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β-lactamase-mediated Resistance: A Biochemical, Epidemiological and Genetic Overview

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Abstract: Early after the introduction of the first (narrow spectrum) penicillins into clinical use, penicillinase-producing staphylococci replaced (worldwide) the previously susceptible microorganisms. Similarly, the extensive use of broad-spectrum, orally administered β-lactams (like ampicillin, amoxicillin or cefalexin) provided a favorable scenario for the selection of gram-negative microorganisms producing broad spectrum β-lactamases almost 45 years ago. These microorganisms could be controlled by the introduction of the so called "extended spectrum cephalosporins". However, overuse of these drugs resulted, after a few years, in the emergence of extended-spectrum β-lactamases (ESBLs) through point mutations in the existing broad-spectrum β-lactamases, such as TEM and SHV enzymes. Overuse of extended-spectrum β-lactams also gave rise to chromosomal mutations in regulatory genes which resulted in the overproduction of chromosomal AmpC genes, and, in other regions of the world, in the explosive emergence of other ESBL families, like the CTX-Ms. Carbapenems remained active on microorganisms harboring these extended-spectrum β-lactamases, while both carbapenems and fourth generation cephalosporins remained active towards those with derepressed (or the more recent plasmidic) AmpCs. However, microorganisms countered this assault by the emergence of the so called carbapenemases (both serine- and metallo- enzymes) which, in some cases, are actually capable of hydrolyzing almost all β-lactams including the carbapenems.

Although all these enzyme families (some of them represented by hundreds of members) are for sure pre-dating the antibiotic era in environmental and clinically significant microorganisms, it was the misuse of these antibiotics that drove their evolution. This paper describes in detail each major class of β-lactamase including epidemiology, genetic, and biochemical evaluations.

Keywords: Penicillinases, Cephalosporinases, Broad-spectrum β-lactamases, Extended-spectrum β-lactamases, Inhibitor-resistant β-lactamases, Carbapenamases.

HISTORIC PERSPECTIVES

Shortly after the introduction of the first β-lactams into clinical practice (Penicillin G, Penicillin V) it was clear that most infections caused by gram-negative bacteria could not be controlled by penicillins. However, control of infections caused by gram-positive bacteria (opportunistic staphylococcal infections accounting for a good part of them) using penicillins was very effective, and, lacking what could be considered modern microbiological diagnosis, led to a false sensation of controlling infectious diseases. Over prescribing patients suffering any "potential" infectious disease was observed and in just a few years resistant staphylococci emerged.

Natural and emerging resistance gave way to further research for β-lactam development that could treat these resistant organisms. The first wide spectrum penicillins (ampicillin followed by amoxicillin) could be used for serious opportunistic infections caused by different enterobacteria in hospital settings. Cephalosporins arrived to the clinical setting by the mid 1960s with the introduction of cephalothin (followed by other first generation cephalosporins, and together with penicillinase-resistant penicillins reintroduced this family in staphylococcal infection control).

Extensive use of broad-spectrum, orally administered β-lactams (like ampicillin, amoxicillin or cefalexin) provided the selective pressure for the selection of gram-negative microorganisms producing broad spectrum β-lactamases such as TEM in *E. coli* and SHV (initially in *K. pneumoniae*). The genes encoding these enzymes were extensively disseminated globally by plasmids not only into *E. coli* and *K. pneumoniae*, but also in several other genera of hospital (and community as well) associated microorganisms.

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Microbial resistance to classical broad spectrum β-lactams resulted in the development of extended spectrum cephalosporins (oxyimino cephalosporins like cefotaxime, ceftazidime and ceftriaxone) and the monobactam, aztreonam in the 1980s. Overuse of these drugs resulted, after a few years, in the emergence of extended-spectrum β-lactamases (ESBLs) through point mutations in the existing broad-spectrum β-lactamases, TEM and SHV. Overuse of extended-spectrum β-lactams also gave rise to chromosomal mutations in regulatory genes which resulted in the overproduction of the chromosomal AmpC gene for example resulting in fully resistant strains. About half a decade later, cefotaxime resistant microorganisms harboring enzymes totally unrelated to those preexisting started to be sporadically isolated. These corresponding genes, known as the $bla_{\text{CTX-M}}$ types, are currently the most prevalent family of ESBLs worldwide.

Carbapenems are active on microorganisms harboring these extended-spectrum β-lactamases, while 4th generation cephalosporins towards those with derrepresed (or the more recent plasmidic) AmpCs. However, microorganisms countered this assault by emergence of the so called carbapenemases (which are actually capable of hydrolyzing almost all β-lactams including the carbapenems).

A related area (search for inhibitory molecules) rendered sulbactam, clavulanic acid and tazobactam, able to inhibit BSBLs (broad-spectrum β-lactamases) and ESBLs. However, they diminish minimal inhibitory concentrations (MIC) only to a certain amount and if the acquired resistance is too high, they are not able to recover basal susceptibility. In addition, both the TEM and SHV families have accumulated mutations leading to resistance to β-lactamase inhibitors in different amino acid residues than those necessary for extending the hydrolytic profile.

It is clear that the evolution and explosive emergence of β-lactamases has been a direct result of the counter measures used to

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ward off resistance to the initial introduction of the penicillins. Although these enzymes described above were all present in the environment, it was the misuse of these antibiotics that drove their evolution. The following sections describe in detail each major class of β-lactamase including epidemiology, genetic, and biochemical evaluations.

CLASS A B-LACTAMASES

The Classical models (TEMs and SHVs)

TEM β-lactamases

TEM β-lactamase production in gram-negative bacteria became apparent in the 1960s when the first enzyme responsible for aminopenicillin resistance in an *E. coli* isolate was described. The corresponding encoding gene for TEM-1 β-lactamase was located in Tn*3*, a transposable element described in different conjugative plasmids [1]. The rapid increase in its prevalence was associated to its location [1, 2].

The extended-spectrum and inhibitor resistant TEM β-lactamases, most of which are located on plasmids, are derivatives of TEM-1 (Fig. **1**) and/or TEM-2. TEM enzymes belong to Ambler class A and are characterized by an active-site serine [3]. The premature protein consists of 286 amino acids (the first 23 amino acids correspond to the signal peptide), with the mature enzyme having a molecular mass of approximately 29 kDa.

At the amino acid sequence level, the TEM and SHV β-lactamase families share 67% identity, while the CTX-M family is more distant (40% sequence identity with both TEM and SHV)[4, 5].

Broad-spectrum β-lactamases (BSBL)

The broad-spectrum TEM enzymes are mainly represented by TEM-1 (Fig. **1**), -2 and -13 [6-8] with TEM-1 being the most prevalent. They have the same hydrolytic profile and are able to hydrolyze ampicillin at a greater rate than carbenicillin, oxacillin, or cephalothin, and have negligible activity against extended-spectrum cephalosporins. They are inhibited by clavulanic acid. A few additional mutational variants are included in this subgroup, such as -55, -57, -90, -95, -110, -127, -128, -135 and -141 among others (http://www.lahey.org/Studies/). Amino acid changes summarized in (Table **1**) do not expand the spectrum of hydrolysis of broad-spectrum TEMs. They are generally single amino acid changes not preserved in other variants.

Extended-spectrum β-lactamases (ESBL)

While TEM-3 is thought to be the first TEM-type ESBL described in 1988, a ceftazidime-resistant TEM-12 –producing *Klebsiella oxytoca* was isolated in England in 1982, [9]. The gradual acquisition of mutations has led to an unexpected increase in the number of observed β-lactamase variants. Of the natural mutants described for TEM-type β-lactamases a number of amino acid residues are especially important for producing the ESBL phenotype (http://www.lahey.org/Studies/). The observed changes mainly include Gln39Lys (glutamine to lysine at position 39), Glu104Lys, Arg164Ser/His/Cys, Gly238Ser, and Glu240Lys (Table **2**).

Although position 104 is part of a conserved loop, changes to several amino acids like Ser, Thr, Lys, Arg, Tyr or Pro (with conserved activity) have been observed. The Glu104Lys substitution has been frequently associated with an ESBL profile along with other accompanying mutations in the active site cavity [10], as that mutation is unable to confer full resistance by itself. The Gly238Ser substitution is frequently associated with ESBL-TEMs, providing a catalytic efficiency one-order of magnitude greater than TEM-1 towards cefotaxime and ceftazidime [11]. Analysis of the crystal structure suggests that substitution at the Gly238 residue is involved in a β3 strand displacement, improving the active-site area to accommodate bulkier substrates.

Another variable residue is Arg164 which is located in the Ω-loop; Arg164Ser replacement results in more flexibility within the loop (involved in the substrate specificity of the enzyme), widening the cavity for bulky side chains in the extended-spectrum cephalosporins. Therefore, Arg164Ser TEM mutants increase enzymatic activity towards ceftazidime, and an additional mutation, Glu240Lys, further enhances this effect. Although these substitutions expand the spectrum of β-lactamase activity towards third-generation cephalosporins, they are detrimental to the activity on ampicillin [12].

Although TEM-type β-lactamases are most often found in *E. coli* and *K. pneumoniae*, TEM-type ESBLs are also found in other species of *Enterobacteriaceae* and non-fermenting gram-negative

Table 1. Amino Acid Changes Observed in the Broad-spectrum TEMs

Position	21	34	39	92	115	145	157	158	182	218	265
TEM-1	**L**	**K**	**Q**	**G**	**D**	**P**	**D**	**H**	**M**	**G**	**T**
TEM-2			K								
TEM-13			K								M
TEM-55										E	
TEM-57				D							
TEM-90					G						
TEM-95						A					
TEM-110	F										M
TEM-127								N			
TEM-128							E				
TEM-135									T		
TEM-141		E									

Table 2. Aminoacid Changes Observed in Extended Spectrum TEMs, Compared to TEM-1

Position*	6	21	39	104	153	164	182	237	238	240	265	268
TEM-1	**Q**	**L**	**Q**	**E**	**H**	**R**	**M**	**A**	**G**	**E**	**T**	**S**
2be (ESBL)	K	F	K	K	R	SHC	T	TG	SDN	KRV	M	G
% changed	2,6	22	31	46	3,8	55	22	10	41	32	19	2,6
N (nt=78)	2	17	24	36	3	43 (24,15, 4)	17	8 (7, 1)	32 (30, 1, 1)	25 (23, 1, 1)	15	2

*Relative to TEM-1 amino acid sequence
2be: Possible amino acid changes in ESBL-TEM
% Changed: Relative abundance of mutations occurring in each position.
N: Total number of ESBL-TEM carrying specific site mutation; numbers in parenthesis indicate the distribution of each amino acid change.
nt: Total number of ESBL-TEM according to Lahey's Institute website

bacilli [13, 14]. TEM-type ESBLs are (and have been) frequently associated with hospital outbreaks, standing alone as the sole mechanism for the ESBL phenotype, or accompanied by other resistance plasmids encoding other ESBLs and MBLs (IMP- or VIM-like). TEM-type ESBLs observed in Polish hospitals were thought to be the result of selective pressure from several β-lactams used within a given institution, in the absence of proper infection control measures [15]. TEM-3, TEM-4 or TEM-47 producing-*K. pneumoniae* have caused clonal outbreaks in different European Union countries; TEM-24 was associated with outbreaks of *E. aerogenes* and TEM-24, TEM-52 and TEM-92 were related with clonal dissemination of *P. mirabilis*, as well as prolonged outbreak of TEM-21-producing *P. aeruginosa* in nursing homes in France [14, 16-20].

Inhibitor Resistant TEM (IRT)

According to the functional classification of β-lactamases, IRT enzymes belong to group 2br [21] and comprise a group of plasmid-encoded TEM- variants with altered interaction with irreversible suicide inhibitors such as clavulanate, sulbactam and tazobactam. Isolates that only produce IRT are not considered ESBL-producers because susceptibility to extended-spectrum cephalosporins, cephamycins, carbapenems and piperacillin–tazobactam remains unaltered. IRTs were initially found in *E. coli* [22], and later on, in many other enterobacteria [23, 24]. However, the presence of IRTs in *P. aeruginosa* or other non-fermenters has not been reported. Amino acid replacements responsible for the resistance profiles are summarized in (Table **3**).

Analyzing the IRTs described (http://www.lahey.org/Studies/), it appears that the majority of the amino acid changes within the TEM enzyme leading to the inhibitor- resistant phenotype occur at residues Met69, Arg244, Arg275, and Asn276. The sites of these amino acid substitutions are different from those that lead to the ESBL phenotype. Clavulanate, sulbactam and tazobactam act in a similar way to inhibit the TEM β-lactamase, involving a covalent cross-linking reaction between Ser70 and Ser130. Structural analysis suggests that Met69 or/and Arg244 substitutions (frequently present in IRTs) result in accommodations that protect the local environment of Ser130, leading to inhibitor resistance. The way these amino acids perturb this region differs for each substitution; either by conformational changes in Ser130 that attenuate the cross-linking, or by displacing the structural water molecule that stabilizes the substrate in the region of Ser130 [25, 26].

Complex Mechanism TEM β-lactamases (CMT)

Among resistance mechanisms, one of the most interesting evolutionary aspects observed in the last decade was shown by the TEM-type enzymes that combine both hydrolysis of oxyimino cephalosporins and resistance to β-lactamase inhibitors. These enzymes are known as complex mechanism TEMs (CMT), requiring the concomitant appearance of substitutions frequently observed for ESBLs and IRTs (Table **4**). These new β-lactamases confer different levels of resistance to oxyimino-cephalosporins and to clavulanic acid, depending on the specific amino acid substitution. The CMT enzymes have been identified in different species of *Enterobacteriaceae*, including *E. coli*, *K. pneumoniae*, *P. mirabilis* and *E. aerogenes* [23, 27-29].

Emergence of CMT-type β-lactamases has also been associated with a novel promoter region involved in their expression [27]. Most CMT enzymes, as well as IRTs, have been recovered from

Table 3. Amino Acid Residues Observed in IRTs Compared to TEM-1

Position*	21	39	69	127	130	165	244	265	275	276
TEM-1	**L**	**Q**	**M**	**I**	**S**	**W**	**R**	**T**	**R**	**N**
2br (IRT)	FI	K	LVI	V	G	RC	SCHG	M	QL	D
N (nt=34)	4 (3,1)	4	18 (8,6, 4)	2	2	3 (2, 1)	12 (5, 4, 2, 1)	2	6 (4, 2)	7

*Relative to TEM-1 amino acid sequence
2br: Possible amino acid changes in IRT
N: Total number of IRT carrying specific site mutation; numbers in parenthesis indicate the distribution of each amino acid change.
nt: Total number of IRT according to Lahey's Institute website

Table 4. Amino Acid Residues Observed in CMTs Compared to TEM-1

Position*	39	69	104	164	238	240	276
TEM-1	**Q**	**M**	**E**	**R**	**G**	**E**	**N**
2ber (CMT)	K	LV	K	HS	S	K	D
N (nt=10)	2	7 (5, 2)	4	6 (3,3)	3	3	5

*Relative to TEM-1 amino acid sequence
2ber: Possible amino acid changes in CMT
N: Total number of CMT carrying specific site mutation; numbers in parenthesis indicate the distribution of each amino acid change.
nt: Total number of CMT according to Lahey's Institute website

urinary tract infections which correlates with the site of infection where the β-lactam antibiotics and inhibitors reach high concentrations. In general, IRT and CMT enzymes have not been associated with outbreaks; however an IRT-2 producing *K. pneumoniae* has been associated with an outbreak in a geriatric institute in France in February of 1998 [30].

Biochemical Properties of IRTs and CMTs:

When compared to TEM-1, most IRT enzymes have low catalytic efficiencies for most substrates, due to the reduced k_{cat} and increased K_m values (see example in Table **5**). IC50 values for inhibitors are higher than those for TEM-1. In general, clavulanic acid is a more effective inhibitor than sulbactam, while tazobactam may retain some inhibitory activity against these β-lactamases [23, 31]. CMT enzymes usually present a lower hydrolytic activity (k_{cat}) against penicillin than TEM-1, as well as their corresponding parental ESBLs. However, substrate affinity (K_m) values are similar to TEM-1. Values of kinetic constants obtained for TEM-151 and TEM-152 enzymes are also shown [32]. Catalytic efficiencies of these TEM enzymes against oxyimino-β-lactams are closer to those of their corresponding ESBLs. Hydrolytic activitiy of CMTs against cephalothin is similar to that of the IRT enzyme TEM-36, while that observed in the parental ESBLs were considerably higher. In contrast, K_m values were similar for CMTs, ESBLs and IRT enzymes. Finally, these and other CMT-enzymes are also less resistant to inhibitors than their respective IRTs; their increased susceptibility to inhibitors may explain the small difference observed between the MICs of β-lactams compared to the same antibiotics associated with inhibitors [27, 29].

SHV β-lactamases

SHV-1, a broad-spectrum β-lactamase, was initially described in *K. pneumoniae* and later in other members of the *Enterobacteriaceae* [33-35]. Like TEM β-lactamases, SHV-variants derived from plasmidic SHV-1 have emerged with changes in the spectrum of activity towards different substrates, forming part of groups 2be and 2br. There are several published reviews summarizing the kinetic properties of SHV enzymes [36-38].

Until the early or mid 1990´s, SHV-type ESBLs were the most frequently described ESBLs [39, 40]; however they have now been displaced by the CTX-M enzymes [36]. Although 128 SHV variants are described in the Lahey's Institute webpage: (http://www.lahey.org/Studies/), not all of them have a defined

Table 5. Kinetic Parameters of Different TEM β-lactamases [32]

	TEM-1			TEM-36			TEM-29			TEM-151			TEM-28			TEM-152		
	BSBL			IRT			ESBL			CMT			ESBL			CMT		
Antibiotic	k_{cat}	K_m	k_{cat}/K_m	k_{cat}	K_m	k_{cat}/K_m	k_{cat}	K_m	k_{cat}/K_m	k_{cat}	K_m	k_{cat}/K_m	k_{cat}	K_m	k_{cat}/K_m	k_{cat}	K_m	k_{cat}/K_m
PEN	1,5	34	44	800	60	13.3	235	12	19.6	251	25	10	243	17	14.3	196	15	13
AMX	1,125	15	75	624	113	5.5	91	26	3.5	149	40	3.7	88	12	7.3	258	48	5.4
TIC	135	36	3.8	176	130	1.3	42	14	3	60	21	2.9	38	9	4.2	51	36	1.4
PIP	1,25	55	2.3	1,216	170	7.1	336	39	8.6	172	44	3.9	213	27	7.9	306	50	6.12
CEF	165	242	0.7	18.4	335	0.05	126	205	0.61	15	330	0.04	194	288	0.67	4	260	0.02
AZT	<0.1	ND	-	<0.1	ND	-	2.1	34	0.06	8.3	83	0.1	43	75	0.57	3.5	22	0.16
CAZ	<0.1	ND	-	<0.1	ND	-	10	215	0.05	5	250	0.02	64	240	0.27	16	230	0.07
CTX	<0.1	ND	-	<0.1	ND	-	14	345	0.04	5	304	0.02	9	50	0.18	1	34	0.03
FEP	<0.1	ND	-	<0.1	ND	-	17	145	0.11	2.5	126	0.02	8	72	0.11	2.8	45	0.06

(PEN) Penicillin G, (AMO) Amoxicillin, (TIC) Ticarcillin, (PIP) Piperacillin, (CEF) Cephalothin, (AZT) Aztreonam, (CAZ) Ceftazidime, (CTX) Cefotaxime, (FEP) Cefepime.
(ND) No detectable hydrolysis, (-) Not determinable.
k_{cat} (s-1), K_m (μM), k_{cat}/K_m (s^{-1}. μM^{-1}).

