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Commentary

Metallo-β-lactamases (classification, activity, genetic organization, structure, zinc coordination) and their superfamily

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ABSTRACT

One strategy employed by bacterial strains to resist β-lactam antibiotics is the expression of metallo-β-lactamases requiring Zn^{2+} for activity. In the last few years, many new zinc β-lactamases have been described and several pathogens are now known to synthesize members of this class. Metallo-β-lactamases are especially worrisome due to: (1) their broad activity profiles that encompass most β-lactam antibiotics, including the carbapenems; (2) potential for horizontal transference; and (3) the absence of clinically useful inhibitors. On the basis of the known sequences, three different lineages, identified as subclasses B1, B2, and B3 have been characterized. The three-dimensional structure of at least one metallo-β-lactamase of each subclass has been solved. These very similar 3D structures are characterized by the presence of an αββα-fold.

In addition to metallo-β-lactamases which cleave the amide bond of the β-lactam ring, the metallo-β-lactamase superfamily includes enzymes which hydrolyze thiol-ester, phosphodiester and sulfuric ester bonds as well as oxydoreductases. Most of the 6000 members of this superfamily share five conserved motifs, the most characteristic being the His116-X-His118-X-Asp120-His121 signature. They all exhibit an αββα-fold, similar to that found in the structure of zinc β-lactamases. Many members of this superfamily are involved in mRNA maturation and DNA reparation. This fact suggests the hypothesis that metallo-β-lactamases may be the result of divergent evolution starting from an ancestral protein which did not have a β-lactamase activity.

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1. β-Lactams

The β-lactam antibiotics comprise six different structural subtypes, including penams, cephems, monobactams, clavams, penems, and carbapenems. The penams include benzylpenicillin and ampicillin. The cephems include classical cephalosporins such as cephaloridine, nitrocefin, and cefotaxime, as well as cephamycins, which are 7-α-methoxy-cephalosporins. The monobactams are monocyclic β-lactams and include aztreonam. The penems have a 2, 3-double bond

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Abbreviations: MBL, metallo-β-lactamase; BBL, class B β-lactamase; ISCR, insertion sequences common regions

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in the fused thiazolidine ring (hence dihydrothiazole), similar to the carbapenems (e.g. imipenem, biapenem), which also have an unsaturated fused five membered ring, with carbon in place of sulfur at the 1-position.

2. Metallo-β-lactamases

β-Lactamases are the major cause of resistance of bacteria to β-lactam antibiotics. These enzymes cleave the amide bond of the β-lactam ring thus inactivating the antibiotic. β-Lactamases fall into four classes. Classes A, C and D are serine-β-lactamases which employ an active-site serine to catalyze hydrolysis, while metallo-β-lactamases or class B β-lactamases are metallo-enzymes requiring one or two zinc ions for their activity. Metallo-β-lactamases can degrade all classes of β-lactams except monobactams and are special for their constant and efficient carbapenemase activity. This is a most worrisome characteristic because carbapenems, which are stable against the vast majority of serine-β-lactamases produced by resistant pathogens, are the antibiotics with the broadest spectrum of activity. Moreover, metallo-β-lactamases are not susceptible to therapeutic β-lactamase inhibitors. The first of these enzymes, the *Bacillus cereus* metallo-β-lactamase, was identified in 1966 when Sabbath and Abraham showed that the cephalosporinase activity produced by a *B. cereus* isolate was inhibited by EDTA [1]. During the two following decades, it was the only known example of metallo-β-lactamase and was merely considered as a biochemical curiosity. The situation changed after 1980. Indeed, zinc β-lactamases have been discovered in an increasing number of nosocomial strains such as *Bacteroides fragilis* [2], *Pseudomonas aeruginosa* [3], *Aeromonas hydrophila* [4], *Serratia marcescens* [5], *Elizabethkingia meningoseptica* (formely *Chryseobacterium meningosepticum*) [6] and a silent gene coding for a metallo-β-lactamase was discovered in *Bacillus anthracis* [7]. The emergence and dissemination of acquired metallo-β-lactamases, encoded by genes carried on mobile DNA elements, among major Gram-negative pathogens, including members of the family Enterobacteriaceae, *P. aeruginosa*, and *Acinetobacter* species made the situation even more worrying [8]. Moreover, many metallo-β-lactamase genes are present in environmental species which thus constitute reservoirs of β-lactam resistance genes [9–12]. Metallo-β-lactamases are now regarded as a therapeutic challenge and the comprehension of their frightening properties is necessary in the fight to overcome them.

Table 1 – Metallo-β-lactamases

Subclass	Enzyme	Strain	Discovery in	GenBank accession no.	Accession no. (protein)	Structure
B1	BcII	*B. cereus*	1966	M11189	AAA22276	Mono-zinc [48], Di-zinc [50], Apo-form [50]
	CcrA	*B. fragilis*	1990	M63556	AAA22904	Di-zinc [49]
	BlaB	*E. meningoseptica*	1998	AF189298	O08498	Di-zinc [52]
	IND-1	*Chryseobacterium indologenes*	1999	EF394437	ABO21411	
	EBR-1	*E. brevis*	2002	AF416700	AAN32638	
	SFB-1	*Shewanella frigidimarina*	2005	AY590119	AAT90847	
	SLB-1	*Shewanella livingstonensis*	2005	AY590118	AAT90846	
Acquired-B1	IMP-1	*S. marcescens*, *P. aeruginosa*	1994	S71932, AY168635	AAB30289, AAN87168	Di-zinc[51]
	VIM-1	*P. aeruginosa*, *A. baumannii*	1999	Y18050	CAE46717	
	VIM-2	*P. aeruginosa*, *A. baumannii*	2000	AF191564	AAK26253	Mono-zinc 1KO2, Di-zinc 1KO3
	IMP-2	*A. baumannii*, *S. marcescens*	2000	AB182996	BAD26594	
	SPM-1	*P. aeruginosa*	2002	AY341249	AAR15341	Mono-zinc [53]
	VIM-4	*P. aeruginosa*, *K. pneumoniae*, *Enterobacter cloacae*	2003	AY135661	AAR22402	
	GIM-1	*P. aeruginosa*	2004	AJ620678	CAF05908	
	SIM-1	*A. baumannii*	2005	AY887066	AAX76774	
B2	CphA	*A. hydrophila*	1991	X57102	P26918	Mono-zinc [36]
	ImiS	*Aeromonas veronii*	1996	Y01415	CAA71411	
	Sfh-1	*S. fonticola*	2003	AF197943	AAF09244	
B3	L1	*Stenotrophomonas maltophilia*	1991	AB294542	CAB75346	Di-zinc [22], Mono-zinc 2H6A, Apo-form 2FU6
	GOB-1	*E. meningoseptica*	2000	AF090141	ABO21417	
	FEZ-1	*L. gormanii*	2000	Y17896	CAB96921	Di-zinc [54]
	THIN-B	*J. lividum*	2001	AJ250876	CAC33832	
	Mbl1b	*C. crescentus*	2001	AJ315850	CAC48262	
	CAU-1	*Caulobacter vibrioides*	2002	AJ308331	CAC87665	
	BJP-1	*B. japonicum*	2006		NP_772870	Di-zinc 2GMN

They are separated into the three subclasses and classified by year of discovery. All the variants are not included in this table.

