α-face of the β-lactam ring (e.g. cefoxitin and moxalactam, Table 2). As mentioned above (section 7), kinetic and modelling studies performed with various class A enzymes [72] suggested that the very low acylation (Table 2) and deacylation ($k_3 = k_{cat} < 0.01$ s^{-1}) rates measured with these compounds are due to a major displacement of the catalytic water molecule, caused by the α-methoxy group of the substrate. Molecular modelling with imipenem and cefoxitin indicated that the 6-α-hydroxyethyl substituent of imipenem and the 7-α-methoxy group of cefoxitin adopt similar positions in the active site of NmcA [175], which could explain the hydrolytic activity of this enzyme on (oxa)-cephamycins. Table 2 indicates that these enzymes also inactivate third-generation antibiotics, with a remarkably high efficiency for aztreonam. We note that the three class A carbapenemases exhibit a hydroxylated residue at position 237 (Table 7), as found in most ES β-lactamases (see above).

Finally, on the basis of a sequence alignment [167], a few additional positions were identified which might contribute to the carbapenemase activity of these three enzymes, namely 104, 105, 207 and 244. In particular, the absence of arginine at position 244, which would serve as an 'electrostatic anchor' for a water molecule involved in the inactivation mechanism by imipenem (section 8.3.), was suggested to be an important factor [167]. However, Arg-220 in the three class A carbapenemases is likely to play the same role as proposed for Arg-244 in TEM-1 (see section 8.3 and Table 6).

10. CONCLUSIONS

The tertiary structures of class A β-lactamases which have been determined to date exhibit a high degree of similarity, which contrasts with the diversity of the primary structures and the spectacular variations of the specificity profiles. Since the latter can also be profoundly modified by a limited number of mutations, bacteria producing adequate enzymes are easily selected by the intensive utilization of any given antibiotic.

Future research directions should try to counter the genetic fluidity of bacteria, which is the cornerstone of their adaptability. Since one can expect that new β-lactamases will emerge in answer to the introduction of new β-lactams, it would probably be interesting to try to synthesize non-β-lactam inhibitors or inactivators of the DD-transpeptidases, which remain choice targets for antibacterial drugs. Similarly, non-β-lactam inactivators of β-lactamases, which would completely escape hydrolysis, might be quite useful in combined chemotherapies.

Note added in proof (received 20 January 1998)

Recently, active efflux pumps have also been implicated in β-lactam resistance, most markedly in *Pseudomonas aeruginosa* [206].

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