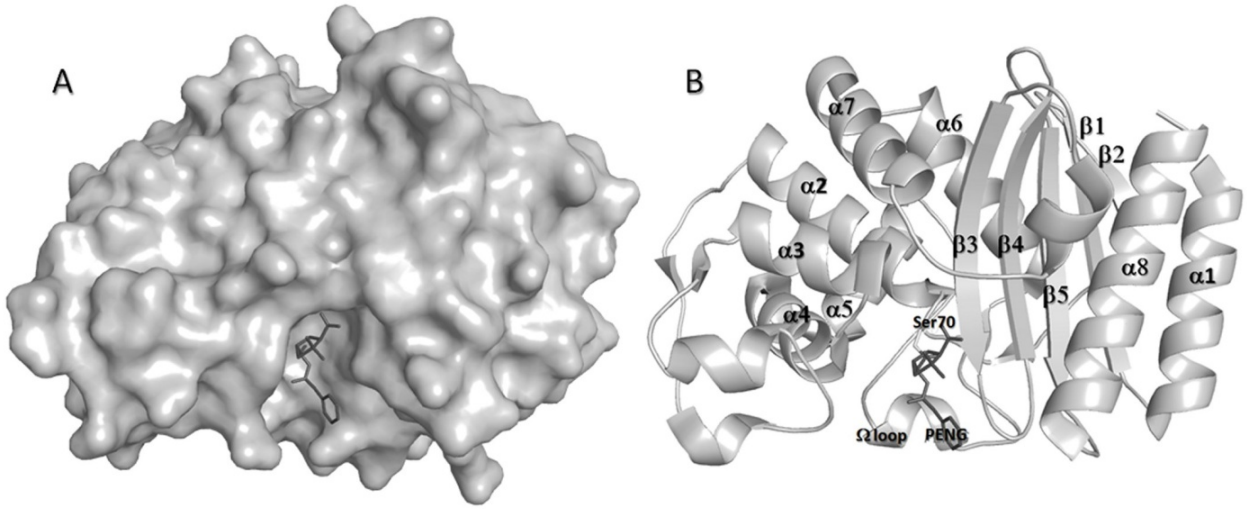

Fig. (1). A) TEM-1 β-lactamase in association with benzyl-penicillin, showing the dimension and location of the active cavity; **B**) Cartoon representation of TEM-1 β-lactamase in association with benzyl-penicillin (PENG), displaying the specific location of the active site serine Ser70 (S70). Secondary structures are also represented: alpha helices (a), β sheets (β), and the Ω loop.

phenotype. Of these, 37 are considered 2be enzymes, 30 are 2b enzymes and only 6 are described as 2br [41]. The most common amino acid changes are summarized in Table **6**.

The first plasmidic extended-spectrum SHV was recovered in Germany in 1983, from *Klebsiella ozaenae* and displayed a Gly238Ser substitution. This substitution alone accounts for the extended-spectrum properties of this ß-lactamase, designated SHV-2 [42]. From that point, the same ESBL and other allelic variants (mainly SHV-5) were found in *Klebsiella pneumoniae* isolated worldwide [40]. Even though SHV-type ESBLs were initially found in *K. pneumoniae*, case reports and outbreaks involving different *Enterobacteriaceae* and non-fermenting gram negative bacilli producing SHV-ESBLs are well documented [43-51].

SHV-1 (and other close related enzymes like PIT-2 and LEN-1) [34, 52] genes are found on the chromosome of *K. pneumoniae* and are considered the ancestor of SHV-ESBLs. However, it cannot be completely ruled out that microevolution within their chromosomal location has occurred for example, SHV-11 [53]) or even the carbapenem hydrolyzing SHVs (SHV-38, [54]) which have been found on chromosomal loci. In silico analysis of the fully sequenced *K. pneumoniae* (GenBank: CP000647) reveals the presence of bla_{SHV-11}.

The conserved nucleotide changes in bla_{SHV} that result in different phenotypes for the SHV variants occur at fewer positions within the structural gene compared to TEM ESBLs. The majority of SHV variants possessing an ESBL phenotype are characterized by the Gly238Ser substitution. A number of variants related to SHV-5 also contain a Glu240Lys substitution. Both changes are critical in determining the phenotype 2be; Ser-238 and Lys-240 have an important role in the hydrolysis of ceftazidime and cefotaxime by SHV-type ESBLs, respectively [55]. Interestingly, both substitions are frequently present among TEM-type ESBLs.

SHV-10 has been reported as the first SHV ß-lactamase with an inhibitor-resistant phenotype and is designated as IRS (**I**nhibitor **R**esistant **S**HV). This enzyme has the same nucleotide substitutions present in the extended-spectrum SHV-9 (also called SHV-5a) but contains an additional Gly130Ser substitution [56]. It is interesting to note that currently, none of the 2br SHV enzymes show this amino acid change. These data suggest that Ser-130 has an important role in the interaction with cephalosporins [56]. Changes associated with IRTs (i.e. Met to Ile, Leu, or Val at position 69, Arg to Cys, Ser, His or Gly at position 244, Arg to Glu and Leu at position 275, and Asn to Asp at position 276) have not been observed in the currently described inhibitor-resistant SHVs.

Sequence data (in addition with evolutionary analysis) confirms the chromosomal origin of bla_{SHV} genes, whose mobilization probably involves IS*26*, on at least two separate occasions. In addition, these two main ESBL bla_{SHV} branches probably evolved from separate chromosomal ancestors [57]. Analysis of genetic environments associated with plasmidic bla_{SHV} genes include in most cases the presence of the insertion sequence IS*26* often associated with transposons or integrons. The presence of IS*26* has been associated with the most common SHV ESBL genes: bla_{SHV-2}, bla_{SHV-2a}, bla_{SHV-5} and bla_{SHV-12} [58-61]. In a strain of *K. pneumoniae*, the plasmidic bla_{SHV-5} gene was flanked on both sides with a defective copy of an IS*26*-like element, reminiscent of Tn*2680*. In addition, the plasmidic bla_{SHV-5} was associated with a 7.9 kb region homologous to part of *K. pneumoniae* chromosome. These data strongly suggest that the orign of bla_{SHV} clearly points to the chromosome of *K. pneumoniae* [62]. bla_{SHV-5} has also been associated with a class 1 integron (In-t3) containing different genes that confer aminoglycoside resistance (*aacA4*, *aacC1*, and *aadA1*) in addition to IS*26* [63].

THE NATURAL EXTENDED SPECTRUM β-LACTAMASES

The CTX-M Family

First reports and Global dissemination: CTX-M Family of ESBLSa

This family was named following the first report of CTX-M-1 (after "CefoTaXimase Munich") and MEN-1, that (both having the same sequence) were found independently in Germany and France in different bacterial species [64-67]. Although CTX-M-1/MEN-1 were the first sequenced bla_{CTX-M} type genes, the first identification of a CTX-M type enzyme is most likely the report of FEC-1, by Matsumoto *et al*, which was able to hydrolyze cefotaxime more efficiently than ceftazidime [68, 69].

Since the initial identification of CTX-M enzymes, at least five genetically distinct groups have been identified from isolates around the world, including over 120 representatives, both plasmid-borne and chromosome-encoded enzymes (www.lahey.org/Studies; [5, 69a]). Although the first isolates were reported in the late 80s, the majority of isolates producing CTX-M enzymes were not detected until the CLSI (formerly NCCLS) changed its recommendations for the detection of ESBLs. Initially, ESBL confirmatory tests did not recommend the use of both cefotaxime and ceftazidime, but

Table 6. Most Frequent Amino Acid Substitutions Occurring in Broad-spectrum, Extended-spectrum and Inhibitor-resistant SHV β-lactamases

Position SHV-1	8 I	35 L	43 R	129 M	234 K	238 G	240 E
2be (n=37)	F (5)	Q (10)	S (5)	V (2)	-	S, A (19, 3)	K, R (17, 1)
2b (n=30)	F[a] (1)	Q (14)	S[a] (1)	V (2)	R (1)	S[b] (3)	K[b] (1)
2br (n=6)	F (1)	Q (1)	-	-	R (3)	S[c] (1)	K[c] (1)

[a] Simultaneous changes in SHV-14, [b] SHV-22, and [c] SHV-10

only ceftazidime. Because the majority of CTX-M enzymes hydrolyze cefotaxime more efficiently than ceftazidime these ESBLs went undetected. The new CLSI guidelines resulted in the identification of several types of CTX-M ESBLs.

Plasmid-borne CTX-M ESBLs are class A serine β-lactamases conferring high-level resistance to most β-lactams, including amino-penicillins (ampicillin and amoxicillin), carboxy-penicillins (carbenicillin and ticarcillin) and the ureido-penicillin, piperacillin, and first- and second-generations cephalosporins such as cephalothin, cephaloridine and cefuroxime. Regarding the extended-spectrum cephalosporins, or oxyimino-cephalosporins, CTX-M producing organisms are generally resistant (or have elevated MICs) to cefotaxime or ceftriaxone, while ceftazidime may remain in the susceptible range even when some CTX-M variants can hydrolyze ceftazidime. Generally, CTX-M producers are associated with increased MIC values for aztreonam and cefepime, although they may remain in the susceptible range. CTX-M producing organisms remain susceptible to cephamycins (cefoxitin) and carbapenems (imipenem, meropenem, ertapenem). As other class A ESBLs, they are well inhibited by β-lactamase inhibitors like clavulanate, sulbactam and tazobactam.

The majority of CTX-M enzymes can be found in frequent hospital- and community-associated pathogens as *E. coli*, *P. mirabilis*, *K. pneumoniae*, *E. cloacae*, *M. morganii* and other species of *Enterobacteriaceae*, but CTX-M enzymes have been found in a variety of other species including *P. aeruginosa*, *Aeromonas* sp, and even *Vibrio cholerae* [70-73].

Today, CTX-M-producing organisms are easily detected as potential ESBL producers by the CLSI screening methods [74], and most automated methods can detect these isolates efficiently. In our hands, the same methods used to identify CTX-M-producing enterobacteria have been used for other species in addition to *E. coli*, *K. pneumoniae* and *Proteus*, thus extending the utility of the tests. However, organisms with an inducible AmpC can be more easily characterized if other indicator drugs are used such as cefepime or cefpodoxime [75].

In the paragraphs that follow each CTX-M cluster will be discussed with regards to the number of family members and the epidemiology of CTX-M producers. These sections will be followed by a brief discussion of the molecular and kinetic characteristics of this large group of enzymes.

General, Clinical and Epidemiological Aspects of the CTX-M Clusters (Table 7 and Fig. 2)

CTX-M-1 (-3) cluster

CTX-M-1-type enzymes display a typical "cefotaximase" profile, with efficient hydrolysis (measured originally V_{max}/K_m) towards aminopenicillins, first- and second-generation cephalosporins (not cephamycins), and some oxyimino-cephalosporins such as cefotaxime and ceftriaxone, with ceftazidime, ceftizoxime and imipenem the most stable tested antibiotics.

CTX-M-3, which is identical to the mature enzyme FEC-1 and CTX-M-66 (GenBank ABQ45409), is a variant of CTX-M-1 which differs at only four amino acid positions, and was first recognized in Poland in 1996 from *Enterobacteriaceae* isolates [76]. From this first appearance, CTX-M-3 began to disseminate within Poland and was quickly detected in a wide range of countries. Many enterobacterial species served as hosts including *E. coli*, *K. pneumoniae*, *E. cloacae*, *C. freundii*, *M. morganii*, *S. marcescens*, and *Salmonella* Typhimurium [5, 77], showing that the gene could be easily disseminated [78]. Today, CTX-M-3 is found all over the world including many countries in Europe, Asia, Oceania and Africa. Other prevalent CTX-M-1-related β-lactamases include CTX-M-1 [79-82], CTX-M-32 [80, 83, 84], and CTX-M-15 (also known as UOE-1), which has been described in many European, Asian, African, North and South American countries, as well as Australia [80, 85-113].CTX-M-1, CTX-M-3 and CTX-M-15 have been associated with well described outbreaks in several locations [78, 87, 90, 114-118]. Other members of the CTX-M-1 cluster include CTX-M-10-12, -22, -23, -28-30, -32-34, -36, -37, -42, -52-55, -57, -58, -60-62, -64, -66, -68, -69, -71-73, -79, -80, -82, -88, KLUC-2, with CTX-M-55 and -57 having identical mature proteins.

Although KLUC-1 (from *Kluyvera cryocrescens)* was originally proposed as an ancestor from which this cluster evolved [119], other enzmes have been found to have direct chromosomal counterparts; a $bla_{CTX\text{-}M\text{-}3}$ gene was found in the chromosome of an environmental *K. ascorbata* [120], and $bla_{CTX\text{-}M\text{-}37}$ as a chromosomal gene in a *K. cryocrescens* strain isolated from a urinary tract infection from an outpatient [121]. Therefore, it is more likely that the genes encoding these individual enzymes were directly recruited from their chromosomal counterparts in different kluyveras (and, possibly, microevolution afterwards). KLUC-2, a plasmid-borne β-lactamase found in an *Enterobacter cloacae* has been described as a "mutant" of KLUC-1 [122]).

CTX-M-2 Cluster

CTX-M-2 was the first reported member of this group of related enzymes, and was present in isolates as early as the late 1980s. CTX-M-2 enzymes were first detected by phenotypic analysis in different cefotaxime-resistant *Salmonella* serovars from different pediatric hospitals in Argentina that produced an enzyme of pI > 8 that hydrolyzed cefotaxime very efficiently and was susceptible to inhibitors. The gene encoding CTX-M-2 was reported as plasmid-borne, having 84% identity with CTX-M-1, and 78% identity with the chromosome-encoded β-lactamase from *Klebsiella oxytoca* [66, 123].

CTX-M-2 is considered one of the most prevalent ESBLs in South America especially in Uruguay, Peru, Bolivia, Paraguay and

Argentina. This enzyme is associated with both hospital and community settings, and found in different enterobacterial species, *Pseudomonas* spp., as well as many less prevalent species [75, 124].

Today, nearly 85% of the oxymino-cephalosporin resistant Gram-negative pathogens which are isolated in different hospitals from Argentina and neighboring countries produce at least one CTX-M-derived β-lactamase, mainly CTX-M-2 (SIR, AAM: http://www.aam.org.ar). These enzymes have also been found in isolates collected from very different geographic regions [5, 40, 75, 125-135]. Nosocomial outbreaks involving CTX-M-2-producing strains have also been documented [124, 136-139]. Other CTX-M-2-derived ESBLs include CTX-M-44 (formerly known as TOHO-1), CTX-M-4 [140], CTX-M-5 [141], CTX-M-6 [142] and CTX-M-31 [75]. TOHO-1 was first detected in a cefotaxime-resistant *Escherichia coli* strain isolated in 1993, from patients in Japan, and was 83% identitcal to MEN-1) [69],

Additional plasmid-borne members of the CTX-M-2 group which are not prevalent include: CTX-M-7, -20, -35, -43, -56, -59, -74, -77. Several chromosome-encoded cefotaximases from *Kluyvera* also belong to this cluster including KLUA-1-6, -8-12 [143] and, more recently, CTX-M-76 and CTX-M-77 (all from *K. ascorbata*) [144]. However, the mature form of the enzymes KLUA-1, -3, -4 and -12 are identical and the mature forms of KLUA-2, -6, CTX-M-5, and CTX-M-2, CTX-M-75 are also identical.

CTX-M-8 Cluster

This cluster has only a few representatives of which CTX-M-8 was the first to be isolated. It was detected in three AmpC-producing enterobacteria (*E. cloacae*, *E. aerogenes* and *C. amalonaticus*) isolated in Brazil [145]. At the present time members of the group include CTX-M-40 [146], CTX-M-41 [147], and CTX-M-63 (AB205197). However, the amino acid sequences for the two latter are identical, therefore CTX-M-63 should be removed and renamed, CTX-M-41. KLUG-1, a chromosomal β-lactamase from *Kluyvera georgiana* has been proposed as the putative origin of this cluster [148].

CTX-M-9 Cluster

CTX-M-9 related enzymes are the second major cluster, representing 25 plasmid-encoded members at the present time. The dominance of these enzymes began by the end of the 1990s. CTX-M-9 was the first reported enzyme of this group and was detected in *E. coli* isolated in 1996 in Spain. One year later, four *Salmonella* Virchow isolates were collected [149, 150], while, at the same time CTX-M-9-type producers were collected in Brazil, including a novel member: CTX-M-16 [151].

The other widely disseminated member of this group is CTX-M-14, which has been found in isolates collected from Europe, North and South America, Asia and Africa [80, 81, 100, 107, 130, 152-161]. Although initially some of these enzymes were named differently such as UOE-2 and TOHO-3 these names are no longer used. Additional plasmidic members of the CTX-M-14 group in-

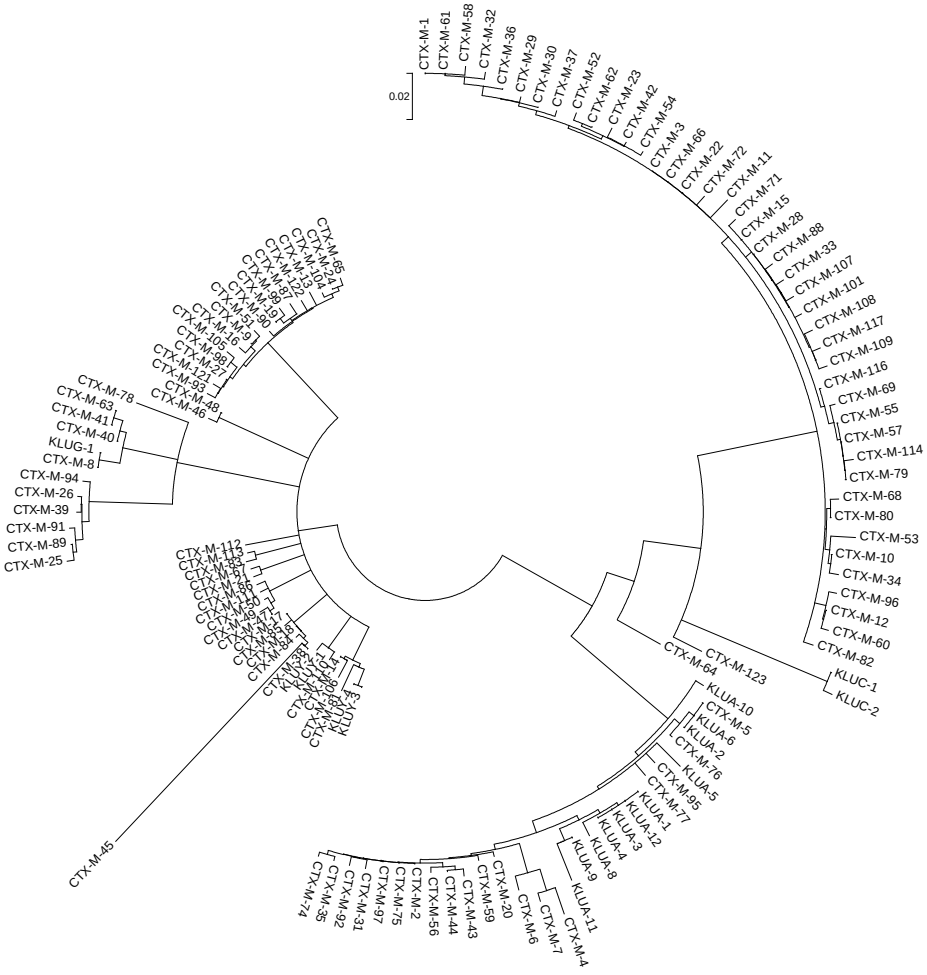

Fig. 2. Phylogenic relationship within chromosome- and plasmid-encoded CTX-M/KLU β-lactamases showing the five clusters: CTX-M-3, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25, as well as un-clustered members.

clude CTX-M-13, -16-19, -21, -24, -27, -38, -46-51, -65, -67, -81, and -83-87. This group includes as chromosomal-encoded counterparts KLUY-1-4, observed in isolates of *Kluyvera georgiana* from Guyana [162]. Identical mature β-lactamases clustered in this group which deserve nomenclature re-examination are CTX-M-14, -18 and KLUY-1; and KLUY-3-KLUY-4.Well characterized outbreaks with isolates producing these enzymes have been reported from humans as well as poultry [163-165] [166].