Table 2 – Minimal Inhibitory Concentrations (μg/ml) for *E.coli* strains producing some metallo-β-lactamases

	E. coli DH10B (reference)	*E. coli* DH10B (pBlaB-1) (B1)	*E. coli* DH10B (pEBR-1) (B1)	*E. coli* DH10B (pSLB-1) (B1)	*E. coli* DH10B (pSFB-1) (B1)	*E. coli* DH10B (pVIM-2) (acquired-B1)	*E. coli* DH10B (pGOB-1) (B3)	*E. coli* DH5α (Ref.)	*E. coli* DH5α (pGIM-1) (acquired-B1)	*E. coli* DH5α (pFEZ-1) (B3)	*E. coli* XL-1 (Ref.)	*E. coli* XL-1 (pSIM-1) (acquired-B1)
Amoxicillin	2	128	>512	512	16	>512	64	–	–	–	–	–
Amoxicillin-CLA	2	–	>512	512	16	512	–	–	–	–	–	–
Ticarcillin	2	256	>512	512	16	> 512	64	–	–	–	4	>128
Piperacillin	1	4	8	4	1	4	2	1	16	1	1	1
Cephalothin	2	16	32	8	4	256	32	4	–	32	4	>128
Cefuroxime	4	–	128	32	2	–	–	4	–	32	4	>128
Ceftazidime	0.06	–	0.5	32	0.12	16	–	0.12	256	0.5	0.12	32
Cefotaxime	0.06	0.12	0.5	0.5	0.12	8	0.25	0.06	–	4	≤0.06	32
Cefepime	0.06	0.03	0.12	0.06	0.06	0.06	0.06	0.06	4	–	≤0.06	2
Cefpirome	0.06	0.06	0.12	–	–	–	0.5	–	–	–	–	–
Ceftriaxone	0.06	–	0.25	–	–	–	–	0.125	128	–	≤0.06	32
Cefoxitine	4	2	8	4	4	128	16	2	–	32	2	>128
Moxalactam	0.12	0.12	1	0.06	0.06	–	1	–	–	–	–	–
Aztreonam	0.12	0.25	0.12	0.06	0.12	0.12	0.25	0.12	0.125	0.12	0.25	0.25
Imipenem	0.06	0.5	0.5	0.5	0.25	1	0.5	0.12	0.5	0.25	0.12	1
Meropenem	0.06	0.12	0.25	0.12	0.25	0.25	0.12	0.03	0.125	0.25	≤0.06	2
	[15]	[20]	[15]	[25]	[25]	[40]	[20]	[21]	[41]	[21]	[42]	[42]

–, not determined.

3. Classification

The class B β-lactamases are classified into three subclasses B1, B2 and B3 on the basis of sequence alignments [13]. The classification task was complicated by the generally low degree of similarity between subclass sequences but facilitated by the availability of X-ray structures (see below), which allowed the identification of corresponding secondary structures elements, even when the sequence similarity was not obvious. The standard BBL numbering of class B β-lactamases allows easy comparisons of the sequences of these enzymes [13].

Subclass B1 enzymes share more than 23% identity. These enzymes include the prototypical BcII from *B. cereus* [14], CcrA from *B. fragilis* [2], BlaB from *E. meningoseptica* [6] and EBR-1 from *Empedobacter brevis* [15]. The acquired IMP-type MBLs found in some clinical isolates of *P. aeruginosa* [16], *S. marcescens* [5], *Klebsiella pneumoniae* (GenBank EMBL accession no D29636) and *Acinetobacter baumannii* [8], as the VIM-type or SPM-1 enzymes produced by clinical isolates of *P. aeruginosa* [8,17], belong also to this subclass (Table 1).

The members of subclass B2 show only 11% of identity with subclass B1 members. This subclass includes the enzymes produced by different species of *Aeromonas* (the most studied are CphA produced by *A. hydrophila* [4] and ImiS produced by *A. veronii* [18]) as well as the Sfh-I enzyme produced by *Serratia fonticola* [9] (Table 1).

Subclass B3 metallo-β-lactamases have only nine conserved residues when compared with the other metallo-β-lactamases. Subclass B3 includes the L1 and GOB-1 metallo-β-lactamases produced by clinical strains of *S. maltophilia* [19] and *E. meningoseptica* (formely *C. meningosepticum*) [20] and FEZ-1 isolated from *Legionnella gormanii* [21]. To date, 18 variants of GOB-1 have been reported, but none outside the *E. meningoseptica* species. In GOB enzymes, His116 is replaced by a glutamine. THIN-B [10], Mbl1b [11], and BJP-1 [12] produced by environmental bacteria (*Janthinobacter lividum*, *Caulobacter crescentus* and *Bradyrhizobium japonicum*, respectively) belong also to this subclass (Table 1). With the exception of L1 which is a homo-tetramer [22], all these enzymes are monomeric as are the B1 and B2 enzymes.

4. MICs

Compared to the reference *Escherichia coli* strains, strains producing a metallo-β-lactamase exhibit decreased susceptibility to a broad array of β-lactam antibiotics, including penicillins, narrow- and expanded-spectrum cephalosporins, and carbapenems (Table 2), indicating that metallo-β-lactamases have a broad overall substrate specificity profile. Only the MICs of aztreonam are unchanged, suggesting that theses enzymes are not active against this compound. It has also been observed that *E. coli* expressing the *cphA* gene is resistant only to carbapenems and only when high inocula (10^8 CFU) are used for MIC determinations [23]. However, a rational approach of the correlation between the MICs and the kinetic parameters of the β-lactamase is made difficult by the absence of knowledge of outer membrane permeability for many of the usually studied β-lactam antibiotics and by the fact that most authors do not estimate the amount of enzyme produced.

Table 3 – Kinetics parameters of some metallo-β-lactamases

	Benzylpenicillin			Nitrocefin			Imipenem			
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($M^{-1}\,s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($M^{-1}\,s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($M^{-1}\,s^{-1}$)	
B1										
BcII	680	1500	4.5×10^5	45	70	6.4×10^5	>100	>1000	1×10^5	[24]
CcrA	190	40	4.8×10^6	200	20	1×10^7	200	270	7.4×10^5	[24]
EBR-1	120	50	2.4×10^6	–	–	–	190	780	2.4×10^5	[15]
BlaB	280	30	9×10^6	20	70	2.8×10^5	350	370	9.5×10^5	[6]
Acquired-B1										
IMP-1	320	520	6.2×10^5	60	30	2×10^6	50	40	1.2×10^6	[16]
VIM-1	30	840	3.5×10^4	100	20	5×10^6	2	2	1×10^6	[8]
VIM-2	60	50	1.2×10^6	–	–	–	10	10	1×10^6	[8]
SPM-1	110	40	2.8×10^6	0.50	4	1.2×10^5	30	40	7.5×10^5	[53]
GIM-1	7	50	1.4×10^5	6	10	6×10^5	30	290	1×10^5	[41]
B2										
CphA	0.03	870	35	0.003	1200	2.5	1200	340	3.5×10^6	[26]
CphA N116H	0.4	910	440	0.09	30	3×10^3	150	1400	1.1×10^5	[26]
CphA N116H-N220G	3	150	2×10^4	0.7	4	1.8×10^5	20	190	1×10^5	[27]
ImiS	0.50	250	2×10^3	0.06	20	3×10^3	160	180	9×10^5	[18]
B3										
L1	1100	50	2×10^7	20	10	2×10^6	65	90	7.2×10^5	[24]
FEZ-1	70	590	1.2×10^5	90	100	9×10^5	>200	>1000	2×10^5	[88]
GOB-1	200	110	1.8×10^6	–	–	–	40	60	6.6×10^5	[20]
Mbl1b	770	20	3.8×10^7	1800	100	1.8×10^7	940	200	4.7×10^6	[11]

–, not determined.

5. Activity profiles

The metallo-β-lactamases belonging to subclasses B1 and B3 are broad spectrum enzymes and hydrolyze most β-lactam antibiotics including carbapenems [24] (Table 3). Only the monobactams are neither hydrolyzed nor recognized by these enzymes which are not inhibited by classical inhibitors of serine-active β-lactamases such as clavulanic acid and tazobactam. These compounds in fact behave as poor substrates. SFB-1 from the psychrophilic *Shewanella frigidimaria* is an unusual subclass B1 β-lactamase which does not significantly hydrolyze benzylpenicillin, ticarcillin, third generation cephalosporins and meropenem [25].

The subclass B2 enzymes are strict carbapenemases [18,23,24]. They only hydrolyze carbapenems efficiently and show a very weak activity, if any, towards penicillins and cephalosporins (Table 3). In B2 enzymes, the conserved His116 of B1 and B3 enzymes, is replaced by an asparagine. The presence of this asparagine in position 116 is far from being the only reason which explains the narrow spectrum of B2 metallo-β-lactamases, since, although increased, the activity of the N116H CphA mutant towards penicillins and cephalosporins remains low [26]. A mutant of CphA with a considerably broadened activity spectrum, N116H-N220G, was recently obtained by site-directed mutagenesis [27]. In contrast to the wild-type enzyme, it is able to efficiently hydrolyze penicillins and cephalosporins in addition to carbapenems although with a reduced efficiency towards the latter (Table 3).