CTX-M-25 Cluster

CTX-M-25 and CTX-M-26 were the first enzymes described in *E. coli* isolated in Canada, and a *K. pneumoniae* from the United Kingdom, respectively [167, 168]. This group of β-lactamases contains six members including CTX-M-25, CTX-M-26, CTX-M-39, -89, -91, and CTX-M-78, and a chromosome-encoded β-lactamase from a strain of *Kluyvera georgiana* isolated from a bloodstream infection in Louisville, USA, in 2002 [106, 128, 147, 169].

Difficult to Cluster CTX-Ms

Some CTX-M β-lactamases are difficult to group into the existing sub-families based on protein alignments. The most peculiar of these is CTX-M-45, formerly known as TOHO-2[170]. This enzyme displays a frame shift mutation and successive micro deletions that recover the natural reading frame after the sixth base is lost. This set of amino acid changes and deletions results in a protein with an internal short peptide with no amino acid identity with other CTX-M family members. Nucleotide alignments, however, place it close to CTX-M-14 (CTX-M-9 cluster). CTX-M-64 has been described as a "hybrid" β-lactamase. This enzyme may be the result of homologous recombination between a $bla_{\text{CTX-M-14}}$-like (CTX-M-9 cluster) and a $bla_{\text{CTX-M-15}}$-like (CTX-M-3 cluster) [171].

Molecular Aspects of the CTX-M β-lactamases:

Unlike the classical "mutation-born" ESBLs (TEM, SHV), pre-existing chromosome-encoded ESBLs residing in the genome of different members within *Kluyvera* are the main source of plasmidic CTX-M β-lactamases. Low level expression of CTX-M/KLU β-lactamases in *Kluyvera*, in which the $bla_{\text{CTX-M}}$ genes are chromosomaly located, accounts for the susceptible phenotype when tested against gram negative active β-lactams. However, transformation of these chromosomal genes into *E. coli* led to the discovery that these enzymes were "natural" cefotaximases [120].

In general, horizontal transfer of plasmid-borne $bla_{\text{CTX-M}}$ genes is mediated by conjugative plasmids carrying additional resistance markers, and generally associated with transposons and/or integrons [5]. Several mechanisms have been reported and are involved in both recruitment and mobilization of these genes (Table **7**).

Genes encoding CTX-M-1 family members have been found associated with IS*Ecp1*-like insertion sequences (belonging to the IS*1380* family), commonly plasmid-harbored elements that are located upstream of the $bla_{\text{CTX-M}}$ and are able to mediate transposition of flanking DNA regions by a one-ended mechanism [174]. This is the only experimentally evaluated transposition mechanism involved in the mobilization of $bla_{\text{CTX-M}}$ genes [174, 175]. $bla_{\text{CTX-M}}$ genes associated with the IS*Ecp1*-like insertion sequence include $bla_{\text{CTX-M-1, -3, -10, -12, -15, -28, -32, -42, -54, -55, -57, -62, -64, -79}}$ (group 1); $bla_{\text{CTX-M-5, -20}}$ (group 2); $bla_{\text{CTX-M-9, -13, -14, -17, -19, -21, -24, -27, -65}}$ (group 9), $bla_{\text{CTX-M-25, -26, -89}}$ (group 25).There are several IS*Ecp1*-linked genes that are also associated with the IS*903D* insertion sequence located downstream of the CTX-M gene. This arrangement (IS*Ecp1*-$bla_{\text{CTX-M}}$-IS*903D*) is thought to be part of a potential transposon structure that probably facilitates the dissemination of the embedded $bla_{\text{CTX-M}}$ [80]. Known IS*903D*-associated $bla_{\text{CTX-M}}$ genes belong to group 9 and include $bla_{\text{CTX-M-17, -19, -24, -27, -65}}$, and $bla_{\text{CTX-M-54}}$ (cluster 1) [176].

The second most prevalent genetic element associated with $bla_{\text{CTX-M}}$ genes is the IS*CR1* element, formerly known as the orf513-common region, which is normally found within the backbone of unusual or complex class 1 integrons [177, 178]. The IS*CR1* element is usually located upstream of $bla_{\text{CTX-M}}$ genes and has been found associated with $bla_{\text{CTX-M-2,}}$ and $bla_{\text{CTX-M-59}}$ from group 2 [71, 179-182], (GenBank EU622856) and $bla_{\text{CTX-M-9}}$ and $bla_{\text{CTX-M -14}}$ from group 9 [178, 183-185], and $bla_{\text{CTX-M-1}}$ from group 1 [186]. Structures harboring IS*CR1*-associated integrons are usually related to *Tn*402 derivatives, and sometimes linked with *Tn*21 or *Tn*1696 transposons. These transposons are widely disseminated among both clinical and environmental Gram-negative bacteria and carried by large conjugative plasmids [187]. Furthermore, mobilization of some *Tn*21-harbored $bla_{\text{CTX-M}}$ genes has been suggested by some authors [71, 188] and is probably mediated by insertion sequences associated with the transposon, such as IS*4321* (IS*1111* family).

Both IS*Ecp1* and IS*CR1* (orf513) elements are thought to play important roles in the recruitment and mobilization of resistance markers by conjugative plasmids. The $bla_{\text{CTX-M-10}}$ gene (cluster 1) was found in association with both IS*Ecp1* and phage-related sequences, suggesting that the occurrence of phage-mediated recruitment/mobilization mechanisms was also possible [189]. Finally, $bla_{\text{CTX-M-53}}$ (cluster 1) was found associated with a unique structure deduced as a putative relaxase/mobilization nuclease with unknown function in the recruitment or mobilization of the associated gene [190].

Gene Expression of bla_{CTX-M}

Experimental determination of $bla_{\text{CTX-M}}$ promoters has been limited, although *in silico* putative promoters have been identified. In general, experimentally determined promoters have similar structures as the promoters examined *in silico*. For example, expression of IS*Ecp1*-associated $bla_{\text{CTX-M}}$ genes seems to be enhanced by promoter's -10 and -35 sequences embedded in the 3' region of the insertion sequence [191]. For some $bla_{\text{CTX-M-2}}$ family members related to IS*CR1*, two putative promoters have been identified [192].

Biochemical and Functional Properties of CTX-M/KLU β-lactamases

Mature CTX-M β-lactamases contain 291 amino acids, resulting in proteins of about 30 kDa, and basic isoelectric points between 7.4 and 9.0. They possess the typical conserved motifs for the class A serine-β-lactamases [3, 193], including the active site serine motif, Ser^{73}-Thr-Ser-Lys^{76}, a Ser^{133}-Asp-Asn^{135} (however for CTX-M-81 the conserved sequence is Ser-Asp-His), and a Lys^{238}-Thr-Gly^{240}. In addition, the Ω-loop seems to be located between Arg^{164}-Asp^{182}, containing the sequence Glu^{169}-X-Thr-Leu-Asn^{173}, in which the Glu^{169} is directly involved in the hydrolysis of the β-lactam moiety. Kinetic data available for members of the five clusters include CTX-M/KLU: CTX-M-1, -3, -12, -15, -32, -54, -64, -71, -72, -80 (cluster 1); CTX-M-2, -5, -43, -44 (TOHO-1), KLUA-9 (cluster 2); CTX-M-8 (cluster 8); CTX-M-9, -14, -16, -18, -19, -27, -65, -81, -87 (cluster 9); CTX-M-25, -26, -78 (cluster 25); CTX-M-45 (TOHO-2) (un-clustered members). Some representative enzymes are shown in (Tables **8**-**10**).

As described above, CTX-M β-lactamases exhibit a natural oxyimino-cephalosporinase activity which, when produced in clinical isolates other than their natural host can result in high MICs values for cefotaxime/ceftriaxone and for some variants, ceftazidime. These enzymes also efficiently hydrolyze penicillins and first-generation cephalosporins [5]. However, compared to other ESBLs, CTX-M β-lactamases hydrolyze penicillins less effectively than TEM/SHV ES-variants [5]. In addition, the hydrolysis of ceftazidime is also much higher in TEM, SHV or PER β-lactamases compared to CTX-M enzymes [24, 207], even though these variants are considered "ceftazidimases". Cefotaxime is a much better substrate for CTX-M enzymes than ceftazidime (even in the so called

Table 7. CTX-M-family of β-lactamases. Worldwide Reports, Origin, Genetic Association and Biochemical Characterization

	CTX-M cluster					
	CTX-M-1/3	**CTX-M-2**	**CTX-M-8**	**CTX-M-9**	**CTX-M-25**	**Un-clustered**
First report						
Enzyme	FEC-1[a]/CTX-M-3[a]	CTX-M-2	CTX-M-8	CTX-M-9	CTX-M-25, CTX-M-26	CTX-M-45 (TOHO-2)
Year	1986	1990	1996-1997	1996	2000 and 2002	1998
Country	Japan	Argentina	Brazil	Spain	Canada and UK	Japan
Bacterial species	*Escherichia coli*	*Salmonella* Typhimurium	*E. cloacae*, *E. aerogenes* and *C. amalonaticus*	*Escherichia coli*	*Escherichia coli* / *K. pneumoniae*	*Escherichia coli*
Clinical aspects						
First clinical report						
Enzyme	CTX-M-1/MEN-1	CTX-M-2	CTX-M-8	CTX-M-9	CTX-M-25, CTX-M-26	CTX-M-45 (TOHO-2)
Year	1989	1990	1996-1997	1996	2000 and 2002	1998
Country	Germany/France	Argentina	Brazil	Spain	Canada and UK	Japan
Bacterial species	*Salmonella Typhimurium/K. oxytoca*	*Salmonella* Typhimurium	*E. cloacae*, *E. aerogenes* and *C. amalonaticus*	*Escherichia coli*	*Escherichia coli* / *K. pneumoniae*	*Escherichia coli*
Outbreak implication						
Enzyme (Country)	CTX-M-1 (Spain), CTX-M-3 (Poland, Japan), CTX-M-15 (France, Sweden, Norway, Tunisia, Korea)	CTX-M-2 (Argentina, Japan, Brazil)		CTX-M-9 (France), CTX-M-14 (Korea, Canada), CTX-M-27 (Tunisia)	CTX-M-26 (UK)	
Epidemiological data[$]						
Dissemination (countries)	Europe, Asia, Africa, Oceania, America (see text)	South America, Japan, Israel, Europe (Italy, France, Belgium, UK), Turkey, South Africa	Brazil, Israel, UK, Thailand, Tunisia	Europe, Asia, Africa, North and South America (see text)	UK, Israel	
Enzymes	CTX-M-1/3[a]/10-12/15[b]/22/23/28-30/32/33[c]/34/36/37/42/52-55[a]/57[a]/58/60-62/64/66[a]/68/69/71-73/79/80/82/88	CTX-M-2/4-7[d]/20/31/35/43/44[e]/56/59/74-77	CTX-M-8/40/41/63	CTX-M-9/13/14/16-19/21/24/27/38/46-51/65/67/81/83-87/90	CTX-M-25/26/39/78/89/91	CTX-M-45/64
Most prevalent β-lactamase	CTX-M-1, CTX-M-3, CTX-M-15, CTX-M-32	CTX-M-2	CTX-M-8	CTX-M-9, CTX-M-14, CTX-M-16		
Recruitment / genetics						
Association with (genetic element)	IS*Ecp1* / IS*26* / IS*CR1* / phage-related sequences	IS*CR1* / IS*Ecp1*		IS*CR1* / IS*Ecp1* / IS*903D*	IS*Ecp1*	
Available data on genetic environment	$bla_{CTX\text{-}M\text{-}1, -3, -10, -12, -15, -28, -32, -42, -54, -57, -62, -64, -79}$ (IS*Ecp1*)	$bla_{CTX\text{-}M\text{-}5, -20}$ (IS*Ecp1*)		$bla_{CTX\text{-}M\text{-}9, -13, -14, -17, -19, -21, -24, -27, -55, -65}$ (IS*Ecp1*)	$bla_{CTX\text{-}M\text{-}25, -26, -89}$ (IS*Ecp1*)	
	$bla_{CTX\text{-}M\text{-}1}$ (IS*CR1*)	$bla_{CTX\text{-}M\text{-}2, -59}$ (IS*CR1*)		$bla_{CTX\text{-}M\text{-}9, -14}$ (IS*CR1*)		
	$bla_{CTX\text{-}M\text{-}10}$ (phage-related sequences)			$bla_{CTX\text{-}M\text{-}17, -19, -24, -27, -65}$ (IS*903D*)		
	$bla_{CTX\text{-}M\text{-}53}$ (relaxase/mobilization nuclease?)					

(Table 7) Contd....

	CTX-M cluster					
	CTX-M-1/3	**CTX-M-2**	**CTX-M-8**	**CTX-M-9**	**CTX-M-25**	**Un-clustered**
Origin						
Kluyvera spp.	*K. ascorbata* / *K. cryocrescens*	*K. ascorbata*	*K. georgiana*	*K. georgiana*	*K. georgiana*	*K. cryocrescens*
Known chromosome-encoded counterpart	$bla_{CTX-M-3}$ (*K. ascorbata*)	$bla_{CTX-M-76}$ (*K. ascorbata*)	bla_{KLUG-1} (*K. georgiana*)	$bla_{KLUY-1-4}$ (*K. georgiana*)	$bla_{CTX-M-78}$ (*K. georgiana*)	bla_{KLUC-1} (*K. cryocrescens*)
	$bla_{CTX-M-37}$ (*K. cryocrescens*)	$bla_{CTX-M-77}$ (*K. ascorbata*)				
		$bla_{KLUA-1-6, -8-12}$ (*K. ascorbata*)				
Biochemical characterization						
Availability of kinetic data	CTX-M-1, -3, -12, -15, -32, -54, -64, -71, -72, -80	CTX-M-2, -5, -43, -44, KLUA-9	CTX-M-8	CTX-M-9, -14, -16, -18, -19, -27, -65, -81, -87	CTX-M-25, -26, -78	CTX-M-45

*Isolation or description
[$] References: [5, 24, 80, 104, 172, 173], and www.lahey.org/studies.
[a]Identical mature β-lactamases: CTX-M-3 and CTX-M-66; CTX-M-55 and CTX-M-57
[b]Alternative name: UOE-1
[c]Previously known as CTX-M-27GR
[d]Previously given the name CTX-M-5
[e]Also known as TOHO-1

Table 8. Kinetic Constants for CTX-M-1 (-3) Cluster [a]

		Amoxicillin			Piperacillin			Cephalothin			Cefotaxime			Ceftazidime			Aztreonam		
		k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)
CTX-M-1		87	10	8.7				2,450	115	21.3	317	125	2.5	1	50[b]	0.02			
CTX-M-3	$	160	185	0.86	180	66	2.7	2,800	96	29	380	113	3.4	<0.01	>3,000	ND	190	188	1
	#							114.7	163.7	0.7	47.1	71.1	0.66	ND	ND	ND	33.7	66.4	0.51
	§							408	91.3	4.47	22.5	10.3	2.18	ND	1,670[b]	0.001[d]	3.67	122	0.03
CTX-M-12								146	806	0.182	312	99.7	3.13	1.8	464.5	0.004	17.1	177.4	0.096
CTX-M-15		20	38	0.52	35	13	2.7	35	43	0.8	150	54	2.8	2	1,760	0.001	1.5	11	0.14
CTX-M-32		3[c]	8[c]	0.4				928	211	4.4	320	322	1	0.91	271	0.003	1	31	0.03
CTX-M-54		4[c]	33[c]	0.1				11	325	0.03	34	182	0.2	0.13	48	0.003	9	244	0.04
CTX-M-64		37[c]	19.5[c]	1.9				185	37.9	4.9	197	103	1.9	ND	>10,000	ND			
CTX-M-71		49	5.2	9.4	27	3.2	8.4	68[e]	18[e]	3.8	65	130	0.5	0.69	180	0.004	0.84	10	0.084
CTX-M-72								150.7	153.3	0.98	47.7	71.6	0.67	ND	ND	ND	49.4	74	0.67
CTX-M-80								189.6	160.7	1.18	47.2	49.3	0.96	ND	ND	ND			

[a]References: CTX-M-1 [65]; CTX-M-3 $[5], #[194], and §[195]; CTX-M-12 [196]; CTX-M-15 [197]; CTX-M-32 [83]; CTX-M-54 [176]; CTX-M-64 [171]; CTX-M-71 [198]; CTX-M-72 [194]; CTX-M-80 [199]
[b] Apparent K_m was determined as K_i in competitive assays using a reporter substrate
[c] Kinetic parameters for ampicillin
[d] Obtained by initial hydrolysis rates at low substrate concentrations
[e] Kinetic parameters for cephaloridine
ND: not determinable

"ceftazidimases"), due to a more favorable environment within the active site for the recognition and interaction for those substrates as compared to the bulky ceftazidime moiety [205, 208]. Stable substrates for which negligible hydrolysis is observed include the carbapenems and 7-α-methoxy-cephalosporins such as cefoxitin. Even though CTX-M production has been associated with carbapenem resistance for *P. aeruginosa*, these isolates also show decreased permeability and/or upregulated efflux systems. Lastly, inhibition by class A β-lactamase inhibitors is stronger for tazobactam than clavulanate (although for some enzymes a comparable inhibition is observed) and sulbactam [24].

The strictly conserved Ser237 amino acid has been suggested as important for the hydrolytic activity observed for the oxyimino-cephalosporins, especially cefotaxime [140, 209] In addition, another residue that seems to be involved in the modulation of oxy-

Table 9. Kinetic Constants for CTX-M-9 Cluster [a]

	Amoxicillin			Piperacillin			Cephalothin			Cefotaxime			Ceftazidime			Aztreonam		
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)
CTX-M-9	90	20	4.5	110	20	5.5	3,000	150	20	450	120	3.7	2	600	0.003	10	220	0.04
CTX-M-14	100	20	5	200	48	4	2,700	175	15.4	415	130	3.2	3	630	0.004	10	200	0.05
				39	20	2	510	27	19	1,400	41	34	nd	13,000	ND	nd	41	ND
CTX-M-16	40	10	4	45	8	5.6	2,800	83	33.7	1,400	150	9.3	15	350	0.04	3	17	0.18
CTX-M-18	10	105	0.09	15	23	0.65	7[b]	216[b]	0.03	20	54	0.37	ND	ND	-	2	286	0.007
							1,190	51	23.4	74.5	1.68	44.3	ND	5,610	ND	18.5	278	0.07
CTX-M-19	1	100	0.01	8	10	0.75	30[b]	123[b]	0.25	3	60	0.06	0.02	25	0.0001	ND	ND	ND
							882	54.8	16.1	84.8	58	1.47	ND	2,720[c]	ND	0.42	313[c]	0.0013
CTX-M-27	5	10	0.5	9	8	1.1	232	83	2.8	113	150	0.75	3	330	0.009	0.4	17	0.02
CTX-M-81							176.4	148.9	1.19	47.6	47.3	1.01	ND	ND	ND			

[a]References: CTX-M-9 [81, 151]; CTX-M-14 [81, 200, 201]; CTX-M-16 [151]; CTX-M-18 [5, 202, 203]; CTX-M-19 [202, 203]; CTX-M-27 [200]; CTX-M-81 [199]
[b] Kinetic parameters for cephaloridine
[c] Apparent K_m was determined as K_i in competitive assays using a reporter substrate
nd: Not detectable
ND: not determinable

imino-cephalosporinase activity is Arg276 located at a position equivalent to Arg244 in TEM and SHV ESBLs [210]. Insights into the structural properties of the CTX-M β-lactamases reveal some distinct characteristics compared to other class A enzymes, and supports the previous hypotheses about the relative importance of some amino acid residues. In CTX-M-44 (TOHO-1), for example, the hydroxyl group of the Ser^{237} seems to rotate due to interactions with the carboxyl group located on the substrate. Another noteworthy difference is the presence of three sulfate ions located in the vicinity of the catalytic site, one of which is tightly bound to the active site, and the other two interact with the positively charged region containing two arginine residues (Arg^{274} and Arg^{276}). This could allow the interaction of the methoxyimino moiety of the third-generation cephalosporins prior to its proper binding to the active site resulting in further hydrolysis [205]. Lys^{73} seems to be able to adopt two different and alternative conformations, one of which is the close interaction with Glu^{166} [205]. This characteristic was also observed in other CTX-M β-lactamases such as CTX-M-9 and CTX-M-14 [208].

There are a number of emergent CTX-M variants that produce up to 8-fold higher MIC values for ceftazidime, such as CTX-M-15 and -16, compared to related enzymes such as CTX-M-3 and -9, respectively Several amino acids substitutions in CTX-Ms have been suggested as responsible for the increased ceftazidime hydrolysis. One of these mutations, Asp240Gly, seen in enzymes such as CTX-M-15, -16 and -27 [151, 197, 200], leads to increased flexibility of the β3-strand remodeling the active site in order to become more accessible to the bulkier ceftazidime moiety [208]. CTX-M-15, -16 and -27 are closely related to CTX-M-3, -14 and -

Table 10. Kinetic Constants for other CTX-M Clusters [a]

	Amoxicillin			Piperacillin			Cephalothin			Cefotaxime			Ceftazidime			Aztreonam		
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)
Cluster 2																		
CTX-M-2							839	8.74	9.6	6.58	3.13	2.1	ND	15,000[d]	0.0003[e]	3.97	205	0.02
CTX-M-5							1,500[b]	350[b]	4.3	210	95	2.2	7.4	440	0.017	21	730	0.03
CTX-M-43	14.8[c,d]	21[c]	0.7	4.1	9.7[d]	0.4	11.3	10.2	1.1	70	30	2.3	ND	1,600[d]	ND	28	4.5	0.6
CTX-M-44				13	8	1.7	480	39	12	250	120	2.1	21	7,900	0.0013			
KLUA-9	7	15	0.47	5	11	0.46	56	49	1.15	3.3	43	0.08	ND	>1,000	ND	0.24	390	0.0006
Cluster 8																		
CTX-M-8	55	12	4.6	74	19	3.9	1,600	87	18.4	72	74	0.97	2	>500	ND	13	800	0.02
Cluster 25																		
CTX-M-25	5.9	7.7	0.8				230	190	1.2	101	28	3.6	33	13	2.6	84	120	0.7
CTX-M-26	12	24	0.5				530	110	4.7	120	150	0.77	0.015	3,300	ND	100	130	0.78
CTX-M-78	29.2[c]	46[c]	0.63	16.6	19	0.88	84.1	26	3.24	9.5	30	0.32	ND	ND	ND	5.3	396	0.013
Un-clustered																		
CTX-M-45	1.2	12	0.1	130	84	1.6	12,000	470	25.5	220	66	3.4	1.3	160	0.008	0.1	140	0.0007
KLUC-1	ND	30	-	ND	20	-	ND	140	-	ND	110	-	ND	5,700	-	ND	150	-

[a]References : CTX-M-2 [195]; CTX-M-5 [141]; CTX-M-43 [204]; CTX-M-44 [205]; KLUA-9 [206]; KLUC-1 [119]; CTX-M-25, CTX-M-26 [167]; CTX-M-45 [170]
[b] Kinetic parameters for cephaloridine
[c] Kinetic parameters for ampicillin
[d] Apparent K_m was determined as K_i in competitive assays using a reporter substrate
[e] Obtained by initial hydrolysis rates at low substrate concentrations
ND: not determinable

9, respectively, and are thought to have evolved through selective pressure by ceftazidime [5, 173].

Interestingly, unlike TEM or SHV ESBLs, the active site of the CTX-M β-lactamases has not been enlarged to interact with bulkier molecules like the oxyimino-cephalosporins. The expansion of their hydrolytic activities seems to rely on an enhanced mobility of the β3-strand, expanding the activity towards ceftazidime, or a direct interaction of specific amino acids (probably Ser[237] and Asn[104]) with the oxyimino side chains of third-generation cephalosporins, such as what is seen for CTX-M-27 [208]. Furthermore, the β3-strand includes both residues essential for catalytic activity (Lys[234] and Ser[237] as part of the "oxyanion" hole), and amino acids at positions 231 and 240, at either end of this secondary structure. Substitutions associated with increased hydrolytic activity towards ceftazidime, like Asp240Gly and Val231Ala, appear to result from the higher flexibility of the β3-strand, but these substitutions were also correlated with lower stability [208]. Recent studies revealed "breathing" of CTX-M β-lactamases and the implication that the Asp240Gly replacement accommodated ceftazidime. The insertion of the ceftazidime side chain deep in the catalytic domain, along with a coordinated movement of Ser[70], the β3-strand and the Ω-loop, facilitates the interaction with the antibiotic [211].

Mutations at position 167 (generally Pro[167], or 142 in the mature protein) occur in the immediate vicinity of the Ω-loop, apparently modifying the interaction with the antibiotic, especially the oxyimino-cephalosporins [202]. Nevertheless, even when mutations occurring in these positions generally lead to large increases in the MICs to ceftazidime for the producing strains, only discrete catalytic efficiency towards ceftazidime is obtained [5, 173]. Therefore, the term "ceftazidimases" that is currently applied for these CTX-M variants should be used carefully. It has also been noted that Asp240Gly substitutions have been selected more frequently than mutations in Pro[167] (generally Pro167Ser or Pro167Thr), probably because modifications in residues comprising the Ω-loop result in a significant decrease in the catalytic efficiencies [173].

PER β-lactamases:

At the present time there are 6 members of the PER β-lactamase family with PER-1 and PER-2 being the most prevalent family members (Fig. **3**). PER-1 was identified in a *Pseudomonas aeruginosa* strain ("*Pseudomonas* Extended Resistance") isolated from the urinary tract of a hospitalized patient in France in 1991 [212]. PER-1 has been responsible for oxyimino-cephalosporin resistance in clinically-important enterobacteria and non-fermenter Gram-negative bacilli isolated in different locations worldwide [212-225].

PER-2 shares 86% amino acid sequence identity with PER-1 and accounts for 10% and 5% of the oxyimino-cephalosporin resistance observed for *K. pneumoniae* and *E. coli* isolates respectively from Argentina [75]. The first PER-2 producing isolate can be traced back to a *Proteus mirabilis* strain isolated in Argentina in 1989, which was at that time named ARG-1 (M. A. Rossi, G. Gutkind, M. Quinteros, *et al*., Abstr. 31[st] ICAAC, abstr. 939, 1991). However, the gene sequence was described as bla_{PER-2} in a ceftibuten-resistant *Salmonella* Typhimurium isolate whose gene was harbored by a transferable plasmid [226]. Since its first report, PER-2 has been found in other species including *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Enterobacter aerogenes*, and *Vibrio cholerae* and community-acquired enteropathogenic *Escherichia coli* (EPEC) isolates in Argentina and Uruguay [75, 227-229], and

PER-producing isolates have also been found in other countries worldwide [127, 230, 231].

Biochemical and genetic analyses in addition to the crystal structure of PER-1 have been reported [212, 218, 232, 233]. The gene encoding PER-1 is 927 bp and encodes for a 308 amino acid protein which has less than 30% amino acid identity with the known TEM or SHV ESBLs [234]. The enzyme is a 29kDa protein with a pI of 5.4 and displays high catalytic efficiencies for both penicillins and cephalosporins (classical and oxyimino-cephalosporins), with high K_m values for cephaloridine and third-generation cephalosporins (particularly ceftazidime) and high k_{cat} values; resulting in catalytic efficiencies comparable to those for TEM or SHV ESBLs [232, 235]. This explains the high level resistance to β-lactam drugs, especially ceftazidime, in PER-1 producing isolates.

The mature PER-2 β-lactamase possesses a molecular mass of 30,780 Da and a pI of 5.4, with a predicted signal peptide of 26 amino acids [207]. Like PER-1, PER-2 has high catalytic efficiencies (k_{cat}/K_m) towards most of the tested antibiotics, generally characterized by low K_m and high k_{cat} constants (Table **11**). PER-2 has similar catalytic efficiencies for both ceftazidime and cefotaxime although the mechanism for this high efficiency differs. PER-2 has a 7-fold higher affinity for cefotaxime compared to ceftazidime but the turnover constant (k_{cat}) for ceftazidime is 4-fold higher [207]. According to Bouthors *et al*. [232], PER-1's k_{cat}/K_m values for both cephalosporins are one order magnitude lower than PER-2 (0.093 and 0.026 $\mu M^{-1}.sec^{-1}$ for cefotaxime and ceftazidime, respectively), due to 10-fold higher K_m values. The most poorly hydrolyzed antibiotics were cefoxitin, cefepime and imipenem. PER-2 was strongly inhibited by lithium clavulanate and tazobactam, displaying IC_{50} values of 0.068 and 0.096 μM, respectively [207]. As observed in (Table **11**), PER-6 displays overall lower catalytic efficiencies than PER-2 for most of the β-lactams, due to significant differences in K_m values (especially for cephalosporins), and less susceptibility to inhibitors [207, 236].

The main difference observed in the 3D-structure of PER-1 and PER-2 compared to other class A β-lactamases is the new fold in the Ω loop and insertion of four amino acids residues at the S3 strand, leading to an expansion or broadening of the catalytic cavity which better accommodates the bulky moieties of some cephalosporins [233]. In addition, a modification at position 242 in both PER-1 and PER-2, representing the counterpart of the Glu240 residue in TEM or SHV β-lactamases, does not seem to result in changes in its kinetic properties as observed for TEM/SHV [207, 232].

The genetic environment of bla_{PER-1} has been elucidated from different species. In some strains it is part of composite transposons flanked by different arrangements of insertion sequences, depending on whether it is found on the chromosome or a plasmid [213, 237]. In an *Alcaligenes faecalis* strain, it was found associated with a Tn*3*-family transposon-like structure, named Tn*5393d*, which contains the *strAB* genes typical of other Tn*5393* derivatives. The authors postulate the occurrence of consecutive insertion of two composite transposons, one of them (Tn*4176*) including two non-identical and interrupted copies of the IS*1387* element (IS*1387a* and *b*) [237]. In other gram-negative microorganisms, bla_{PER-1} was identified as part of either a chromosome composite transposon, bracketed by IS*Pa12* and IS*Pa13* (equivalent to IS*1387a* and IS*1387b*, respectively), as observed in *Pseudomonas aeruginosa*, *Providencia stuartii* and *Acinetobacter baumannii*, or as a plasmid-borne gene that was associated with an upstream IS*Pa12*, in *Salmonella* Typhimurium and *A. baumannii* [213].

The genetic environment of the bla_{PER-2} gene is homologous to those associated with plasmid-borne bla_{PER-1} in *Salmonella* Typhimurium and *Acinetobacter baumannii* isolates [207, 213, 215, 237, 238]. One difference observed was the location of the upstream position of the IS elements with respect to the structural bla_{PER} genes. This difference in the positioning of the IS element could impact bla_{PER} expression [207, 213]. Downstream of bla_{PER-2} and some plasmid-encoded bla_{PER-1} lies a *gst*-like gene encoding a hypothetical glutathione-S-transferase identified in aquatic microorganisms [213], and a putative ABC-transporter encoding gene similar to that from *Shewanella oneidensis* [207]. The presence of similar structures upstream of bla_{PER} suggests a common history of recruitment and mobilization.

The lack of reports of PER-2 producing isolates outside of South America is intriguing. It is possible that detection of the gene or its enzyme is problematic thus underestimating its occurrence. Sequence differences in bla_{PER-2} may result in less efficient or no amplification using primers designed from the bla_{PER-1} sequence. Additionally, the pI of 5.4 correlates with the TEM-1 β-lactamase making detection by IEF very unlikely. The variety of genetic backgrounds (including both chromosomal and plasmid locations) could dictate the transmissibility of this gene compared to the gene encoding PER-1.

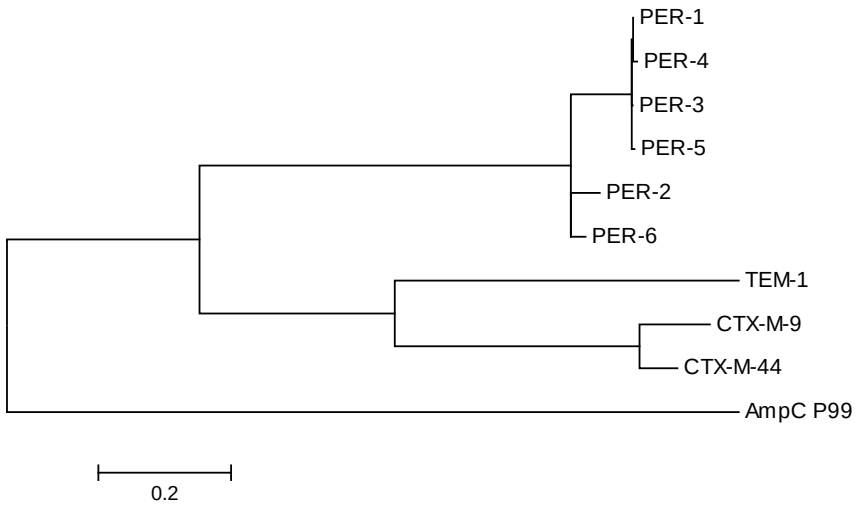

Fig. 3. Phylogenic relationship among members of PER family, compared to other types of β-lactamases.

Table 11. Main kinetic parameters of PER β-lactamases [a]

Substrate	PER-1			PER-2			PER-6		
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)
Benzylpenicillin	8	27	0.29	2	16	0.12	5	200	0.025
	4.6	24	0.19						
Ampicillin	ND	ND	ND	12	38	0.33	1	20	0.05
Piperacillin [b]	ND	ND	ND	0.04	0.2	0.2	0.1	4	0.025
Cephalothin	8	23	0.35	6	9	0.67	8	55	0.145
	9.5[c]	75[c]	0.127						
Cefoxitin [b]	ND	ND	ND	< 0.001	0.14	-	ND	ND	ND
Cefuroxime	ND	ND	ND	6	21	0.3	ND	ND	ND
Cefotaxime	41	441	0.093	34	46	0.76	40	900	0.045
	8.15	30	0.27						
Ceftazidime	109	4150	0.026	140	320	0.43	12	1,000	0.012
	24.5	806.4	0.030						
Cefoperazone	ND	ND	ND	0.5	5	0.10	ND	ND	ND
Cefepime	ND	ND	ND	0.39	16	0.02	10	2,000	0.005
Aztreonam [b]	11	147	0.075	0.23	2	0.12	3	40	0.075
	2.33	45.3	0.051						
Imipenem [b]	ND	ND	ND	<0.001	0.06	-	0.006	1.5	0.004

[a]References: PER-1 [218, 232], PER-2 [207], PER-6 [236]
[b]K_m determined as K_i
[c]Kinetic parameters for cephaloridine
ND: not determinable

Recently, the PER-6-encoding gene was detected on the chromosome of an *Aeromonas allosaccharophila* environmental isolate from France with an amino acid identity with PER-2 of 92%. The identification and evaluation of PER-6 may give some clues as to the family origin and evolution of PER enzymes [236].

Little is known about the other PER family members. PER-3 and PER-4 are closely related to PER-1, sharing more than 99% amino acid sequence identity whereas PER-5 shares only 76.9-88.3% amino acid identity with the other variants. This 12-13% difference in sequence idenity probably represents a new subcluster within the PER family. bla_{PER-3} has been found associated with an IS*CR1* element in a complex class 1 integron (In39) from an *Aeromonas caviae* strain (GenBank AY740681). The position of bla_{PER-3} is equivalent to that of other IS*CR1*-associated resistance genes including PER-4 (GenBank EU748544) and PER-5 (GenBank EU687473) detected in *Proteus vulgaris* and *Acinetobacter baumannii* strains, respectively.

Class A Carbapenemases:

The class A carbapenemases have gained wide notoriety with the identification of KPC-producing organisms [239]. These enzymes can be clustered in divergent groups: SME, NMC/IMI, KPC, GES, SFC-1 and SHV-38, displaying 30 to 70% identity in amino acid sequence between the different groups [240]. These monomeric enzymes are mainly included in Bush-Jacoby-Medeiros functional group 2f. Functionally, GES-1, GES-2 and SHV-38 are assigned to group 2be, because of their hydrolytic activity on oxyimino-cephalosporins. Crystal structures of some of these enzymes (SME-1, NMC-A and SHV-38) share overall structures of other class A β-lactamases [240]. The substrate profile of these enzymes includes penicillins, cephalosporins, aztreonam and carbapenemes. As expected, they are inhibited by clavulanic acid although KPC enzymes are only partially inhibited by β-lactamase inhibitors [41, 241]. Class A carbapenemases have been detected in members of *Enterobacteriaceae* such as *E. cloacae*, *S. marcescens*, *K. pneumoniae* and to a lesser extent *K. oxytoca*, *C. freundii and E. coli. S*ome (GES-2 and KPC-2) have also been reported in pseudomonads [242, 243]. Carbapenem MICs for class A carbapenemase producers are variable and the MICs can range from highly resistant to fully susceptible.

SME, NMC-A and IMI are chromosomally encoded class A carbapenemases, and their coding genes have not been associated with mobile genetic elements to date, except for the allelic variant IMI-2 [244]. Although these enzymes are encoded in the chromosome of *Serratia marcescens* and *Enterobacter* spp. they are not found in every isolate of these species [240]. Isolates producing the chromosomal carbapenemase can display resistance to carbapenems (higher for imipenem than meropenem) while remaining susceptible to expanded spectrum cephalosporins [245].

SME stands for *Serratia marcescens enzyme*. Three SME enzyme variants have been reported, SME-1, SME-2, and SME-3 [246-248]. IMI-1 (named for *imipenemase*), was identified from an *E. cloacae* isolated in the United States in 1984, before the approval of carbapenem usage. This enzyme shares more than 95% identity to NMC-A [249] and is 99% identical to IMI-2. IMI-2 was identified from both environmental and clinical isolates of *E. asburiae* [244] and *E. cloacae*, respectively [250]. Surprisingly, bla_{IMI-2} and its regulator, bla_{IMI-R}, were located on a self-transferable 66-kb plasmid, flanked downstream by an open reading frame that shared 97% nucleotide identity with *tnpA* of Tn*2501* and further upstream by an IS2-like element. NMC-A was the first class A carbapenemase reported and was identified in an *E. cloacae* isolated in 1990 in France [251, 252]. Expression of NMC-A is inducible [246]. The carbapenemase coding gene is preceded by a divergently transcribed LysR-type regulatory gene, *nmc-R*, similar to those found upstream in some genera of AmpC-β-lactamase producers. The NmcR regulator increased enzyme production in the presence of imipenem and cefoxitin. Constitutively high production of NMC-A is associated with mutations in the regulator gene, *nmc*R or in an amidase coding gene (*ampD*) [253, 254]. A similar LysR-type transcription factor is encoded upstream of bla_{SME-1}. Evaluation of the *Serratia* system revealed only a slight increase in β-lactamase production after induction due to a high basal level of the enzyme [253]. bla_{IMI-1} and bla_{IMI-2} were also found associated to *imiR*, located in the chromosome or in a plasmid, respectively [244, 249]. The IMI-2 producing isolates indicated a derepressed phenotype with little induction observed due to a high basal level of enzyme production [250].