6. Phylogeny

Subclasses B1 and B2 are descended from a common ancestor and really form a single group within which the sequences exhibit significant similarities, with subclasses B1 and B2 forming two distinct clades within the group. Subclass B3 shares structural but not sequence similarities with the B1/B2 group [28]. A phylogenetic tree with representative members of all the three subclass is given in the previous Ref. [28]. Hall and Barlow have proposed, on this basis, to revise the classification of metallo-β-lactamases [29]. However, the presently accepted classification reflects other properties of these enzymes. Functional and mechanistic factors clearly distinguish the B1, B2 and B3 enzymes from each other and, on this basis, B2 is not more related to B1 than B3 (see activity profile, zinc coordination points). Thus it seems unnecessary to modify the broadly accepted nomenclature [30].

7. Catalytic mechanism

Bounaga et al. (1998) [31] have proposed a mechanism for the monozinc form of the subclass B1 BcII enzyme. In this mechanism, the zinc ion interacts with His116, His118, His196 and a water molecule. The zinc ion behaves as a Lewis acid and decreases the pK of the water molecule so that it exists as a hydroxide ion at neutral pH. This ion performs a nucleophilic attack on the carbon of the carboxyl group of the β-lactam, leading to the formation of a tetrahedral intermediate stabilized by its interaction with the zinc ion. Asp120, acting as a general base, deprotonates the –OH group to generate a dianionic tetrahedral second intermediate which is also stabilized by the zinc ion. In a subsequent step, Asp120 gives the proton to the nitrogen of the β-lactam ring and takes part in cycle opening.

A mechanism based on the study of the hydrolysis of nitrocefin (a chromogenic cephalosporin) by the CcrA and L1 enzymes has been proposed for the dizinc form of metallo-β-lactamases. A ring-opened intermediate containing an anionic nitrogen was identified, the protonation of which was rate-limiting [32-34]. But the mechanism observed for nitrocefin seems to be atypical, due to its highly conjugated bis-nitro-substituted styryl substituent which is ideally suited to stabilize the negatively charged nitrogen. When other substrates are used, the cleavage of the tetrahedral intermediate, which occurs concomitantly to the protonation of the nitrogen of the β-lactam ring, appears to be the rate-limiting step [35].

A different reaction mechanism has been proposed for the monozinc subclass B2 enzymes [36,37]. The nucleophilic hydroxyde is generated by the Asp120 and His118 residues. This nucleophile attacks the β-lactam carbonyl, possibly activated by His196, to generate a tetrahedral intermediate. As observed for B1 and B3 enzymes, the rate-limiting step in the reaction is the C–N bond cleavage. The crystal structure of CphA shows the presence of a bicyclic product of biapenem in the active site of the enzyme [36]. Garau et al. proposed that, after β-lactam bond cleavage, a substantial bond rotation occurs that allows the nucleophilic attack of C3 by the oxygen of the 6-hydroxyethyl substituent with concomitant transfer of its proton to C2. This cyclization occurs after C–N bond cleavage. Some results indicate that the final product of imipenem hydrolysis by ImiS is different from that by L1 but the NMR spectra of both products are the same [37].

A very recent study of D120N, D120E, D120Q and D120S BcII mutants indicates that Asp120 is not the proton donor, nor does it play an essential role in nucleophilic activation. It is proposed that the role of Asp120 is to act as a strong Zn2 ligand, locating this ion optimally for substrate binding, stabilization of the development of a partial negative charge in the β-lactam nitrogen, and protonation of this atom by a zinc-bound water molecule [38].

8. Genetic organization

Some members of subclass B1 are encoded by the chromosome such as BcII from *B. cereus* [14] and BlaB from *E. meningoseptica* [6]. The gene coding for CcrA (*B. fragilis*) is often quiescent and requires a surrogate sequence to provide an adequate promoter. Insertion elements, such as IS*942*, IS*1186* and IS*4351*, have been shown to lie immediately upstream of the ribosome-binding site, providing enhanced transcriptional capabilities for the CcrA gene [39].

Five plasmid-borne sets of transferable B1 metallo-β-lactamase genes have presently been identified of which the IMP- and VIM-types occur most frequently (Table 1). The IMP-type enzymes, produced by *P. aeruginosa* [16], *S. marcescens* [5], *K. pneumoniae* (GenBank accession no. D29636), have now

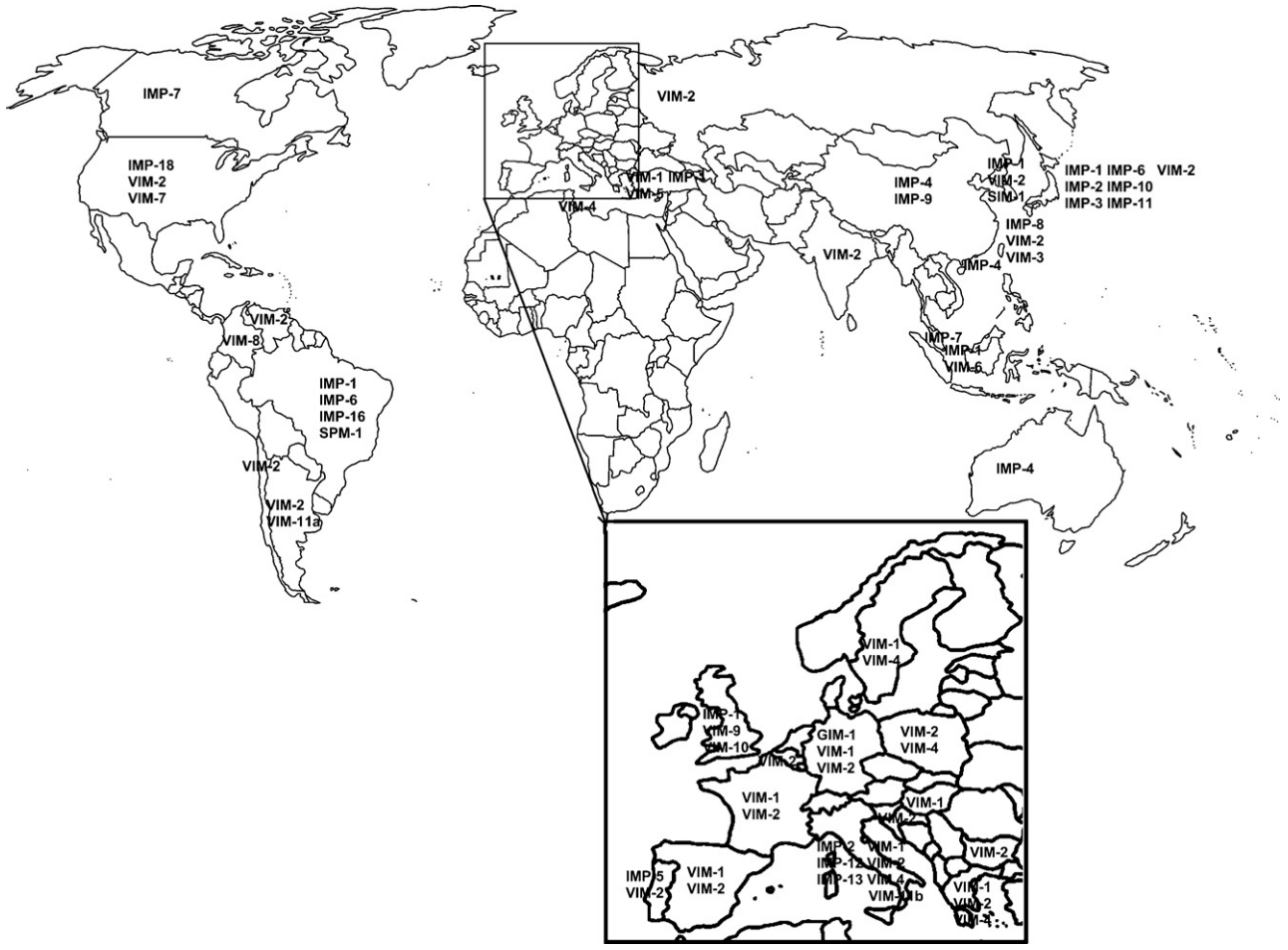

Fig. 1 – Current spread of acquired metallo-β-lactamases. Data are obtained from Ref. [8] and from the 17th European Congress of Clinical Microbiology and Infectious Diseases, 25th International Congress of Chemotherapy (Munich, Germany, 31 March–3 April 2007).