SME, NMC-A and IMI enzymes display a broad hydrolytic profile that includes penicillins, classical cephalosporins, aztreonam and carbapenems, but not cefoxitin or oxyimino cephalosporins. Rates of hydrolysis (k_{cat}) for imipenem are higher than for meropenem and ≥ 200% that of benzyl-penicillin. Kinetic parameters for representative members of these enzymes are shown in (Table **12**). The overall hydrolytic activity is not affected by substitutions observed between the different allelic variants [248, 249, 255, 256].

KPC (named for *K. pneumoniae carbapenemase)* enzymes are a major concern. Unlike previously mentioned class A carbapenemases, KPC are able to hydrolyze oxyimino-cephalosporins. Despite the ability to hydrolyze all β-lactams including carbapenems using *in vitro* assays, KPC-producing organisms do not always display a carbapenem resistant phenotype. These organisms often display MICs for imipenem and meropenem that do not exceed the susceptibility breakpoints. This phenotype makes detection of KPC-producing organisms difficult and ertapenem seems to be a better indicator of KPC production than the other carbapenems [257]. Carbapenemase screening tests have been proposed regarding those *Enterobacteriaceae* displaying reduced susceptibility [74].

KPC were first reported from *K. pneumoniae* isolated in the United States in 2001, in North Carolina [239]. Very shortly after, KPC producing isolates were described in multiple locations in the US and worldwide [242, 258-271]. bla_{KPC} has been identified in multiple *Enterobacteriaceae* isolates as well as *Pseudomonas* spp. and *Acinetobacter* [262, 270, 272-277]. There are twelve variants of KPC, KPC-2-13 (KPC-1 has been resequenced and shown to be KPC-2) [239, 278]. KPC-2, initially reported from *Klebsiella* spp. [257, 279] seems to be the most common KPC-enzyme detected in *Enterobacteriaceae* and *Pseudomonas* spp.; KPC-3 was also detected first in *K. pneumoniae* [259] while KPC-5 [242] and KPC-6 [280] were reported from non fermenters (*P. aeruginosa* and *Acinetobacter* spp., respectively) These three allelic variants displayed single but different amino acid substitutions with respect to KPC-2 while KPC-4 [242] showed two substitutions. Genes for the other allelic variants include: KPC-7 (EU729727), KPC-8 (FJ234412), KPC-9 (FJ624872), KPC-10 (GQ140348), KPC-11 (HM066995), KPC-12 (HQ641422), and KPC-13 (HQ342890).

KPC-2 shares 63% amino acid identity with SFC-1 (see below), 57% to SME-1, 55% to NMC-A and IMI [239, 257]. The characteristics of KPC-2 are typical for Class A carbapenemases with a decrease in the size of the water pocket and the placement of the catalytic serine [281]. Purified KPC-2 hydrolyzes efficiently penicillins and classical cephalosporins while imipenem, meropenem, cefotaxime and aztreonam are hydrolyzed 10-fold less. Cefoxitin and ceftazidime are only slightly hydrolyzed. The kinetic studies also revealed that clavulanic acid and tazobactam particially inhibited KPC-2 [239]. Substrate profiles for different KPCs are similar, however differences in the hydrolytic profiles have been observed ([242, 282], and Table **12**).

bla_{KPC} genes are associated with a novel Tn*3*-based transposon, Tn*4401*. This transposon is flanked by a 5-bp target site duplication, the signature of a recent transposition event, and is inserted in different open reading frames located on plasmids that vary in size and nature. Tn*4401* was considered the origin of bla_{KPC} acquisition and dissemination to various-sized plasmids identified in non-related *K. pneumoniae* and *P. aeruginosa* isolates from the United States, Colombia and Greece [283]. Although the genetic environment of bla_{KPC} from isolates recovered in China is different they are still associated with a transposable element [284].

GES-type β-Lactamases (for Guiana Extended Spectrum)

This family has 15 recognized members, including ESBLs and a cephamycinase. The ability to hydrolyze carbapenems is not a common feature of GES enzymes, but GES-2 [285], -4 [286], -5, -6 [287] and -11 [288, 289] are capable of carbapenem hydrolysis. GES-2 was identified in a *P. aeruginosa* isolated in 2000, from South Africa. The isolate was resistant to expanded-spectrum cephalosporins and displayed intermediate susceptibility to imipenem. The GES-2 amino acid sequence differs from GES-1 by a single amino acid substitution (Gly170Asn) in the omega loop of class A enzymes. This modification seems to be critical for carbapenemase activity [287]. GES-2 hydrolyzes expanded-spectrum cephalosporins and imipenem and is less inhibited by clavulanic acid and tazobactam compared to GES-1 (Table **12**). GES-2 enzymes have been predominantly identified in *P. aeruginosa* while GES-4 [286], -5 and -6 [287] have been reported from *Enterobacteriaceae* and GES-11 from *Acinetobacter* spp. [288]. GES-2 producing isolates have been implicated in nosocomial outbreaks [290, 291]. All bla_{GES}, except for bla_{GES-7}, have been found as gene cassettes in class 1 integrons located in transferable plasmids, non-transferable plasmids or the chromosome [286-288].

SFC-1 and SHV-38 are chromosomally encoded class A carbapenemases identified from an environmental isolate of *S. fonticola* and a clinical isolate of *K. pneumoniae*, respectively [54, 292]. SFC-1 enzyme was not present in other members of the species and could have been acquired by horizontal gene transfer, whereas SHV-38 is a single point variant of the chromosomally encoded SHV-1 of *K. pneumonia*, being the only class A carbapenemase with a known origin. An Ala146Val substitution in SHV-38 is responsible for the hydrolytic spectrum that includes ceftazidime and imipenem [54].

AmpC β-lactamases

Chromosomally-encoded AmpCs

AmpC serine β-lactamases can be classified as Ambler class C or group 1 enzymes according to Bush-Jacoby-Medeiros [3, 41]. The first recognized AmpC was the chromosomally encoded enzyme from an *Escherichia coli* strain capable of hydrolyzing penicillin [293, 294]. AmpC β-lactamases are widely distributed among members of different phyla including several enterobacteria, and pseudomonads, which are the most frequent producers of this type of enzyme in the clinical setting [295]. Recently, an excellent review of AmpC β-lactamases was written by George Jacoby. Therefore, this section on AmpC β-lactamases will only highlight critical aspects of these important enzymes [301].

Susceptibility profiles of organisms that produce a chromosomally encoded AmpC depend on the level of enzyme production. When enterobacteria overproduce the AmpC β-lactamase they may become resistant to broad-spectrum penicillins, cephalosporins (except cefepime and cefpirome), β-lactam/β-lactamase inhibitor combinations, and aztreonam. In the case of *P. aeruginosa*, overproduction of AmpC also negates the use of the 4th generation cephalosporins. The carbapenems are not affected unless the enterobacteria display a down regulation of its porins in addition to AmpC overproduction [296-298].

The over expression of chromosomal *ampC* genes can occur by two different mechanisms. For organisms such as *E. coli* that do not have an inducible *ampC* gene, mutations in the promoter or attenuator region result in higher level expression of *ampC*. Organisms such as *E. cloacae*, *S. marcescens*, *C. freundii* or *P. aeruginosa* carry an inducible *ampC* gene on the chromosome and over expression of the gene results from modifications in genes involved in regulatng induction. Phenotypically these isolates are referred to as derepressed mutants [299, 300].

The expression of inducible *ampC* is regulated by AmpR, a LysR-family regulator divergently transcribed from *ampC* [301]. Under β-lactam-mediated induction, hydrolyzed murein products gain access into the cytoplasm through the AmpG permease [302, 303]. During normal growth, muropeptides are shuttled into the cytoplasm and recycled for use during cell wall synthesis. A major recycling enzyme is the cytoplasmic AmpD amidase which specifically cleaves the anhydro moetiy from the muropeptides preparing the peptides for recycling [304, 305]. During the induction of *amp*C, the intracellular concentration of anhydro-*N*-acetylmuramyl-L-Ala-γ-D-Glu-*meso*-DAP (anhMurNAc-tripeptide) increases, and acts as a cofactor for the modification of AmpR resulting in an increase in *ampC* transcription. Removal of the inducing β-lactam from the medium reduces the amounts of muropeptide in the cytosol allowing low basal levels of *ampC* transcription due to the displacement the muropeptide from AmpR by the cofactor in greater concentration, UDP-*N*-acetylmuramyl-L-Ala-γ-D-Glu-*meso*-DAP-D-Ala-D-Ala (UDP-MurNAc-pentapeptide), the peptide required for cell wall synthesis [299]. Mutations in *ampD* also lead to an accumulation of muropeptides, and increasing the synthesis of AmpC independently of inducers, and giving rise to the acquired resistance to oxyimino-cephalosporins and more stable penicillins such as piperacillin and ticarcillin.

Plasmid-encoded AmpC β-lactamases

In the late 1980's, genes encoding chromosomal AmpC β-lactamases became mobilized and found on plasmids. The threat from these plasmid-encoded AmpC genes was the mobilization of the gene and their capcity to spread to organisms that did not produce these enzymes as part of their natural genome. (Table **13**) (adapted from reference [295]) summarizes different chronological and epidemiological data of AmpC β-lactamases.

Plasmid-mediated *ampC* genes can be grouped based on their genomic origin and divided into six different families (http://www.lahey.org/studies, and (Fig. **4**)). The nomenclature of plasmid-encoded AmpCs can be confusing as there are two distinct groups with the same name, CMY. By nucleotide sequence analysis, the majority of CMY enzymes belong to the cluster of CMY-2, including CMY-2-7, CMY-12-18, CMY-20-41, CMY-43-46, CMY-49, and CFE-1. These plasmid-encoded genes are derived from the closely related chromosome-encoded AmpC of *Citrobacter freundii*. In addition, CMY-34, 35, 37, 39, 41, 45, 46, and 49 are chromosome-encoded β-lactamases found in *C. freundii* (http://www.lahey.org/studies). With sequencing methodologies improving, some CMY-2-group members have been found to have the same sequence and duplicate names can be found on the lahey website (http://www.lahey.org/studies). CMY-2 is the most prevalent plasmid-encoded AmpC worldwide [295, 341-343].

The other CMY family seems to be mostly derived from aeromonads (*A. hydrophila*, *A. sobria*), including CMY-1, -8-11, -19 and MOX β-lactamases, although MOX-4 was found on the chromosome of *Vibrio fluvialis.*

Derivatives of the *Enterobacter* genome include ACT and MIR β-lactamases; ACT-1 from *E. asburiae* and MIR-1 from *E. cloacae*, while MIR-2 and MIR-3 are actually chromosomal enzymes from *Aeromonas* sp.

DHA-1 and DHA-2 were recruited from the chromosomal gene of *Morganella morganii*; both are inducible as the structural gene and the *ampR* gene required for induction were mobilized together. The same inducible phenotype has been described for organisms producing ACT-1 and CMY-13 [311, 321, 323, 326]. ACC enzymes are derived from *Hafnia alvei* while FOX enzymes are closely related to the chromosomal gene of *A. caviae* CAV-1. Plasmid-encoded AmpC producers such as ACC-1, CMY-2, and DHA have been implicated in nosocomial outbreaks [308, 322, 324, 327].

The most common mobile genetic element associated with plasmid-encoded AmpC β-lactamases is the insertion sequence IS*Ecp1*, associated with many CMY-encoding genes (CMY-2, 4, 5, 7, 12, 14-16, 21, 31, and 36), and ACC-1 and ACC-4 [295]. IS*Ecp1* has been shown to participate in the mobilization of chromosomal *bla* genes from the reservoir to a plasmid location [344], and to provide strong promoters from which the genes can be properly expressed, as demonstrated for the $bla_{\text{CMY-7}}$ gene [345].

The other element that has been associated with *ampC* genes is the IS*CR1* from complex class 1 integrons [177]. Plasmid-borne *ampC* genes linked to this structure are some $bla_{\text{CMY-1-type}}$ genes (1, 8-11, 19), $bla_{\text{DHA-1}}$, and $bla_{\text{MOX-1}}$ [295, 328, 346]. Finally, $bla_{\text{CMY-13}}$ has been shown to be associated with two copies of IS*26* [321], while other *ampC* genes are linked to different genetic backgrounds [333, 337, 340].

Recently, a new group of AmpC β-lactamases (both chromosomaly-encoded and plasmid-borne) with extended-spectrum activity has been described and are refered to as ESACs for extended spectrum AmpC. These enzymes display an enhanced activity towards oxyimino-cephalosporins due to different genetic modifications including deletions, insertions and substitutions [347]. For example, among the plasmidic AmpC, CMY-10 displays a 3-amino acid deletion in the R2 loop compared to *E. cloacae* P99, increasing both k_{cat} and k_{cat}/K_m parameters (in spite of the increased K_m) for ceftazidime and imipenem [348]. In general the modifications for ESACs are located in either the **Ω**-loop or in the R2-loop, leading to a redistribution of the active site that improves the accessibility of substrates bearing bulkier R1 side chains (oxyimino-cephalosporins) or an expansion of the R2 binding site, respectively [348].

Biochemical and Structural Features of AmpC β-lactamases:

The biochemical features of AmpCs include molecular masses between 34-40 kDa and pIs ranging from 6.6 to >8.0 [295, 320]. The structure of AmpC β-lactamases is similar to other serine-β-lactamases and DD-peptidases, having an open active cavity capable of accommodating the bulkier side chain of cephalosporins [295]. The active site serine is located at position 64 (Ser^{64}), and other important residues for the catalytic activity include Lys^{67}, Tyr^{150}, Asn^{152}, Lys^{315} and Ala^{318} [349]. Compared to class A β-lactamases, class C enzymes belong to a more homogeneous group with respect to their kinetic behavior [350]. Class C β-lactamases are able to recognize a wide range of antibiotics but are typically described as cephalosporinases. Generally, these enzymes have strong affinities for cephalosporins (very low K_m) but low k_{cat} values and are less efficient in deacylation ($k_{+2} >> k_{+3}$) which is the limiting step of the hydrolytic reaction [351, 352].

Table 12. Kinetic Parameters of Representative Class A Carbapenemases

Antibiotics	SME-1			IMI-1			NMC-A			SFC-1			SHV-38			KPC-1			GES-2		
	K_m (µM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	K_m (µM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	K_m (µM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	K_m (µM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	K_m (µM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	K_m (µM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	K_m (µM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)
Benzylpenicillin	16.7	19.3	1.15	64	36	0.56	28	260	9.3				13	100	7.70				4	0.4	0.096
Ampicillin	488	181	0,37	780	190	0.24				176	155	0.881				130	110	0.9			
Cephaloridine	770	980	1.25	1,070	2,000	1.9	185	2,820	15.2				150	40	0.27	560	340	0.6	0.5	7.7	0.065
Cefoxitin	NC	< 0.15	NC	45	0.3	0.0067	93[a]	5.0	0.062	77	4.2	0.054				120	0.3	0.002	NH	NH	NH
Ceftazidime	NC	<0.07	NC	270	0.0068	2.4×10^{-5}	90[a]	4.7	0.052	52	2.1	0.040	3,800	110	0.030	94	0.1	0.001	>3,000	ND	ND
Cefotaxime	NC	< 0.98	NC	190	3.4	0.018	956	286	0.30	89	8.3	0.093	800	1	0.001	160	14	0.1	890	2.2	0.0025
Aztreonam	259	108	0.42	93	51	0.55	125	707	5.60	484	162	0.33	5,500	3	0.0005	310	20	0.07			
Imipenem	202	104	0.52	170	89	0.52	92	1,040	11.30	82	54	0.66	24	0.01	0.0005	81	12	0.2	0.45	0.004	0.009
Meropenem	13.4	8.9	0.66	26	10	0.38	4.35	12	2.75	26	6.5	0.250				12	3	0.3	NH	NH	NH

References: SME-1 [248]; IMI-1 [249]; NMC-A [255]; SFC-1 [256]; SHV-38 [54]; KPC-1 [239] ; GES-2 [285]
NC: not calculated because too low initial rate hydrolysis. [a]: K_m determined as K_i
NH: not hydrolyzed
ND: Hydrolysis not detectable due to very high K_m values

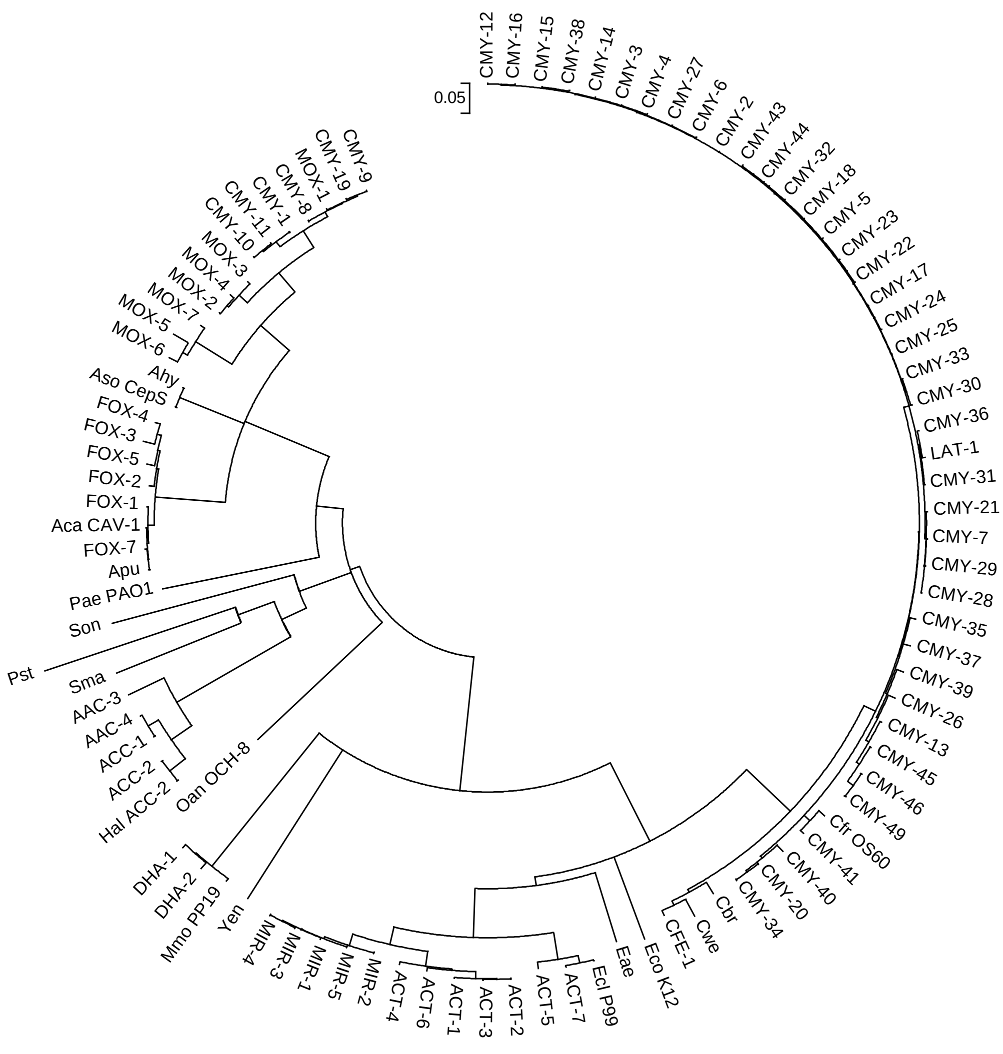

Fig. 4. Phylogenic relationship between plasmid-encoded and selected chromosome-encoded AmpC β-lactamases.

Table 13. Plasmid-encoded AmpC β-lactamases. First Report, Origin, Genetic Environment, and Availability of Structural/Biochemical Data

AmpC families	First report (country / year)	Species of first isolate	Most probable origin	Other representatives	Mode of expression: C (constitutive); I (inducible)	Outbreak implication (year, source)	Known genetic association	Available kinetic (K) and/or structural (S) analyses	References
ACC-1	Germany / 1999	*K. pneumoniae*	*H. alvei*	ACC-2 to -4	C	ACC-1 (1998-1999, *K. pneumoniae*)	IS*Ecp1* (ACC-1, ACC-4)	ACC-2 (K)	[295, 306-309]
ACT-1	USA / 1997	*K. pneumoniae*	*E. cloacae*	ACT-2 to -7	ACT-1: I ACT-2-7: C			ACT-1 (K,S)	[297, 310-314]
CFE-1	Japan / 2004	*E. coli*	*C. freundii*		C				[315]
CMY-1	South Korea / 1989	*K. pneumoniae*	*A. hydrophila*	CMY-8-11, CMY-19	C		IS*CR1* (CMY-1, 8-11, 19)	CMY-1 (K)	[295, 314, 316-318]
CMY-2	Greece / 1996	*K. pneumoniae*	*C. freundii*	CMY-3-7, CMY-12-18, CMY-20-41, CMY-43-46, CMY-49	CMY-13: I Remaning: C	CMY-2 (2001, Taiwan / *Shigella sonnei*)	IS*26* (CMY-13) IS*Ecp1* (CMY-2, 4, 5, 7, 12, 14-16, 21, 31, 36)	CMY-2 (K)	[295, 314 , 319, 320, 321 , 322]
DHA-1	Saudi Arabia / 1997	*S.* Enteritidis	*M. morganii*	DHA-2	I	DHA-1 (2004, Korea / *K. pneumoniae*; 2006, Belgium, *K. pneumoniae*)	IS*CR1*		[323-328]
FOX-1	Argentina / 1994	*K. pneumoniae*	*A. caviae*	FOX-2 to -7	C			FOX-5 (K)	[329-334]
LAT-1	Greece / 1993	*K. pneumoniae*	*C. freundii*		C				[335, 336]
MIR-1	USA / 1990	*K. pneumoniae*	*E. cloacae*	MIR-4, MIR-5	C			MIR-1 (K)	[314, 337, 338]
MOX-1	Japan / 1993	*K. pneumoniae*	*A. hydrophila*	MOX-2	C		IS*CR1*	MOX-2 (K)	[339, 340]

Preferred substrates include benzyl-penicillin followed by aminopenicillins [350, 351], first and second generation cephalosporins and cephamycins. AmpC enzymes also have high affinity for the oxyimino-cephalosporins. However resistance arises when the organism over produces the enzyme [299, 300, 352]. AmpC β-lactamases are poorly inhibited by mechanism-based β-lactamase inhibitors such as clavulanate. It is noteworthy that the AmpC of *M. morganii* displays unusual inhibition by tazobactam combinations, with lower MIC values when compared to other AmpC producers [300, 353, 354]. This behavior, along with the apparently high catalytic efficiency of *Morganella* AmpC towards piperacillin, could suggest a slight protection of the latter by tazobactam due to a rapid acylation of the active site by tazobactam [41, 350, 354]. (Table **14**) displays a selection of kinetic parameters for different variants of AmpC β-lactamases. A more comprehensive review was recently published by G. Jacoby [295].

CLASS D β-LACTAMASES

The broad-spectrum OXA β-Lactamases, "Oxacillinases", are Ambler class D β-lactamases that possess active site serine groups that were first named by the high relative rates of oxacillin and cloxacillin hydrolysis observed with the initial enzymes analyzed. However, this criterion no longer defines the group. Among the β-lactamases, class D enzymes are the most diverse group in amino acid sequence. Therefore, there can be as little as 20% identities among some members of this family. This diversity is also observed at the biochemical level with enzymes of narrow- to expanded-spectrum activity, including some that can hydrolyze carbapenems. Even with limited amino acid identity between OXA-β-lactamases, most new enzymes can be clustered with one or more pre-existing members of the OXA β-lactamase family. [355]. In general, carboxylation of the ε-amine of Lys-70 by dissolved CO_2 (using the numbering for OXA-10) in the first conserved motif (SXXF) is essential for the acylation and deacylation steps in the class D ß-lactamase, while Val-117 promotes carboxylation. Inhibition of this class of enzymes by chloride ions seems to be due to a competition with the carboxlated Lys (Vercheval, L., *et al*. 49th ICAAC-Abstract C1-1378, 2009). Class D enzymes are not easily inhibited by clavulanic acid, tazobactam or sulbactam. In turn, NaCl can inhibit most of these β-lactamases [356]; a criterion that could be used for preliminary detection of new representatives.

Table 14. Kinetic Parameters of Selected AmpC β-lactamases

	Benzylpenicillin			Amoxicillin			Cephalothin			Cefoxitin*			Cefotaxime*			Imipenem*			Aztreonam		
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	k_2/K ($\mu M^{-1}.s^{-1}$)	$10^4 \times (k_3)ss$ (s^{-1})	$K_{m(calc)}$ (nM)
Chromosome-encoded																					
E. coli K12	45	4.4	10	4.2	3.5	1.2	300	42	7	0.2	0.65	0.3	0.17	1.7	0.1	0.01	0.8	0.012	0.135	1.6	1.2
E. cloacae P99	14	0.6	23	0.74	0.4	1.8	200	9*	20	0.06	0.024	2.5	0.015	0.01	1.5	0.003	0.04	0.075	0.26	4.4	1.2
C. freundii	31	0.4	75	6.5	0.2	30	210	13	16	0.32	0.25	1.3	0.017	0.005	3.4	0.016	0.085	0.19	0.18	3.2	1.4
S. marcescens	75	1.7	44	0.46	0.01	46	1,1	67	16	0.014	0.3	0.04	1.7	12	0.14	0.001	0.06	0.017	0.012	7	58
M. morganii M29	0.007	0.25	0.03	0.07	0.13	0.5	140	148	1	0.04	0.02	2	0.032	0.02	1.6	0.07	2	0.035	1	40	ND
Hafnia alvei ACC-2	8.1	10	0.81	0.2[b]	<1[b]	>0.2	300	13	23	<0.01	ND	ND	0.02	19	0.001	<0.01	ND	ND	ND	ND	ND
P. aeruginosa	76	1.7	45	4.4	0.5	9	430	17	25	0.12	0.05	2.4	0.15	0.2	0.75	0.03	0.026	1.15	0.058	23	50
Aeromonas caviae CAV-1	5	8.7	0.57	ND	ND	ND	540	500	1.08	0.5	0.4	1.25	0.2	0.1	2	ND	ND	ND	ND	ND	ND
Plasmid-borne																					
ACT-1	55	2.1	26	1	1.7	0.6	460	38	12	0.37	0.5	0.74	0.05	0.07	0.7	0.011	0.37	0.03	0.024	21	12
MIR-1	14	0.4	35	0.55	0.16	3.4	160	2.1	76	0.64	0.75	0.7	2.7	4	0.67	0.012	0.15	0.08	0.22	16	8
CMY-1	13	1	13	0.45	2.2	0.2	480	30	16	0.05	0.06	0.9	0.01	0.015	0.67	0.002	0.05	0.04	0.36	<80	<20
CMY-2	14	0.4	35	0.55	0.16	3.4	160	2.1	76	0.23	0.07	3.3	0.004	0.0012	3.3	0.033	ND	ND	2	<60	<3
MOX-2	5	9.7	0.51	ND	ND	ND	250	78	3.2	35	300	0.12	0.05	ND	0.9	ND	ND	ND	ND	ND	ND
FOX-5	11	9.2	1.2	ND	ND	ND	870	71	12	0.7	0.85	0.82	0.08	ND	ND	ND	ND	ND	ND	ND	ND

*Apparent K_m determined as K_i values
[a] Cephaloridine instead of cephalothin was used
[b] Amoxicillin was used
Chromosome-encoded AmpC: *E. coli* K12, *E. cloacae* P99, *C. freundii*, *S. marcescens*, *P. aeruginosa* [351, 352], *M. morganii* [354], *H. alvei* [307], *A. caviae* CAV-1 [329]; Plasmid-borne AmpC: ACT-1, MIR-1, CMY-1, CMY-2 [314], FOX-5 [333]; MOX-2 [340]

The OXA β-lactamases can be divided into broad spectrum β-lactamases, extended-spectrum β-lactamases, and carbapenemases. The broad-spectrum β-lactmases include many OXA enzymes and are listed in (Tables **15** and **16**). OXA-1 (also known as OXA-30) is intriging as it can hydrolyze fourth generation cephalosporins but has limited activity against ceftazidime [357]. OXA-2 shares 30% amino acid identity with OXA-1 [358]. OXA-2 producing microorganisms have been associated with outbreaks but bla_{OXA-2} was associated with integrons in which other ß-lactamases were encoded (PER-1, CTX-M, and metallo β-lactamases, see Table **16**), so their contribution may be marginal.

Table 15. OXA-1 Related Broad Spectrum Enzymes

Enzyme	Clinical isolate	Outbreak Association	Genetic environment	Reference
OXA-1/-30	Eco*, Shi	yes (CTX-M-15)	P, I, GC,	[359-361]
OXA-31	Pae	No	P, I, GC,	[357]
OXA-47	Kpn	No	P, I, GC,	[362]

*Corresponds to the first description
P = plasmid, I = integron, GC = gene cassette
Eco: *E. coli*; Pae: *P. aeruginosa*; Kpn: *K. pneumoniae*, Shi: *Shigella*

Table 16. The OXA-2 Subgroup

Enzyme	Clinical isolate	Outbreak	Genetic environment	Reference
OXA-2	*S.* Typhimurium*, Several ent, Pae, Aba	yes (PER-1, MBL, CTX-M-2)	P, I, GC, Tn	[181, 358, 363, 364]
OXA-3	Pae*, Kpn	no	P, I, GC	[365]
OXA-21	Aba*, Pae	no	P, I, GC	[366]
OXA-34	Pae	no	?	AF350424
OXA-36	Pae	no	?	AF300985
OXA-46/-81*	Pae	no	Cr, I, GC	[367]
OXA-53	Sal	no	P, I, GC	[368]
OXA-56	Pae	Yes (SPM-1)	P, I, GC	[369]

P = plasmid, I = integron, GC = gene cassette, Tn = Transposon, ? = not detemined
ent: enterobacteria, Pae: *P. aeruginosa*, Aba: *A. baumanii*, Sal: Salmonella
*Corresponds to the first description

Other Wide-Spread Broad-Spectrum OXA Enzymes

OXA-10 was first described as PSE-2 and has since been renamed. It is closely related to OXA-5 which was initially isolated from a *P. aeruginosa* isolate recovered in the UK [370]. Although the production of OXA-10 does not increase the MICs for ceftazidime in pseudomonas, this enzyme possess a broader hydrolysis spectrum than other narrow-spectrum class D β-lactamases [371, 372]. Several OXA enzymes of this subgroup possess increased activity towards extended-spectrum cephalosporins. Other OXA-10 derived enzymes with broad spectrum activity include OXA-28, -35/4, -56, -74 and OXA-101 [363, 369, 371, 373, 374]. OXA-9 is unique as it is inhibited by clavulanic acid and cloxacillin but not by NaCl [375]. It shares low amino acid identity with even its most closely related enzymes OXA-12 and OXA-18 (see below).

Most of the broad-spectrum bla_{OXA} genes are located in gene cassettes. The bla_{OXA-5} gene was found as a gene cassette inserted in a class 1 integron, and was also found in association with the bla_{GES-2} gene during an outbreak of carbapenem-resistant *P. aeruginosa* isolates from South Africa [376]. The bla_{OXA-9} gene was first identified as part of Tn*1331* on a plasmid in a *K. pneumoniae* isolate [377]. Therefore it is not surprising that bla_{OXA-9} has been described in several species, including *Salmonella* spp. [378], and *P. putida* where it was associated with the metallo-β-lactamase VIM-2 [379]. OXA-20 and OXA-46 are also integron-borne narrow-spectrum class D β-lactamases that share between 75- 78% identity with their closest relative, OXA-2 [367, 380]. With the exception of OXA-101, described in several enterobacteria, the remaining enzymes have been described in *P. aeruginosa*. Only OXA-74 was associated with outbreaks of *P. aeruginosa* [363, 369, 373, 374].

Extended-spectrum OXA β-lactamases

Most OXA ESBLs identified so far were recovered from *P. aeruginosa* (Table **17**). The OXA-2 variants such as OXA-15 have a single-point mutation as compared to OXA-2 (Asp150Gly). OXA-15 has an increased ability to hydrolyze ceftazidime, and to a lesser extent cefepime and aztreonam. OXA-32 differs from OXA-2 by an Leu169Ile amino acid substitution which is responsible for resistance to ceftazidime but not to cefotaxime when present in *P. aeruginosa* [376]. OXA-ESBLs derived from OXA-10 or OXA-7 are also listed in (Table **17**). They seem to require two amino acid substitutions (Asn73Ser, Gly157Asp) to extend their substrate profile [371, 372]. Most OXA-type ESBLs are relatively resistant to inhibition by clavulanic acid. Some of them confer resistance predominantly to ceftazidime, but OXA-17 (prevalent in Taiwan [381]) seems to increase the MICs of cefotaxime and cefepime when cloned into *E. coli*. [382].

bla_{OXA-18} encodes a 275 amino acid enzyme whose closest OXA-derivatives are OXA-45, OXA-9 and OXA-12 (66%, 45% and 42% amino acid identity, respectively). OXA-45 resembles OXA-18 in its substrate profile including inhibition by clavulanic acid. OXA-18 is unique since it is a class D β-lactamase which confers high resistance to extended-spectrum cephalosporins but its activity is totally inhibited by clavulanic acid [383-386]. OXA-18 has only been found in *P. aeruginosa*, and was associated with an outbreak in Tunisia [387].

All of the OXA ESBLs have been associated with gene cassettes. The bla_{OXA-15} gene was identified as a gene cassette within the variable region of a class 1 integron structure in a *P. aeruginosa* [388]. bla_{OXA-18} is likely to be chromosomally located; it was found bracketed by two duplicated sequences containing IS*CR19*. OXA-45 was described in *P. aeruginosa* and, in this case, chromosomally located and flanked by IS*CR5* elements.

Carbapenem Hydrolyzing Class D β-Lactamases

Most class D enzymes that hydrolyze carbapenems have been found in *Acinetobacter* spp. (Table **18**) and the relatedness of the enzymes and their epidemiology are reviewed in this current issue by Evans *et al*. Therefore, the following section will include the biochemical properties of OXA β-lactamases including the carbapenem hydrolyzing class D β-lactamases.

Biochemical properties OXA Enzymes

The Bush 2d subgroup of β-lactamases are defined as enzymes hydrolyzing oxacillin at a rate >50% of that for benzyl-penicillin [41]. As seen in Tables **19** and **20**, some of the OXA β-lactamases (especially the carbapenem hydrolyzing enzymes) do not fulfill this requirement. Typically, the enzymes efficiently hydrolyze penicillins (benzyl-penicillin, ampicillin, piperacillin and ticarcillin) and the narrow-spectrum cephalosporins, cephalothin and cephaloridine [356].

Table 17. Extended-spectrum OXA β-lactamases

Enzyme	Clinical isolate	Outbreak	Genetic environment	Reference
OXA-2 variants				
OXA-15	Pae	no	P, I, GC,	[388]
OXA-32	Pae	no	P, I, GC,	[376]
OXA-10 variants				
OXA-7	Eco	no	P, I, GC	[370, 389]
OXA-10/PSE-2	Pae*, ent	no	P, I, GC, Tn	[390-392]
OXA-11	Pae,	no	P	[393]
OXA-13	Pae	no	Cr, I, GC	[394]
OXA-14	Pae	no	P	[395]
OXA-16	Pae	no	P	[396]
OXA-17	Pae*, Kpn	no	P	[382, 397]
OXA-19	Pae	no	Cr, I, GC	[372]

ent: enterobacteria, Pae: *P. aeruginosa*, Kpn: *K. pneumoniae*
*Corresponds to the first description

Table 18. Carbapenem Hydrolyzing OXA β-lactamases

Enzyme cluster	Aditional member	Described mainly in	Outbreak	Genetic environment	Reference
OXA-23 (ARI-1)	OXA-27, OXA-49, OXA-73	Aba	yes	Cr, P, IS	[398]
OXA-24/-40	OXA-25, OXA-26, OXA-40,	Aba	yes	Cr, P	[399, 400]
	OXA-72				
OXA-51	**OXA-64 to OXA-71, OXA-75 to**	Aba	no	Cr, IS	[401, 402]
	OXA-78, OXA-83, OXA-84,				
	OXA-86 to OXA-89, OXA-91,				
	OXA-92, OXA-94, OXA-95				
OXA-58	OXA-96, OXA-97	Aba	yes	P, IS	[403]
OXA-55	**OXA-SHE**	Sal	no	Cr	[404]
OXA-48	**OXA-54**	Kpn, Son	yes	P, IS, Cr	[362, 405, 406]
OXA-50	**None**	Pae	no	Cr	[407]
OXA-60	**None**	Rpi	no	Cr	[408]
OXA-62	**None**	Ppn	no	Cr	[409]

Aba: *A. baumannii*, Sha: *S. algae*, Kpn: *K. pneumoniae*, Son: *S. oneidensis*, Pae: *P. aeruginosa*, Rpi: *R. pickettii*, Ppn: *P. pnomenusa*,
Outbreak associated: yes or no
Cr: chromosome; P: plasmid; IS: associated to insertion sequences.
Bold text: chromosome-encoded and naturally-occurring class D β-lactamases

Mature class D β-lactamases contain between 243 and 260 amino acid residues with isoelectric points (pIs) between 5.1 and 9.0. The crystal structures of several class D β-lactamases (OXA-1, -10, -13, -46, -48) reveal a dimeric structure, with a similar monomeric folding observed for class A β-lactamases [410-414]. An additional β-strand mediates the association into dimers. Major differences are found when comparing the molecular details of the active site for class D β-lactamases to the corresponding regions in class A and C β-lactamases. Lys70 is carbamylated in the native structure of OXA-10 [415]. The carbamylated lysine may act as a base to activate the Ser67 hydroxyl group for enzyme acylation. The decarbamylated β-lactamases in degassed buffer are shown as inactive and only recover initial activity at pH 7.0 or higher by the addition of HCO_3^- (as the source of carbon dioxide). Discrepancies in the biochemical behavior of OXA enzymes may be related to the different experimental conditions that modify the carbamylation status.

Post-translational carbamylation occurs between the ε-amine of Lys70 and CO_2 and is promoted by the presence of hydrophobic residues such as Val117 in OXA-10. A classical feature of most class D β-lactamases is the inhibition by NaCl, which is attributed to the YGN motif at position 144 to 146. The inhibition by chloride ions appears to be due to a competition with the carboxlated Lys (L. Vercheval *et al*. 2009. 49th ICAAC. Abs C1-1378). OXA β-lactamases having a FGN element instead of YGN are not or are only weakly inhibited by NaCl. It is the opinion of some researchers that the dimeric structure of the OXA β-lactamases suggests that class D enzymes may bind other substrates besides β-lactams which results in carbamylation of the active site Lys-70 residue [413, 415].