spread worldwide [8] (Fig. 1). The VIM-type enzymes, produced by *P. aeruginosa* and *Acinetobacter*, appear as the most prevalent in Europe with more than 12 allelic variants [8] (Fig. 1). The other foci for the VIM-enzymes appear to be East Asia and Americas [8] (Fig. 1). VIM-2, first discovered in France (Marseille) in 2000 [40], is currently the most widespread acquired MBL. It was found repeatedly in Western (Belgium, Germany, Greece, Italy, Portugal, Spain) and Eastern Europe (Croatia, Poland, Russia), United States, Latin America and Asia (China, India, Japan, Korea, Taiwan). Three other types of acquired metallo-β-lactamases, SPM-1, GIM-1 and SIM-1, have been found in South America [17], Germany [41] and Korea [42] (Fig. 1) which suggests that the capture by bacteria of similar resistance genes in the clinical setting could be an ongoing, relatively common, and rapidly increasing phenomenon. These enzymes are produced by *P. aeruginosa* (SPM-1, GIM-1) and *A. baumannii* (SIM-1).

The transferable metallo-β-lactamases are commonly encoded by genes carried by type 1 or type 3 integrons [8]. These integrons could be carried by large plasmids or be located on the chromosome. The MBL bla_{SPM-1} is not part of a gene cassette, nor is it found in the vicinity of a class 1 integron as found for other metallo-β-lactamase genes, but it is located besides the ISCR variant ISCR4 [8–43]. ISCR (IS common regions), a new type of genetic element, was recently identified as being closely associated with the spread of many antibiotic resistance genes. They can be divided into two groups: ISCRs1 are those that form complex class 1 integrons and ISCRs-2 to -13 are those associated with non-class 1 integrons. ISCRs are also associated with *Salmonella enterica* serovar *Typhimurium* pathogenecity islands. ISCR elements are very unusual in that they can mobilize large sections of adjacent DNA via a rolling circle replication. Normally, termination of replication occurs at a defined site; misreading of this site leads to replication of large sections of DNA to the left-hand end of the element including antibiotic resistance genes. Using a PCR strategy employing degenerate primers designed against the aligned DNA sequences of ISCRs1 to -5, Toleman has detected ISCR elements in several strains of *P. aeruginosa* and *A. baumannii* that harbor metallo-β-lactamase genes e.g. ISCR2 was discovered in a *P. aeruginosa* isolate (Brazil) that harbored bla_{IMP-1} and ISCR3 was discovered in two strains of *P. aeruginosa* (Italy) that have bla_{VIM-1} [43].

The genes coding for subclass B2 enzymes are located on the chromosome and their expression can be regulated by the presence of β-lactam antibiotics in the culture medium *via* a two-components BlrAB system [44].

Subclass B3 metallo-β-lactamases are generally chromosome encoded. The gene coding for L1 could be located on the chromosome or on a large plasmid [45]. The production of this β-lactamase is induced by the presence of β-lactam antibiotics in the medium.

Most natural producers of class B enzymes also produce class A β-lactamases, as do *B. cereus* [46], *S. maltophilia* [45], or class D enzymes as does *L. gormanii* [47]. Several *Aeromonas* species produce a class C and a class D β-lactamases in a co-regulated way with the metallo-β-lactamase [44].

9. Structures

The three-dimensional structure of the mono-zinc form of BcII was the first to be solved [48]. Thereafter, the structures of other subclass B1 enzymes, CcrA [49], the di-zinc form of BcII [50] (Fig. 2a), IMP-1 [51], BlaB [52], VIM-2 (PDB accession no. 1KO2 and 1KO3), SPM-1 [53] (Fig. 2b), and subclass B3 enzymes, L1 [22] (PDB accession no. 2H6A), FEZ-1 [54] (Fig. 2d) and BJP-1 (PDB accession no. 2GMN), were determined. More recently, the structure of a subclass B2 enzyme, CphA, was solved [36] (Fig. 2c). Despite of the low level of identity between the amino acid sequences, the general fold of these enzymes is similar and consists of an αββα structure, composed by two central β-sheets with five solvent-exposed α-helices. The N-terminal

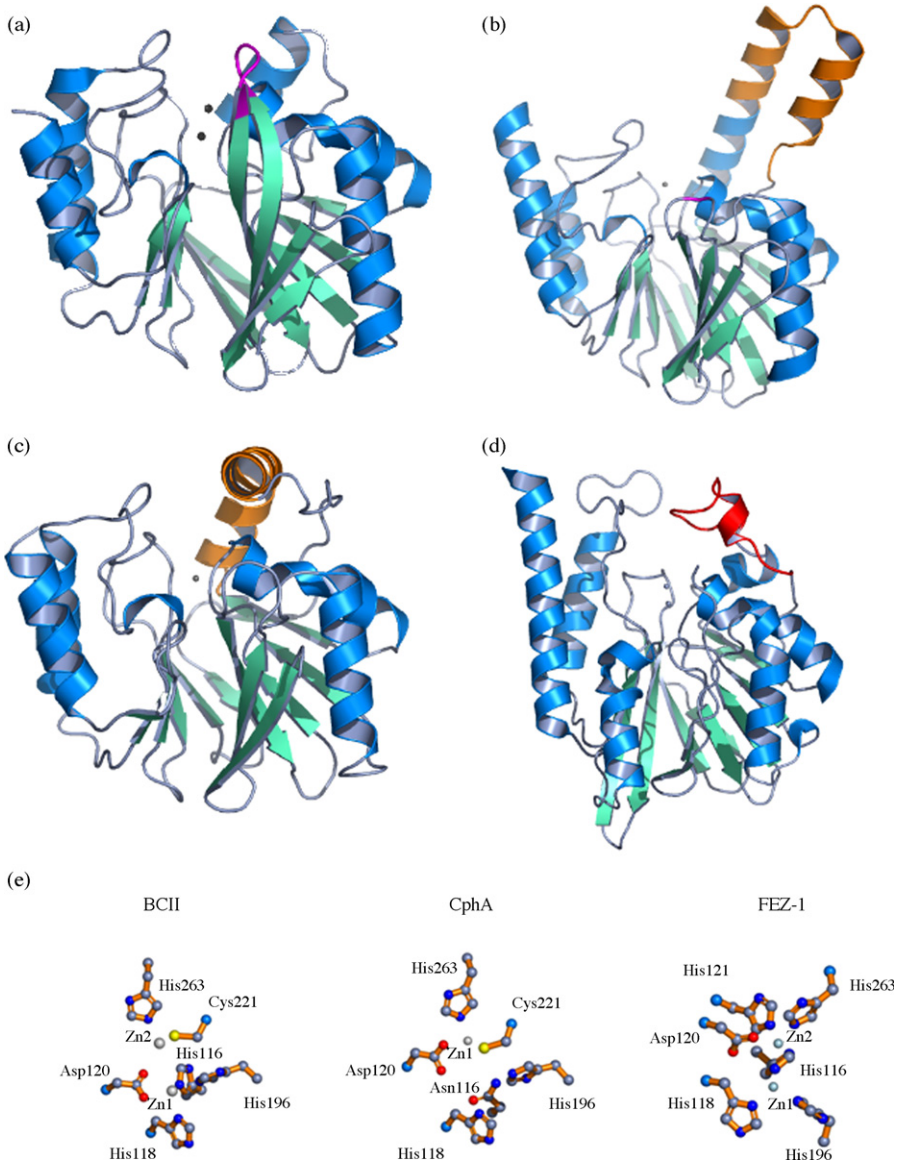

Fig. 2 – Metallo-β-lactamases overall structures. The helices are represented in blue, strands in green and loops in grey. (a) Subclass B1 BcII enzyme with the mobile 61–65 loop in magenta. (b) Subclass B1 SPM-1 enzyme with residues 61–65 in magenta and the extended α3-α4 region in orange. (c) Subclass B2 CphA enzyme with the elongated α3 helix in orange. (d) Subclass B3 FEZ-1 enzyme with the 151–166 loop in red. (e) Representation of the zinc binding sites of subclass B1 (BcII), B2 (CphA), and B3 (Fez-1) β-lactamases.

and C-terminal parts of the molecule, each of them comprising a β-strand and two α helices, can be superposed by a 180° rotation around a central axis, suggesting that the complete structure might have arisen from the duplication of a gene [48]. In all known structures, the active site is located at the external edge of the ββ sandwich.