It must be kept in mind that even with an extremely low k_{cat} value for hydrolysis of a β-lactam, the β-lactam may still be hydrolysed efficiently *in vivo*. The organism may counter balance the low k_{cat} between enzyme and substrate by producing very high periplasmic concentrations of the β-lactamase. This would allow an efficient β-lactam degradation which is directly proportional to the k_{cat} and the enzyme concentration [416]; moreover, if there are restrictions on the penetration of the antibiotic into the periplasmic space, and/or increased efflux through the external membrane, hydrolysis of the entering β-lactam may still result in clinical resistance to that antibiotic.

Carbepenem-hydrolizing class D β-lactamases (CHDLs) may increase carbapenem MICs although they may have low hydrolytic activity on imipenem and especially on meropenem. Most of them do not confer resistance to extended-spectrum β-lactams such as ceftazidime, cefotaxime or aztreonam (Table **19**). Moreover, CHDLs show higher affinities (low K_m) for imipenem than for any other tested β-lactam. Consequently, meropenem is hydrolyzed at a much slower rate than imipenem [417]. The YGN motif at position 144 to 146 is highly conserved among class D β-lactamases, however for the CHDLs, OXA-23 and OXA-24, a Phe for Tyr substitution in the YGN motif has occurred, but the CHDLs OXA-51 and OXA-58 subgroups retain the YGN at this position [243]. Finally, the Phe residue in OXA-40 seems to be responsible for the inhibition by NaCl whereas a Tyr residue in motif YGN was related to susceptibility to NaCl [418].

THE METALLO β-LACTAMASE SUPERFAMILY

In addition to true metallo-β-lactamases, which are able to cleave the amide bond of β-lactams, the MBL superfamily was defined by Neuwald *et al*. in 1997, [420] including related enzymes that hydrolyze thiol-ester, phosphodiester and sulfuric ester bonds as well as oxydoreductases; some members have been shown to play significant roles in nucleic acid processing. This family (that already contains several thousands members) continues to increase in number, and have been found encoded by the chromosome in several bacteria that are susceptible to β-lactam antibiotics, as well as in eukaryotic organisms (including humans), representing perhaps a general scaffold from which metallo-β-lactamases evolved. Surprisingly (or not) some known metallo-β-lactamases seem to be

Table 19. Some Broad Spectrum and Extended Spectrum OXAs

	OXA-46			OXA-10 (Vmax)			OXA-20			OXA-32			OXA-17			OXA-16		
	BSBL			BSBL			BSBL			ESBL			ESBL			ESBL		
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)
PEN	18	48	0.38	89	63	1.412	26	4.4	0.006	3.5	45	0.08	5	34	0.147	48	65	0.738
AMP	6	20	0.3	587	235	2.5	80	33	2.4				26	245	0.106	97	205	0.473
CAR	5	545	0.009	31	195	0.159							2	296	0.021	17	129	0.132
OXA	300	320	0.94	608	222	2.739	116	329	0.35				120	153	0.784	411	960	0.428
CPL				79	2,340	0.033	20	69	0.3	2	360	0.006	23	2,940	0.008	21	424	0.05
CEF	8	23	0.35	6	38	0.158	13	5	2.6	3	60	0.05	5	286	0.017	3	32	0.094
CTX	ND	$>10^3$*	-	9	346	0.026	ND	-	-	ND	-	-	22	2,240	0.01	6	346	0.017
CRO				3	55	0.054							1	544	0.002	1.4	36	0.039
CAZ	ND	$>10^3$*	-	ND	-	-	ND	-	-	H	>3,000	H	ND	-	-	ND	-	-
FEP	ND	$>10^3$*	-															
AZT	ND	$>10^3$*	-				6	69	0.09	ND	-	-						

References: OXA-46 [367]; OXA-10 [396]; OXA-20 [380]; OXA-32 [376]; OXA-17 [382]; OXA-16 [396]
(PEN) Penicillin G, (AMP) Ampicillin, (CAR) Carbenicillin, (OXA) Oxacillin, (CPL) Cephaloridine, (CEF) Cephalothin, (CTX) Cefotaxime, (CRO) Ceftriaxone, (CAZ) Ceftazidime, (FEP) Cefepime, (AZT) Aztreonam.
* Measured as an inhibition constant, (ND) No detectable hydrolysis, (H) Hydrolyzed but kinetic parameters cannot be determined due to high K_m value, (-) Not determinable

more structurally related to different enzymes rather than to other metallo-β-lactamases.

MBLs or class B β-lactamases constitute a heterogeneous family characterized by:

I. Their ability to hydrolyze a broad spectrum of β-lactams, including penicillins, cephalosporins and carbapenems, but lack the ability to hydrolyze monobactams.

II. Their requirement for one or two zinc ions for their activity, making them susceptible to metal ion chelators (EDTA, dipicolinic acid). They remain stable against the therapeutic β-lactamase inhibitors [243, 421].

Initially, MBLs were detected in environmental or opportunistic pathogens as chromosomally encoded enzymes that could not be easily transferred [422]. Unfortunately, an explosive increase in acquired MBLs has occurred in the last ten years; being association with integrons in mobile genetic elements a contributing factor [241]. A limited number of genes, such as bla_{IMP-1}, $_{-3}$, $_{-10}$, and $_{-12}$ and bla_{VIM-2}, have been clearly associated with 31 to 56 kb plasmids found in *P. aeruginosa* or *P. putida* [243, 422].

Class B β-lactamases are classified into three subclasses B1, B2 and B3 on the basis of their known amino acid sequences. Conserved secondary structure as evaluated by X-ray diffraction facilitated their classification even when the sequence similarity was not obvious [423]. Chromosomally encoded subclass B1 enzymes are represented in diverse bacterial lineages, including BcII (*B. cereus)* [424], CcrA (*B. fragilis*) [425], and BlaB (*E. meningosepticum)* [426]. The acquired IMP-type MBLs, VIM-type and SPM-1 enzymes are included in this subclass [427].

Subclass B2 is represented within the genus *Aeromonas*. Good examples of these are CphA (*A. hydrophila*) [428], ImiS (*A. veronii*) [429] and Sfh-I from *Serratia fonticola* [430]. The most heterogeneous and therefore less conserved MBLs are found in subclass B3. These enzymes are chromosomally encoded, and found in environmental isolates. However, L1 enzymes can be responsible for resistance to carbapenems in *S. maltophilia*, an organism causing opportunistic infections in nosocomial settings and therefore a concern [431]. In addition, GOB-1 and its allelic variants are found in strains of *Elizabethkingia meningoseptica* [432] while FEZ-1 was identified from *Legionnella gormani* [433].

All class B enzymes are monomeric with the exception of L1 which is a homo-tetramer. B1 and B3 MBLs are broad spectrum hydrolyzing enzymes while subclass B2 enzymes are strict carbapenemases [423, 427, 434]. Naturally occurring subclass B1 enzymes, such as BcII and BlaB, are encoded by the chromosome, while acquired MBLs have also been identified in plasmids. Most acquired MBLs are associated with gene cassettes of type 1 or type 3 integrons, located on the chromosome or in plasmids. However, bla_{SPM-1} is associated with a different mobile genetic element, IS*CR4* [435].

Despite their great diversity, most MBLs share five conserved motifs: Asp84, His116-X-His118-X-Asp120-His121, His196, Asp221 and His263 and they all exhibit an αββα-fold [427]**.** The conserved motifs are necessary for maintaining appropriate folding (Asp84) or for coordinating the two potential Zn2+ binding sites. The first site ("the histidine site") includes His116/Asn116, His118, His196 and a water molecule or hydroxyl group, the second site (the "cysteine site") requires Asp120, Cys121/His121, His263 and two water molecules (Table **21**).

A big difference between sublasses B1 and B3 with respect to B2, is that B2 types are inhibited upon binding of the second Zn^{2+} ion, whereas the B1 and B2 enzymes increase their activity upon binding the second site [427].

Table 20. A comparison between Broad and Extended Spectrum OXAs with Carbapenem Hydrolizing OXAs

	OXA-46			OXA-32			OXA-17			OXA-40			OXA-48			OXA-58		
	BSBL			ESBL			ESBL			CHDL			CHDL			CHDL		
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)
PEN	18	48	0.38	3.5	45	0.08	5	34	0.147	5	23	0.220	245	40	6.1	5.5	50	0.11
AMP	6	20	0.3				26	245	0.106	5	220	0.02	340	5,200	0.065	1	130	0.008
OXA	300	320	0.94				120	153	0.784	2	876	0.003	25	30	0.85	1.5	70	0.002
PIP				3	155	0.02				1	23	0.05	75	410	0.18	2.5	50	0.05
CEF	8	23	0.35	3	60	0.05	5	286	0.017	3	72	0.050	3	20	0.15	0.1	150	0.001
CFR				ND	-	-				ND	-	-	8	390	0.02	0.1	200	0.0005
CAZ	ND	$>10^3$*	-	H	>3,000	H	ND	-	-	20	2,500	0.01	4	5,100	0.001	ND	-	-
CTX	ND	$>10^3$*	-	ND	-	-	22	2,240	0.01	ND	-	-	11	190	0.06	ND	-	-
FEP	ND	$>10^3$*	-							ND	-	-	1	160	0.005	ND	-	-
AZT	ND	$>10^3$*	-	ND	-	-				ND	-	-	ND	-	-	ND	-	-
IMI										0,1	6.5	0.015	2	14	0.15	0.1	7.5	0.014
MER										ND	-	-	0.1	200	0.0005	<0.01	0.075	<0.15

References: OXA-46 [367]; OXA-32 [376]; OXA-17 [382]; OXA-40 [418]; OXA-48 [406]; OXA-58 [419]
(PEN) Penicillin G, (AMP) Ampicillin, (OXA) Oxacillin, (PIP) Piperacillin, (CEF) Cephalothin, (CFR) Cefpirome, (CTX) Cefotaxime, (CAZ) Ceftazidime, (FEP) Cefepime, (AZT) Aztreonam, (IMI) Imipenem, (MER) Meropenem.
* Measured as an inhibition constant, (ND) No detectable hydrolysis, (H) Hydrolyzed but kinetic parameters not be determined due to high K_m value, (-) Not determinable.

Table 21. Important Zn Binding Residues (Adapted from refs. [423, 436])

β-Lactamase		Ligand(s)					
		First binding site			Second biniding site		
Subclass B1	**consensus**	**His116**	**His118**	**His196**	**Asp120**	**Cys221**	**His263**
	BcII	His86	His 88	His149	Asp90	Cys168	His210
	IMP-1	His77	His79	His139	Asp81	Cys158	His197
	VIM-2	His88	His90	His153	Asp92	Cys172	His214
	SPM-1	His76	His78	His165	Asp80	Cys184	His221
Subclass B2	**Consensus**	**Asp120**	**Cys221**	**His263**	**Asn116**	**His118**	**His196**
	CphA	Asp73	Cys167	His205	Asn69	His71	His148
Subclass B3	**consensus**	**His/Gln116**	**His118**	**His196**	**Asp120**	**His121**	**His263**
	FEZ-1	His71	His73	His149	Asp75	His76	His215

The Imported IMP MBLs

Currently there are 24 IMP variants that are shown in Table **22**. Although IMP-variants diverge from each other by single or several amino acid substitutions (up to approximately 20 % of the amino cid sequence) (Figs. **5** and **6**), the six residues involved in zinc binding are conserved. What is common for all isolates that produce these enzymes is that the coding genes are carried on a gene cassette inserted mainly into class 1 integrons and the resistance profile includes most β-lactams except aztreonam [437].

Kinetics of the IMP MBL cluster

Initial kinetic analysis of IMP-1 soon revealed that this enzyme hydrolyzed classical broad-spectrum and extended-spectrum β-lactams in addition to carbapenems, while aztreonam was relatively stable. Clavulanate or cloxacillin failed to inhibit IMP-1 but the presence of EDTA resulted in a sharp decrease in enzymatic activity [421, 438, 439].

Table **23** summarizes some reported kinetic parameters for IMP-1. As indicated, a common problem in the analysis of kinetic

Table 22. IMP Variants

IMP-variant	Original isolate (species, country, year)	Genetic context and location of *bla*$_{IMP}$	References
IMP-1	*P. aeruginosa*, Japan, 1988	Transferable plasmid, chromosome Integron: integrase-like gene-*bla*$_{IMP-1}$- *aac(6´)-Ib*-like gene (transferable plasmid) In31:*Inti1-bla*$_{IMP-1}$-*aacA4-catB6*-orfN-*qaCG- qacEΔ1-sul1*- orf5	[438-444]
IMP-2	*A. baumannii*, Italy, 1997	In42: *Inti1-bla*$_{IMP-2}$-*aacA4-aadA1- qacEΔ1-sul1* (chromosome)	[445]
IMP-3, originally MET-1	*S. flexneri*, Japan, 1998	class 1 integron (plasmid)	[446, 447]
IMP-4	*Acintetobacter* spp., China, 1994	chromosome class 1 integron: *Int1-bla*IMP-4-*qacG2-aacA4-catB3- qacEΔ1-sul1*(plasmid) class 1 integron: *int1-bla*IMP-4-*orf*II-*orf*III-*qacE_1-sul1-orf5* (plasmid)	[448-451]
IMP-5	*A. baumannii, Portugal, 1998*	In76: *int1-bla*$_{IMP-5}$-*qacEΔ1-sul1*-	[452]
IMP-6	*S. marcescens*, Japan, 1996	integron: *int-bla*$_{IMP-6}$-ORF3 (plasmid)	[453-455]
IMP-7	*P. aeruginosa* Canada, 1995	class 1 integron: *int1-ORF1-aacC4-bla*$_{IMP-7}$-*aacC1-qacEΔ1-sul1*-	[456]
IMP-8	*K. pneumoniae* Taiwan, 1998	class 1 integron: *intII-bla*$_{IMP-8}$-*aac(6´)Ib* (plasmid) class 1 integron: *intII-bla*$_{IMP-8}$-*aadB-cmlA* (chromosome)	[457-459]
IMP-9	*P. aeruginosa*, China, 2000	conjugative plasmid	[460]
IMP-10	*P. aeruginosa*, Japan, 1997 *Alcaligenes xylosoxidans*, Japan, 2000	plasmid, chromosome	[461]
IMP-11	*A. Baumannii*, Japan, 2001	Not evaluated	AB074436
IMP-12	*P. putida*, Italy, 2000	class 1 integron: *int1-bla*$_{IMP-12}$-*aacA4-qacEΔ1-sul1* (plasmid)	[462]
IMP-13	*P. aeruginosa*., Italy, 2001	InPSG: *int1-bla*$_{IMP-13}$-*aacA4- qacEΔ1-sul1* (chromosome) In88: *int1-bla*$_{IMP-13}$- *qacEΔ1-sul1* In89: *int1-orf1-bla*$_{IMP-13}$-*aadB- qacEΔ1-sul1*	[463 , 464-466]
IMP-14	*P. aeruginosa*, Thailand, 2005	class 1 integron: *int1-bla*$_{IMP-14}$-*aacA4- qacEΔ1-sul1*	AY553332 [467]
IMP-15	*P. aeruginosa*, Thailand, 2003	Class 1 integron: *int1-bla*$_{IMP-15}$-*dfr-aacA4-qacEΔ1-sul1* In95: *int1-aacA7-bla*$_{IMP-15}$-*qacH-aacA4-aadA1-oxa-2aadA1-qacEΔ1-sul1* (non transferable plasmid)	AY553333 [468]
IMP-16	*P. aeruginosa*, Brazil, 2002	class 1 integron: *int1-bla*$_{IMP-14}$-*aac(6´)-30/aac(6´)Ib´-aadA1-qacEΔ1-sul1*(chromosome)	[469]
IMP-18	*P. aeruginosa*, USA, 2006	Unpublished	[470]
IMP-19	*Aeromonas caviae*, France, 2006	Class 1 integron: *int1*-IS*Aeca-1-aacA4-bla*$_{IMP-19}$- *qacEΔ1-sul1* (plasmid)	[471]
IMP-20	*P. aeruginosa*, Japan, 2004	Not evalutated	AB196988
IMP-21	*P. aeruginosa*,, Japan, 2005	Not evaluated	AB204557
IMP-22	*P. aeruginosa*, Italy, 2005	Class 1 integron (plasmid)	[472]
IMP-24	*S. marcescens*, Taiwan, 2002-2006	chromosome	[459]

Note : IMP variants implicated in nosocomial outbreaks are marked in bold letter

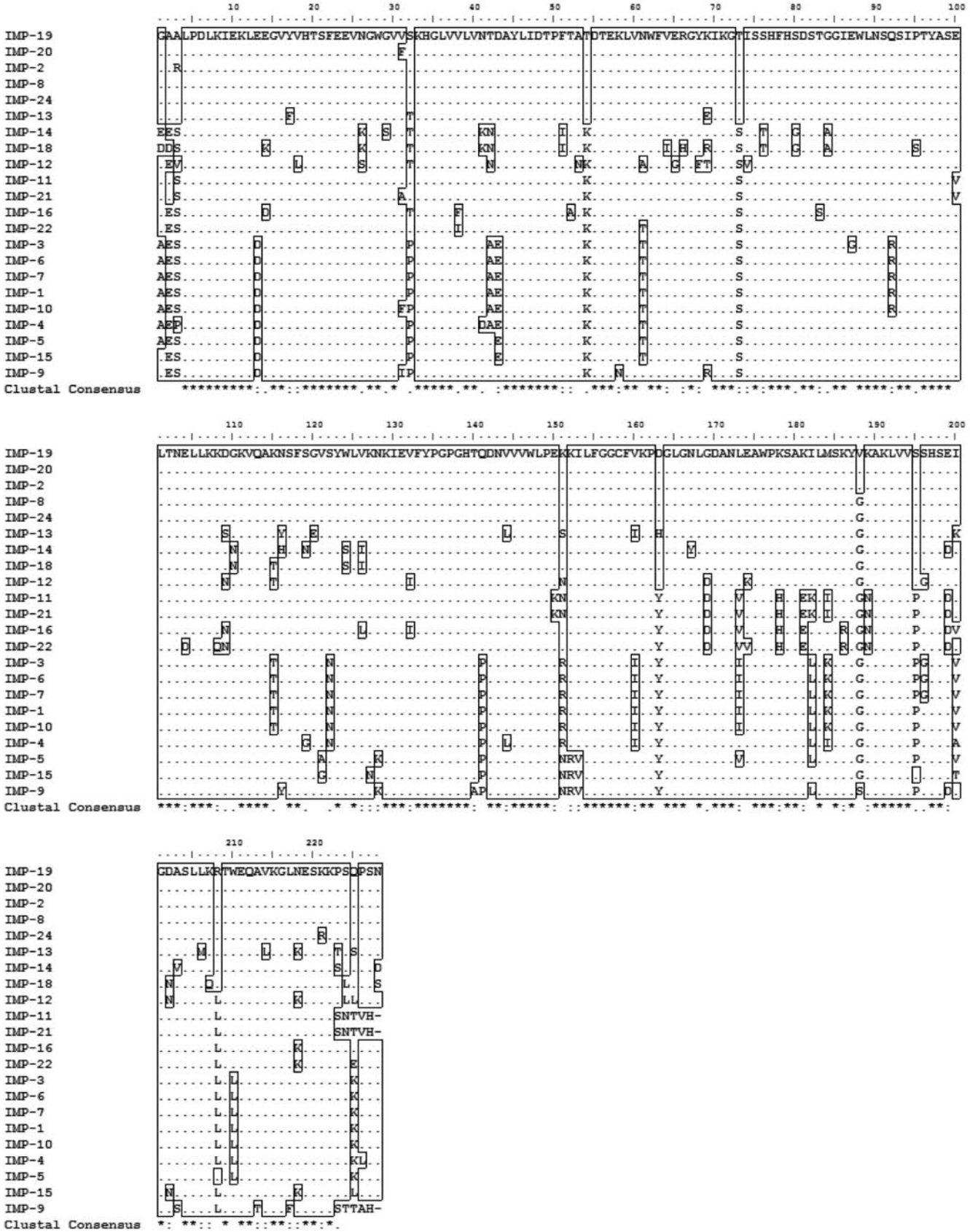

Fig. 5. IMP-MBLs amino acid sequences alignment.