The N-terminal domain of B1 metallo-β-lactamases incorporates a loop (residues 61–65) that can interact with substrate or inhibitor molecules which possess hydrophobic side-chains (Fig. 2a). This loop is very flexible in the native form of the enzyme. When the substrate or the inhibitor diffuses into the active site, the loop moves to block the molecule in the active site. This movement of the loop is caused by the interaction of the side chain of the W64 residue with the hydrophobic side chain of the substrate [55]. The loop is also stabilized upon binding of inhibitors [51,52] and transforms the active site groove into a tunnel-shaped cavity. The deletion of this loop (named flap) seriously affects the enzymatic activity by weakening the binding of substrate to the enzyme, with the exception of imipenem [55]. Actually, the kinetic parameters of this carbapenem compound are barely affected by the absence of the active site loop. The main difference between the carbapenems and other β-lactams is that both penicillins and cephalosporins usually have bulky aromatic ring substitutions which are absent in the former.

The 61–65 flap is absent in β-lactamases of subclasses B2 and B3.

The subclass B2 CphA metallo-β-lactamase possesses an elongated α3 helix (residues Arg140-Leu161) close to the active site groove (Fig. 2c). This helix presents a kink about Trp150 that enables α3 to follow the surface curvature of the protein and provides a hydrophobic face that contributes to the binding of carbapenem substrates [36]. Consequently, CphA exhibits a very well defined active site, which explains the very narrow activity profile of the enzyme.

The structure of the subclass B1 SPM-1 enzyme shows that it lacks the 61–65 mobile loop. In SPM-1, strands β3 and β4 are separated only by a stretch of five amino acid residues that form a tight turn. Moreover, SPM-1 accommodates a central insertion of 24 amino acids in an extended α3–α4 region (residues 150b-165) (Fig. 2b). Deleting this insertion has only marginal effects upon binding and hydrolysis of a range of β-lactam antibiotics [53]. Both structural features of SPM-1 are reminiscent of that found in the subclass B2 CphA enzyme. By these aspects, SPM-1 thus represents a structural hybrid between subclasses B1 and B2. Moreover, when the sequence of SPM-1 is compared to that of other metallo-β-lactamases, the highest identities are seen with IMP-1 (35.5%) and then the subclass B2 enzymes ImiS (32.2%) and CphA (32.1%).

In subclass B3 enzymes, a loop between α3 and β7 formed by residues 151–166 is mobile and close to the active site [22,54] (Fig. 2d). The I166A mutant of L1 exhibited increased K_m values only when cefoxitin was the substrate, suggesting an interaction of the isoleucine side chain with the cefoxitin methoxy group. The F160A mutant of L1 exhibited higher K_m values when using the cephalosporins as substrates, suggesting an interaction of the cephalosporins' substituents with the phenylalanine on the loop that extends over the active site [56]. L1 is a tetramer, a characteristic unique among β-lactamases. One main interaction between two subunits involves the extended N-terminal region [22]. L1 also contains a disulphide bridge in its monomer and a similar one can be seen in the structure of FEZ-1 [22,54], whereas this is no intramolecular disulphide bridge in the enzymes of subclasses B1 or B2.

10. Zinc coordination

Metallo-β-lactamases possess two potential Zn^{2+} binding sites (Fig. 2e). In the case of B1 enzymes, one zinc ion possesses a tetrahedral coordination sphere and is coordinated by His116, His118, His196 and a water molecule or OH^- ion. The other metal ion has a trigonal-pyramidal coordination sphere which involves Asp120, Cys221, His263 and two water molecules (Table 4). One water/hydroxide molecule serves as a ligand for both metal ions. The two binding sites are named the "histidine" and "cysteine" sites, respectively. In the mononuclear BcII, VIM-2 and SPM-1 enzymes, the sole metal ion was shown to be located in the "histidine" site [48,PDB accession no. 1KO2, 53].

In the B3 enzymes, the "histidine" site is the same as that found in B1 enzymes. Cys221 is replaced by a serine and the second zinc ion is ligated by Asp120, His121, His263 and the nucleophilic water molecule which forms a bridge between the two metal ions (Table 4). Ser221 does not directly interact

Table 4 – Composition of the two metal binding sites in the MBL superfamily

Daiyasu's groups	Enzymes	Site 1	Site 2
Group 1	B1 MβLs	His116-His118-His196	Asp120-Cys221-His263
Group 1	B2 MβLs	Asp120-Cys221-His263	His118?-Met146? or His196?
Group 1	B3 MβLs	His116-His118-His196	Asp120-His121-His263
Group 1	B3 MβL GOB	Asp120-His121-His263	?
Group 2	Glyoxalase II	His116-His118-His196-Asp221	Asp120-His121-Asp221-His263
Group 3	ROO	His116-Glu118-His196-Asp221	Asp120-Asp221-His263
Group 3	FprA	His116-Glu118-His196-Asp221	Asp120-His121-Asp221-His263
Group 4	tRNaseZ, ZiPD	His116-His118-His196-Asp221	Asp120-His121-Asp221-His263
Groups 6 and 7	CPSF-73	His116-His118-His196-Asp221	Asp120-His121-Asp221-His(C)
Group 9	Pce	His116-His118-Asn196-Asp221	Asp120-His121-Asp221-His263
Group 12	AiiA lactonase	His116-His118-His196-Asp221	Asp120-His121-Asp221-His263
Group 13	SdsA1	His116-His118-Glu196	Asp120-His121-Glu221-His263
Group 15	MPH	His116-His118-His196-Asp221	Asp120-His121-Asp221-His263

with the zinc ion but with a second water molecule located in the active site and which could serve as proton donor in the catalytic process [22]. In contrast to all other related metallo-β-lactamases, the subclass B3 GOB-18 enzyme is fully active against a broad range of β-lactam substrates as a mono-zinc species. Different spectroscopic techniques, 3D modeling, and mutagenesis experiments, reveal that the zinc ion is bound to Asp120, His121, His263, and a solvent molecule, i.e., in the canonical site 2 of dinuclear B3 metallo-β-lactamases [57] (Table 4). However, L. Horsfall found two zinc ions per GOB-1 molecule after a purification procedure during which no Zn^{2+} was added to the buffers [89]. These two GOB enzymes differ by a few point mutations, apparently far from the active site (Leu94Phe, Ala137Val and Asp282Asn).

While the B1 and B3 enzymes exhibit maximum activity as di-zinc species, the B2 β-lactamases are inhibited in a non-competitive manner upon binding of a second Zn^{2+} ion (for CphA, the dissociation constant Ki is 46 μM at pH 6.5 [58]). In agreement with EXAFS spectroscopy studies [59] and site-directed mutagenesis [26], the crystallographic structure of CphA shows that the first zinc ion is in the ''cysteine'' site [36]. Currently, the identification of the second binding site in subclass B2 enzymes remains unsettled since even in the presence of a large excess of zinc (10 mM) and in spite of the low value of the dissociation constant, Garau and his coworkers did not succeed in obtaining crystals of the di-zinc form. On the basis of spectroscopic studies with the cobalt-substituted ImiS enzyme, Crawford et al. postulate that in subclass B2 the second metal binding site is not the traditional ''histidine'' site [60]. Costello et al. propose that in ImiS the inhibitory zinc ion binds to both His118 and Met146 [61]. Indeed, their EXAFS data show that the inhibitory zinc is bound by a sulfur. The sulfur ligand in the inhibitory site should come from a methionine residue, since the only cysteine residue (Cys221) is a ligand of Zn1. The mutation of Met146 to Ile abolishes inhibition by zinc. However, mutations of other CphA residues, which have not been implicated as Zn2 ligands, can have similar effects (lower inhibition or even activation by excess of zinc) [26,27]. Moreover, a careful examination of the CphA 3D structure indicates that His118 and Met146 are poorly positioned to bind the same zinc ion. The distance between their Cα's is 7.57 Å, while the distances between the sulphur of Met146 and the nitrogens of the imidazole group of His118 are 10.40 and 10.91 Å, respectively. Also, the space between these residues is occupied and does not allow the accommodation of a zinc ion. The Met146 residue is not conserved in the subclass B2 Sfh-I enzyme where it is replaced by a glutamine. Maybe, as proposed by Vanhove et al., the Cys221 side-chain is displaced upon binding of the second metal ion [26]. The role of His118 residue in the catalytic mechanism [36,37] could explain the inhibition by the binding of the second zinc ion, this residue being prevented from playing this role when it is immobilized as ligand of this second zinc ion in the di-zinc form of the enzyme.

11. Zinc affinities

The subclass B2 CphA enzyme exists mainly in the mono-zinc form as the values of the dissociation constants are 20 nM and 46 μM, respectively for the binding of the first and the second zinc ion [58]. Subclass B1 metallo-β-lactamases show distinct stereochemical similarities in their active sites, with identical residues contributing to metal coordination. However, the corresponding zinc affinities appear to be different, so that CcrA and IMP-1 show two high-affinity metal-binding sites, whereas BcII binds Zn1 and Zn2 with very different affinities. Fluorescence spectroscopy studies with a chromophoric chelator show that BcII has very different dissociation constants for the two metal-binding sites. For the loss of metal-ion from the mononuclear enzyme, the dissociation constant, K_{mono}, is 6.2×10^{-10} M and that for the loss of one metal-ion from the dinuclear MBL, K_{di}, is 1.5×10^{-6} M [62]. K_{mono} decreases significantly, from nanomolar to picomolar values, in the presence of substrate, whereas K_{di} decreases only twofold [63]. This suggests that the mono-zinc enzyme is responsible for the catalytic activity under physiological conditions, where the concentration of free zinc ions is in the picomolar range [63]. A single Zn^{2+} ion is found by crystallographic methods in the ''histidine'' site, at pH 5.6, in presence of 100 μM zinc [48]. At this pH, the activity is only three- to fourfold lower than that at pH 7.5 [31]. But the existence of the mononuclear form, where the sole metal ion would be shared between the two binding sites is supported by several kinetic results, PAC (Cd) and spectroscopic (Co) data [62]. However, one cannot completely reject the hypothesis of a strong positive cooperativity between the two zinc ions with coexistence of only the apo- and di-zinc forms. At least, Damblon and his coworkers observe that the inhibitor thiomandelic acid induces positive cooperativity in metal binding [64].

Conversely, and despite the very close similarity with BcII, the class B1 enzyme CcrA binds both zinc ions very tightly. Kinetic studies of CcrA with nitrocefin have shown that only the di-nuclear species is active and that a previously observed mono-zinc CcrA enzyme [65] was a mixture of the di-zinc and the apo-enzyme [33].

BcII is active in the presence of either one or two zinc ions, although full activity is observed when two zinc ions are bound. It has been proposed that the arginine 121 present in BcII, Vim-2, BlaB and in subclass B2 enzymes is responsible for the lower affinities. The CcrA and IMP-1 enzymes, showing high affinity for both zinc ions, have cysteine and serine, respectively, in the corresponding position. The replacement of the positive Arg121 side chain in CphA by a neutral one enhances the affinity for Zn^{2+} [27], as was already suggested for BcII [48,49]. Rasia and Vila [66] have shown that the R121H mutation increases the affinity for the second Zn^{2+} ion in BcII and that the His121 side chain replaces that of His263 as a ligand of the second Zn^{2+}. The C121R mutant of CcrA was isolated in the di-zinc form, although the removal of the second zinc appeared to be facilitated [33]. All these data indicate that the replacement of Arg121 by a neutral residue (a Cys, Ser or His, respectively) in CcrA, IMP-1, and the subclass B3 enzymes increases the affinity for the second zinc. Until recently, no mono-zinc form of subclass B3 enzymes has ever been obtained. However, the subclass B3 GOB-18 was shown to be a mononuclear enzyme [57]. In the subclass B1 SPM-1 enzyme, a neutral Gly121 is present and, however, SPM-1 possesses two metal sites of very different affinities [53], thus

the presence of Arg121 in BcII is surely not the only reason explaining the weaker affinity for Zn2.

As shown by the data described above, the activity and even the existence of mono-zinc forms of B1 and B3 enzymes remain controversial. As yet, it seems impossible to supply a clear answer to these questions and it remains possible that minor sequence differences might result in different behaviours. For B2 enzymes, the mono-zinc form is clearly the active one, but here the location of the inhibitory second zinc remains mysterious (although this is probably biologically unrelevant).

12. Inhibitors of metallo-β-lactamases

All known inhibitors or inactivators of serine β-lactamases are inefficient towards metallo-β-lactamases. Reported inactivators of all metallo-β-lactamases are the metal chelators EDTA, o-1, 10-phenanthroline and dipicolinic acid, however these have no clinical significance.

The propagation of metallo-β-lactamases among nosocomial bacterial strains justifies the search for compounds which can thwart the activity of these enzymes. Unfortunately, the discovery of a clinically useful, specific inhibitor of metallo-β-lactamases is made difficult by the fact that this compound must remain inactive towards the human proteins which are members of the metallo-β-lactamase superfamily, or other metallo-enzymes such as the angiotensin converting enzyme. Another difficulty is to find a compound active on all the three subclasses of metallo-β-lactamases and even on all the enzymes within a same subclass. Currently, the known inhibitors efficiently inhibit one or two metallo-β-lactamases and are much less active against the others. Moreover, they remain impractical to use as therapeutic agents. In 2004, Toney and Moloughney reviewed the literature on inhibitors of MBLs [67], and more recently Walsh et al. devoted a paragraph on experimental inhibitors in their review on transferable MBLs [8]. A few examples are thioesters derivatives, trifluoromethyl alcohols and ketones, sulfonyl hydrazones, tricyclic natural products, succinic acid derivatives, biphenyl tetrazoles, cysteinyl peptides, carbapenem and penicillin derivatives among which an interesting thiol-substituted penicillin inhibitor [68], degradation products of cephalosporins, simple thiol compounds such as mercaptoacetic acid and thioesters, thiomandelic acid [64], captopril [52,59], derivatives of benzohydroxamic acid [69], pyridine carboxylates [70].

13. The metallo-β-lactamases (MBL) superfamily

The MBL superfamily was defined by Neuwald et al. in 1997 [71]. In addition to metallo-β-lactamases, it includes enzymes which hydrolyze thiol-ester, phosphodiester and sulfuric ester bonds as well as oxydoreductases.

Most of the 6000 members of this superfamily share five conserved motifs [72]: Asp84, His116-X-His118-X-Asp120-His121, His196, Asp221 and His263 (in the order to facilitate comparison, the BBL numbering [13] is applied to the MBL superfamily and used throughout this chapter). The fold of these proteins or of one of their domains is of the αββα type, similar to the structure of zinc β-lactamases. Almost all these proteins exhibit a binuclear metal center similar to that of metallo-β-lactamases. These centers occupy topologically similar locations. Among the conserved motifs, Asp84 exhibits a strained secondary conformation and may play a role in maintaining the protein fold. The other conserved residues are involved in metal coordination. For the majority of these enzymes, the first metal ion binding site is composed of His116, His118 and His 196, while the second binding site is composed of Asp120, His121 and His263. A major difference with true metallo-β-lactamases consists in the presence of an aspartate (Asp221) in the position where a cysteine (B1) or a serine (B3) is found in β-lactamases. Asp221 and a water molecule bridge the two metallic ions.

A large number of hypothetic enzymes found in GenBank could also share this fold and bind one or two metal ions, but the majority of these proteins have not been functionally or structurally characterized yet. Daiyasu has classified these proteins in 17 families on the basis of their biological functions [72]. Until recently, only three of the 17 protein groups proposed to contain this fold had been characterized structurally, true metallo-β-lactamases (group 1-see above), glyoxylases II (group 2), and rubredoxin oxidoreductases (group 3). However, more recent crystal structures of other family members include acid phosphorylcholine esterase Pce (group 9), the methyl parathion hydrolase (group 15), the N-acyl homoserine lactone hydrolase (group 12), the alkylsulfatase from *P. aeruginosa* SdsA1 (group 13), the tRNA3′-processing endoribonuclease tRNaseZ (group 4) and proteins members of the β-CASP family (groups 6 and 7) (Fig. 3). The composition of both metal binding sites of these MBL superfamily enzymes of known structures is described in Table 4.

13.1. *Group 2*

Glyoxalase II belongs to the glyoxalase system (formed by glyoxalases I and II) which catalyzes the conversion of toxic 2-oxoaldehydes to 2-hydroxycarboxylic acids using glutathione as coenzyme. Glutathione thiolesters formed in the reactions catalyzed by glyoxalase I are substrates for glyoxalase II and are hydrolyzed by the latter enzyme to the free 2-hydroxyacids and gluthathione. Glyoxalase II has been isolated from numerous mammalian tissues and from higher plants. The structure of human glyoxalase II is composed of two domains: a metallo-β-lactamase domain and a smaller α-helical domain (Fig. 3a). It binds 1.5 moles of zinc per mole of enzyme and 0.7 mole of iron per mole of enzyme [73]. The cytosolic glyoxalase II from *Arabidopsis thaliana* possesses a binuclear center able to bind zinc, iron or manganese and some results indicate that glyoxalase II exhibits positive cooperativity in metal binding [74]. The two metal-binding sites present the classical structural features of the MBL superfamily (Table 4).

Park et al. succeeded in introducing metallo-β-lactamase activity in the αββα scaffold of glyoxalase II by simultaneous incorporation and adjustement of functional elements in conjunction with directed evolution [75]. However, the very low activity versus cefotaxime of the resulting enzyme ($k_{cat}/K_m = 180\ M^{-1}\ s^{-1}$) does not explain the increased MICs of the

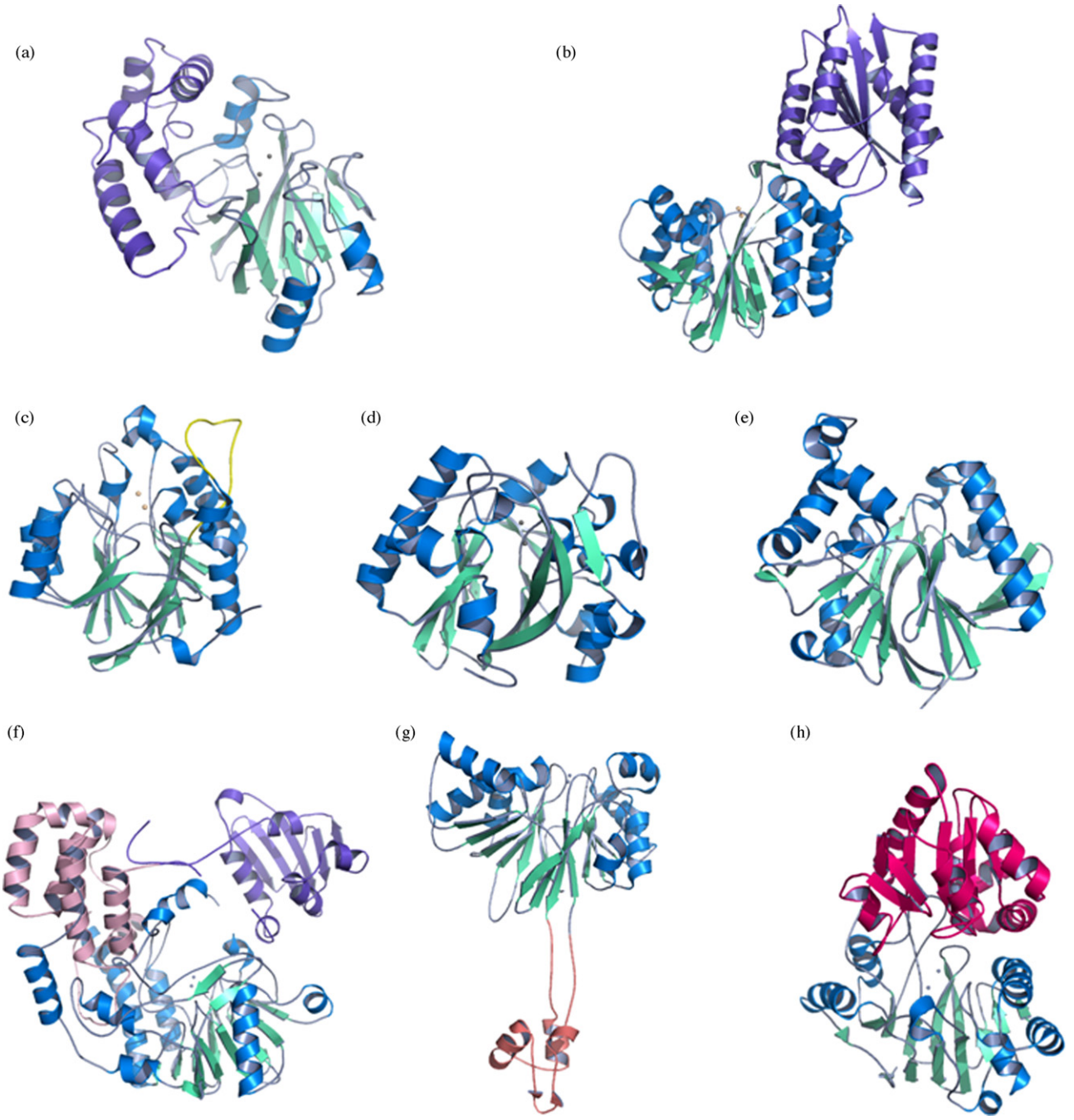

Fig. 3 – Metallo-β-lactamases superfamily overall structures. The metallo-β-lactamase like domains are represented in blue (helices), green (strands) and grey (loops and disordered regions). Zinc ions are represented as grey spheres and iron ions as beige spheres. (a) Glyoxalase II with the additional α-helical domain in violet, (b) ROO with the additional flavodoxin-like domain in violet, (c) phosphorylcholine esterase (Pce) domain of the virulence factor choline-binding protein E with the elongated loop lying on the top of the active site represented in yellow, (d) N-acyl homoserine lactone hydrolase, (e) MPH monomer, (f) SdsA1 monomer with the additional central dimerization domain in pink and C-terminal domain in violet, (g) ZiPD monomer with the 50 amino acids exosite in salmon, (h) TTHA0252 with the β-CASP domain in hotpink.

producing cells which served as a selection tool, unless unrealistic periplasmic concentrations of enzyme are assumed. Moreover, the engineered protein only hydrolyzes cefotaxime and no other cephalosporins or penicillins, a very unlikely property for a β-lactamase.

13.2. *Group 3*

The metallo-β-lactamase fold is also found in a distinct group of cytosolic redox proteins. Rubredoxin:oxygen oxydoreductase (ROO) from *Desulfovibrio gigas* consists of two domains: a

β-lactamase domain and a flavodoxin-like domain (Fig. 3b). ROO is not a zinc-enzyme but contains a di-iron center for dioxygen reduction [76]. The second histidine in the usual H–X–H–X–DH motif is replaced by a glutamate (Glu118). One iron ion is ligated by two histidines (His116, His196), one glutamate (Glu118) and one aspartate (Asp221) while the second iron ion is coordinated by one histidine (His263) and two aspartates (Asp120, Asp221). His121, although present, does not bind Fe2 [76] (Table 4). A comparison of the ROO structure with that of true metallo-β-lactamases shows that the L1 β-lactamase is more similar to the first domain of ROO than to other β-lactamases [28], and FEZ-1 is closer to glyoxalase II and ROO than to BcII or CphA [13].

The structure of FprA from *Moorella thermoacetica*, which likely functions as a scavenging nitric oxide reductase, has also been solved [77]. As in the case of *D. gigas* ROO, FprA contains a solvent-bridged non-heme, non-sulfur di-iron site with five-coordinate iron centers bridged by an aspartate, and terminal glutamate, aspartate, and histidine ligands. However, in contrast to ROO, the FprA di-iron site exhibits four His ligands, two for each iron (Table 4).

13.3. Group 9

The crystal structure of a virulence factor, the phosphorylcholine esterase (Pce) domain of the choline-binding protein E from *Streptococcus pneumoniae* was solved in 2005 [78]. This domain catalyzes the hydrolysis of choline-phosphoester bonds, releasing phosphorylcholine molecules from cell wall-associated teichoic and lipoteichoic acids. A striking feature of Pce, unique among the reported structures of the metallo-β-lactamase superfamily proteins, is the presence of an elongated loop, which lies on the top of the active site (Fig. 3c). This loop would play a role in determining the accessibility of the catalytic cavity for the substrate. Iron ions are essential for catalysis. Site 1 comprises His116, His118, Asn196 and Asp221 residues and site 2 Asp120, His121, Asp221 and His263 (Table 4). A water/hydroxide molecule bridge completes the coordination spheres of the two metals.

13.4. Group 15

The methyl parathion hydrolase (MPH) from *Pseudomonas* sp. WBC-3 catalyzes the degradation of the organophosphate pesticide methyl parathion. MPH is a homodimer, each monomer presenting a typical metallo-β-lactamase fold [79] (Fig. 3e). MPH possesses a binuclear di-zinc center. The coordination sphere of the zinc ions is the same as that found in glyoxalase II (Table 4).

13.5. Group 12

The *N*-acyl homoserine lactone hydrolase from *Bacillus thuringensis* is a quorum-quenching lactonase. More than 50 species of Gram-negative bacteria, *P. aeruginosa* for example, are known to use *N*-acyl-L-homoserine lactones (AHLs) as signal-molecules for intercellular ''quorum sensing'' communication pathways necessary to their pathogenicity. Because of their importance in pathogenicity, these pathways are possible targets in the development of new therapeutic molecules. Nature has already developed several methods to counter these pathways among which the production of quorum-quenching enzymes which hydrolyze the lactone residue of AHLs. Their amino acid sequences exhibit the conserved MBL motifs. The quorum-quenching lactonase AiiA from *B. thuringensis* binds two zinc ions and its activity is Zn dependent. The recently solved 3D structure of this enzyme [80] highlights the typical αββα-fold (Fig. 3d). The coordination sphere of the zinc ions is the same as that found in glyoxalase II (Table 4).

13.6. Group 13

P. aeruginosa is able to degrade and metabolize SDS which is usually a biocide. SdsA1 of *P. aeruginosa* is a secreted SDS hydrolase that allows the bacterium to use primary sulfates such as SDS as sole carbon or sulfur sources. The crystal structure of SdsA1 reveals three distinct domains. The N-terminal catalytic domain with a binuclear Zn^{2+} cluster is a distinct member of the metallo-β-lactamase fold superfamily, the central dimerization domain ensures resistance to high concentrations of SDS, whereas the C-terminal domain provides a hydrophobic groove, presumably to recruit long aliphatic substrates (Fig. 3f) [81]. Zn1 is coordinated by His116, His118, and Glu196. Asp221 is replaced by a glutamic acid residue. Zn2 is coordinated by His121, His263, Asp120 and Glu221. A water molecule bridges the two zinc ions (Table 4).

13.7. Group 4

The crystal structures of *Thermotoga maritima tRNaseZ* [82], *Bacillus subtilis* tRNA maturase tRNaseZ [83] and *E. coli* tRNase Z (ZiPD) [84] (Fig. 3g) have been determined. tRNaseZ belongs to the ELAC1/ELAC2 family within the MBL superfamily. It is found in the vast majority of, if not all, eukaryotes and archea, and in about half of the sequenced bacterial genomes. Two ElaC variants were recently associated with prostate cancer in man. The structure of *B. subtilis* tRNaseZ is characterized by a long flexible arm extending from the core β-lactamase domain, this arm permits RNA recognition and hydrolysis of phosphodiester bonds. The *E.coli* homolog of tRNaseZ, ZiPD, also possesses a specific sequence insertion module of ~50 amino acids, referred to by the authors as ''ZiPD exosite'', located between the zinc ligands His196 and Asp221. This exosite is not required for phosphodiesterase activity but is essential for pre-tRNA processing and tRNA binding. ZiPD is capable of binding two zinc or two iron ions. However, it displays phosphodiesterase activity only in the zinc form. The coordination sphere of the zinc ions is the same as that found in glyoxalase II (Table 4).

13.8. Groups 6 and 7

A separate family of enzymes within those exhibiting the metallo-β-lactamase fold, the β-CASP family, comprises several important proteins acting on nucleic acid substrates, involved in DNA repair (Artemis, Apollo, SNM1 and PSO2) and RNA processing (cleavage and polyadenylation specificity factor subunits CPSF-73 and CPSF-100) [85]. Two regions can be defined within the protein sequence: the MBL domain and a

region specific to members of the MBL superfamily acting on nucleic acids. This region is named the β-CASP motif (metallo-β-lactamases-associated CPSF Artemis SNM1 PSO2). β-CASP family proteins are characterized by three conserved sequence motifs [A (Asp or Glu), B (His) and C (His)], following the four typical metallo-β-lactamase motifs (1–4), whereas other members of the MBL superfamily have motif 5 (His263) just after motif 4 (Asp221).

The crystal structure of TTHA0252 from *Thermus thermophilus* HB8, a RNA degradation protein, was the first report of the tertiary structure of a β-CASP family protein [86]. TTHA0252 comprises two separate domains: a metallo-β-lactamase domain and a ''clamp'' domain (β-CASP domain) (Fig. 3h). The active site of the enzyme is located in a cleft between the two domains, which includes two zinc ions coordinated by seven conserved residues. Although this geometry is similar to those of members of the MBL superfamily, TTHA0252 has one conserved His residue characteristic of the β-CASP family as a ligand.

Most eukaryotic messenger RNA precursors (pre-mRNAs) undergo extensive maturational processing, including cleavage and polyadenylation at the 3′-end. Recent analyses and the resolution of the 3D structure [87] indicated that the 73-kDa subunit of cleavage and polyadenylation specificity factor (CPSF-73) is the endonuclease for this and related reactions. CPSF-73 contains two domains, a metallo-β-lactamase domain and a β-CASP domain. This second domain can be considered as a cassette inserted into the metallo-β-lactamase domain. The active site of CPSF-73, with two zinc ions, is located at the interface of the two domains. The two zinc ions in the active site are each bound in an octahedral environment. Zn1 is coordinated by His116, His118 and His196. Zn2 is coordinated by Asp120, His121 and motif C (His) of the β-CASP domain. A hydroxide ion and Asp221 are bridging ligands (Table 4). CPSF-73 possesses RNA endonuclease activity. Mutations, that disrupt zinc binding in the active site, abolish this activity. CPSF-100 shares sequence conservation and a similar domain architecture with CPSF-73 but lacks the zinc-binding residues in the metallo-β-lactamase domain. Therefore, CPSF-100 cannot bind zinc and is unlikely to possess nuclease activity.

14. Conclusion

The metallo-β-lactamase fold clearly represents a stable scaffold that has repeatedly been used during evolution to catalyze a diverse range of chemical reactions. Structurally, this diversity is achieved by varying the sequence and length of loops near the active site to accommodate various substrates. By combining the metallo-β-lactamase domain with auxiliary substrate- , product- or cofactor-binding domains, the range of functions has been extended yet further.

The catalytic diversity of this superfamily explains how certain members might have easily evolved into metallo-β-lactamases in response to environmental antibiotics. The most recent therapeutic utilization of carbapenems has subsequently favoured the emergence of preexisting enzymes in pathogenic strains. These data support the hypothesis that metallo-β-lactamases may be the result of divergent evolution starting from an ancestral protein which did not have a β-lactamase activity.

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