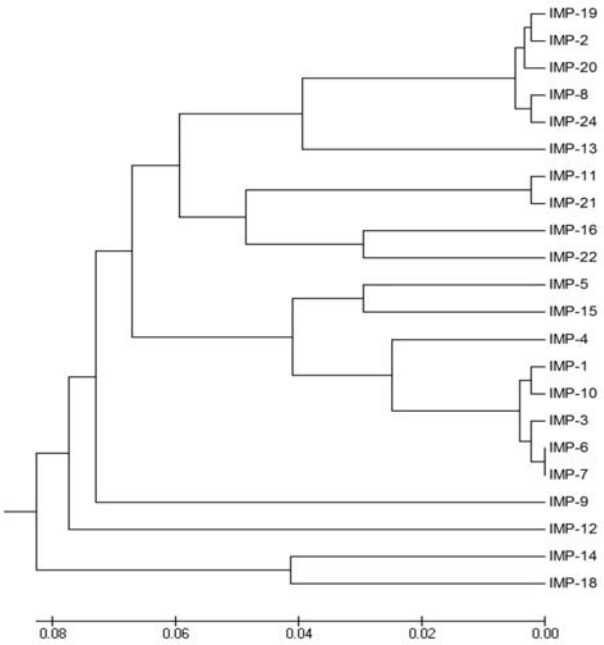

Fig. 6. Phylogenic relationships among IMP-MBLs family.

Table 23. Different Kinetic Parameters Obtained for IMP-1

Antibiotic	Watanabe *et al*		Osano *et al*			Laraki *et al*		
	K_m (μM)	Relative V_{max}	K_m (μM)	Relative V_{max}	Relative V_{max}/K_m	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} .s^{-1}$)
Benzylpenicillin	650	717				520	320	0.62
Ampicillin	335	215	2.15	100	100	200	950	4.8
Carbenicillin	381	391				ND	ND	0.02
Piperacillin	468	145				ND	ND	0.72
Imipenem	24.6	166	7.33	6.9	1.9	39	46	1.2
Meropenem	5.3	37	0.74	1.0	3.0	10	50	5
Panipenem			2.07	2.7	2.8	30	44	1.5
Nitrocefin						27	63	2.3
Cephaloridine	5.7	100	7.74	29.5	8.2	22	53	2.4
Cephalothin	6.1	113				21	48	2.4
Cefuroxime	4.2	35				37	8	0.22
Ceftazidime	46.4	20	1.24	16.1	27.8	44	8	0.18
Cefotaxime	2.3	22				4	1.3	0.35
Cefpirome						14	9	0.64
Cefepime						11	7	0.66
Cefoxitin	6.1	51				8	16	2
Moxalactam	28.9	193	7.55	47.4	13.5	10	88	8.8
Aztreonam	---[a]	< 1	3.97	0.078	0.43	>1,000	<0.01	< 0.00001

References: Watanabe *et al* [438]; Osano *et al* [439]; Laraki *et al* [421]
a: The K_i value determined by using cephaloridine as the substrate, > 100 μM. ND: not determined.
Buffer and experimental conditions as indicated in the corresponding references.

Table 24. Kinetic Constants for the IMP-1 Cluster

Antibiotics	IMP-1			IMP-2			IMP-3			IMP-6			IMP-10			IMP-12			IMP-13 Unpublished			IMP-16			IMP-18			IMP-19		
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)
Benzylpenicillin	520	320	0.62				370^b	14.3^b	0.039	220	51	0.23	ND	ND	0.07							7,805	800	0.10	90	55	0.61	206	1011	4.91
Ampicillin	200	950	4.8	110	23	0.21	464^b	7.4^b	0.016				ND	ND	0.06	1,500	18	0.012	214	82	0,38	1,065	137	0.13						
Carbenicillin	ND	ND	0.02	700	252	0.36							ND	ND	0.18	175	3.7	0.021				3,331	433	0.13						
Piperacillin	ND	ND	0.72							230	22	0.09	ND	ND	0.04	ND	ND	0.023	150	282	1.9	2,804	250	0.09				148	41.2	0.28
Ticarcillin	740	1.1	0.0015													470	6.9	0.015	125	224	1.8							140	683	4.88
Imipenem	39	46	1.2	24	22	0.92	1,140^b	92.3^b	0.08	110	68	0.61	60	220	3.7	920	240	0.26	49	124	2.5	365	133	0.36	7	17	2.4	100	26.5	0.26
Meropenem	10	50	5	0.3	1	3.3				7.6^a	32	4.2	47	64	1.4	7.2	9.5	1.3	10	1.4	0.14	72	23	0.32	8.4	0.05	0.006	7	1.0	0.14
Cephaloridine	22	53	2.4	3	0.8	0.27	248^b	221^b	0.89				28	140	5.0															
Cephalothin	21	48	2.4				9.9	223	22.5	4.7	374	79.6	4.9	230	47	16	118	7.4	31	25	0.81	42	77	1.8				76	11.0	0.14
Cefuroxime	37	8	0.22													7	61	8.7	1	23	23	49	52	1.06	7	0.9	0.15	95	16.4	0.17
Ceftazidime	44	8	0.18	111	21	0.19	128^b	4.5^b	0.035				51	51	1.0	15	6.7	0.45	15	9	0.6	87	13	0.15	1.3	1	0.77	20	6.4	0.32
Cefotaxime	4^a	1.3	0.35				3.1	40.1	12.9	3.8^a	55	14.5	5.7	74	13	22	56	2.5	21	33	1.6	36	35	0.97	3	0.7	0.23	61	20.1	0.33
Cefpirome	14^a	9	0.64																									48	14.3	0.30
Cefepime	11^b	7	0.66	7	4	0.57										26	15	0.58	8	12	1.5	88	20	0.23	0.8	0.35	0.44			
Cefoxitin	8^b	16	2	7	7	1.0													12	35	2.9	CND	NH	CND	11	2	0.18	33	9.7	0.29

References: IMP-1 [421]; IMP-2 [445]; IMP-3 [446]; IMP-6 [454]; IMP-10 [461]; IMP-12 [462]; IMP-16 [469]; IMP-18 (L. Borgianni *et al*, 49th ICAAC, C1-090, 2009); IMP-19 [471]
ND: data could not be determined, NH. No hydrolysis detected
^{a}: K_m was detected as K_i,
^{b}: Vmax was estimated as 2 times the maximum hydrolysis rate observed
Buffer and experimental conditions as indicated in the corresponding references.

data is the variability observed by different research groups; using different methodologies and experimental conditions. With that said, common features that are observed for IMP enzymes include an overall preference for cephalosporins and carbapenems rather than for penicillins (with some exceptions, see K_{cat} for ampicillin in IMP-1 as an example) and a lack of activity toward aztreonam.

IMP-2 exhibited a 20-fold and 10-fold lower K_{cat}/K_m ratio for ampicillin and cephaloridine, respectively, and a much higher affinity for meropenem revealing a functional significance in some of the mutations that differentiated the two IMP variants [445]. IMP-3 preferentially hydrolyzes cephalosporins rather than penicillins or carbapenems while IMP-1 had better hydrolytic activity against imipenem [446]. The $kcat/Km$ value of IMP-6 was approximately 7-fold higher against meropenem than imipenem, and some activity loss on penicillin and piperacillin could be detected when compared to IMP-1 [454]; most likely due to point mutations at the expense of activity against penicillins. IMP-10 differed from IMP-1 in its extremely low hydrolyzing activities for benzylpenicillin, ampicillin, and piperacillin [461]. IMP-12 is less efficient than IMP-1 and other IMP variants for hydrolysis of penicillins. A very high Km is observed for imipenem, similar to that for IMP-3, which is probably due to a Ser262-to-Gly262 substitution not present in IMP variants with higher imipenem affinities [462]. Both amino and ureidopenicillins are good substrates for IMP-13, with a 5-fold higher efficiency in hydrolysis for ureidopenicillins compared to aminopenicillins. Differences were also seen in the hydrolytic profile of piperacillin and ticarcillin compared to other IMP-β-lactamases. IMP-13 is capable of hydrolyzing all the cephalosporins assayed with a 100-fold increase in the efficiency of cefuroxime hydrolysis when compared to IMP-1. Hydrolytic efficiency for imipenem was nearly 20-fold higher compared to meropenem due to the higher catalytic turnover for imipenem (Santella, unpublished). IMP-16 lacks cefoxitin hydrolysis and displays a lower K_{cat}/K_m value for imipenem compared to those for other IMPs [469]. The hydrolytic efficiency of IMP-19 was rather poor for carbapenems, despite an excellent affinity for meropenem **(**[471], and Table **24**).

VIM-MBLs

The VIM-type enzymes are most prevalent in Europe, with 23 allelic variants (Table **25**). They have also appeared in Eastern Asia and the Americas. Sequencing of VIM-1 (Figs. **7** and **8**) revealed it was divergent from the other class B enzymes such as Bc-II (38.7% identity) and IMP-1 (31.4 % identity) [473]. VIM-2 displayed 92.9 % amino acid identity with VIM-1, 32% with Bc-II from *Bacillus cereus*, 31% with IMP-1, and 27% with CcrA from *Bacteroides fragilis* [474].VIM-2, first discovered in France, is currently the most widespread acquired MBL [422].

In general, VIM-MBLs result in a significant decrease in susceptibility to ampicillin, carbenicillin, piperacillin, mezlocillin, cefotaxime, cefoxitin, ceftazidime, cefoperazone, cefepime and carbapenems, in the producing microorganism, even if the producing microorganism is an enterobacterial species [427].

Kinetics of the VIM MBLs Cluster

VIM enzymes have, in general, a broad substrate hydrolysis range, including penicillins, cephalosporins, cephamycins, oxace-

Table 25. VIM Variants

VIM-variant	Original isolate (specie, country, year)	Genetic context and location of *bla*$_{VIM}$	References
VIM-1	*P. aeruginosa,* Italy, 1997	In70.2: IR$_1$-*inti1-bla*$_{VIM-1}$-*aacA4-aphA15-aadA1- qacEΔ1-sul1*- orf5 (chromosome) In70: *res1*- IR$_1$- ISPa7- *inti1- bla*$_{VIM-1}$- *aacA4- aphA15- aadA1- qacEΔ1-sul1*- orf5 (plasmid) In110: 5´-CS- *bla*$_{VIM-1}$-*aacA4-aphA15-aadA1*-3´CS (plasmid)	[473, 475-477]
VIM-2	*P. aeruginosa,* France, 1996	In 56: *inti1-bla*$_{VIM-2}$- *qacEΔ1-sul1*(plasmid) In 58: *inti1-aacCA7-bla*$_{VIM-2}$-*aacC1-aacA4- qacEΔ1-sul1*(chromosome) In 59: *inti1-aacCA29-bla*$_{VIM-2}$-*aacA29- qacEΔ1-sul1*(chromosome) class 1 integron: *inti1-bla*$_{VIM-2}$-*aac(6)IIlike- qacEΔ1-sul1*	[474 , 478, 479]
VIM-3	*P. aeruginosa,* Taiwan, 1997-2000.	class 1 integron: *inti1-bla*$_{VIM-3}$-*orf2-aacA4-aacA4- aadB- aacA4-qacEΔ1*(probably chromosome) class 1 integron: *inti1-bla*$_{VIM-3}$-*orf2-aacA4-qacEΔ1* class 1 integron: *inti1-bla*$_{VIM-3}$-*orf2-aacA4-aadB-aacA4*	[459, 480, 481]
VIM-4	*P. aeruginosa,* Greece, 2001	class 1 integron: *inti1-bla*$_{VIM-4}$- *qacEΔ1-sul1* (probably chromosome) InV4P1: *inti1-bla*$_{VIM-4}$- *bla* $_{P1b}$ *-qacEΔ1-sul1*(chromosome)	[482, 483]
VIM-5	*K. pneumoniae,* Turkey, 2003	class 1 integron: *inti1-bla*$_{VIM-5}$-*orfD- qacEΔ1-sul1* (non conjugative plasmid)	(Midilli, K., *et al*.. A. KLIMIK Congress, Istanbul, Turkey, 2003. Abstract S-21, p. 275) [484, 485]
VIM-6	*P. putida* Singapore, 2000	class 1 integron: *inti1-bla*$_{VIM-6}$-*bla*$_{OXA10}$-*aacA4-* orf-*aadA-qacEΔ1*	[486, 487]
VIM-7	*P. aeruginosa* United States, in 2001	integron: *att1-bla*$_{VIM-6}$ - (transferable plasmid)	[488]
VIM-8	*P. aeruginosa,* Colombia, 1998	Not evaluated	[489]
VIM-9	*P. aeruginosa,* U K, 2004.	Not evaluted	AY524988
VIM-10	*P. aeruginosa,* UK, 2004.	Not evaluated	AY524988
VIM-11	*P. aeruginosa,* Argentina, 2002	class 1 integron: *Inti1-bla*$_{VIM-11}$ class 1 integron: *Inti1-bla*$_{VIM-2}$-*aac(6)IIlike- qacEΔ1-sul1*	[479, 490]
VIM-12	*K. pneumonia,* Greece, 2005	class 1 integron: *inti1-aacCA7-bla*$_{VIM-2}$-*aacA7- qacEΔ1-sul1* (transferable plasmid)	[491, 492]
VIM-13	*P. aeruginosa,* Spain, 2005	class 1 integron: *inti1-bla*$_{VIM-2}$-*aacA4- qacEΔ1* (chromosome)	[493]
VIM-14	*P. aeruginosa,* Italy, 2008	Not evaluated	(AY635904)
VIM-15	*P. aeruginosa,* Bulgaria, 2006	class 1 integron: *inti1-bla*$_{VIM-15}$- *qacEΔ1 -sul1*(chromosome)	[494]
VIM-16	*P. aeruginosa,* Germany, 2005	class 1 integron: *inti1- aac(6´)-Ib´ -bla*$_{VIM-15}$- *aac(6´)-Ib´ - qacEΔ1 -sul1* (chromosome)	[494]
VIM-17 (VIM-2 Homologue)	*P. aeruginosa,* Greece, 2004-2005	In 59.3: *inti1-aacCA29-bla*$_{VIM-2}$-*aacA29- qacEΔ1-sul1*(probably chromosome)	[495]
VIM-18.	*P. aeruginosa,* India, 2006	class 1 integron: *inti1-bla*$_{VIM-18}$- *qacEΔ1 -sul1*(chromosome)	[496]
VIM-23	*E. cloacae,* Mexico, 2009	Not evaluated	GQ242167

Note: VIM variants implicated in nosocomial outbreaks are marked in bold letter

Table 26. Kinetic Parameters of VIM-MBLs

Antibiotics	VIM-1			VIM-2			VIM-5			VIM-7			VIM-11			VIM-13			VIM-15			VIM-16		
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)
Benzylpenicillin	841	29	0.034	49	55.8	1.14	113	29	0.26	17 [a]	430	25	15.3	113	7.4	1,127	757	0.67	25	240	9.6	450	230	0.51
Ampicillin	917	37	0.04				125	14	0.11	15 [a]	190	13	109	190	1.8	197	67	0.34						
Carbenicillin	75	167	2.2							84 [a]	1,200	14												
Piperacillin	3,500	1,860	0.53	72	32.7	0.45	1,753	47	0.03	26 [a]	140	5.4				729	362	0.49						
Ticarcillin	1,117	452	0.41	46	31.7	0.69																		
Cephalothin	53	281	5.3	44	56.2	1.28				45	180	4.0	4.5	81	1.8	76	656	8.6						
Cephaloridine	30	313	10							250	180	0.72							83	190	2.3	1,400	89	0.064
Cefoxitin	131	26	0.2	24	2.8	0.12				68	10	0.15	7.4	6.4	0.86									
Cefuroxime	42	324	7.7	22	12.1	0.55				29	16	0.55	5.5	27	4.9	56	283	5						
Cefotaxime	247	169	0.68	32	27.5	0.86	101	9.2	0.09	22	56	2.6	11.4	41.3	3.6	233	612	2.6	13	90	6.9	240	81	0.34
Ceftazidime	794	60	0.076	98	88.7	0.90	149	0.2	0.001	120	1.4	0.012	110	14	0.13	509	10	0.019	37	1.0	0.027	150	0.22	0.0014
Cefepime	145	549	3.8	184	4.7	0.03	76	0.1	0.001	580	5.3	0.009	>1,200	>100	0.083	1,870	61	0.033	130	9.5	0.076	210	0.31	0.0015
Cefpirome	287	707	2.5	123	9.2	0.07							183	102	0.56									
Cefoperazone				49	29.8	0.61																		
Imipenem	1.5	2.0	1.3	10	9.9	0.99	12	3.5	0.29	27	100	3.7	9.4	20	2.1	18.5	54	2.92	7.3	61	8.4	89	45	0.51
Meropenem	48	13	0.27	5	1.4	0.28	49	2.4	0.05	38	42	1.1	22.7	3.2	0.14	15.5	9	0.59	3.4	6.5	1.9	120	8.4	0.070

References: VIM-1 [497]; VIM-2 [474]; VIM-5 [485]; VIM-7 [498]; VIM-11 [499]; VIM-13 [493]; VIM-15 [494]; VIM-16 [494]
CND: data could not be determined
[a]: K_m were measured as K_i using nitrocefin as reporter substrate
[b]: Vmax was estimated as 2 times the maximum hydrolysis rate observed
Buffer and experimetal conditions as indicated in the corresponding references

phamycins, and carbapenems, but not monobactams (Table **26**). VIM-1 showed the highest k_{cat}/K_m ratios for carbenicillin, azlocillin, some cephalosporins (cephaloridine, cephalothin, cefuroxime, cefepime, and cefpirome), imipenem and biapenem. Kinetic parameters show a remarkable variability within the various penam, cephem, and carbapenem compounds, resulting in no clear preference of the enzyme for any of these β-lactam subfamilies [497]. Kinetic parameters revealed that VIM-2 did not hydrolyze cefepime and cefpirome efficiently [474], while ceftazidime and cefepime were less efficiently hydrolyzed by VIM-5 compared to VIM-1. The behavior of VIM-5 against carbapenems was similar to that of VIM-1 and VIM-2, with greater efficiency for imipenem compared to meropenem [485]. Catalytic efficiencies of VIM-7 for penicillins and carbapenems were higher than VIM-1 and VIM-2. Although VIM-7 exhibits high *Km* values, the higher activity against penicillins, and to some extent carbapenems, correlated with high *k*cat values [498]. VIM-11 showed greater hydrolytic efficiency for imipenem similar to what was observed for VIM-7 [499]. VIM-12 displays unusual characteristics even though the active site is conserved. It has a narrow substrate specificity with activity against penicillin and to a much lesser extent imipenem. In contrast, meropenem was found to act as a noncompetitive inhibitor of the enzyme and ceftazidime was not hydrolyzed due to both a very low affinity and kcat values [500].

Other Acquired MBLs

SPM-1 (**S**ao **P**aulo **M**etallo-β-lactamase) was reported from *P. aeruginosa* isolated in Brazil in 1997. The isolates were resistant to all β-lactam antibiotics, except for aztreonam, which had an MIC of 4 μg/ml. SPM-1 was different from VIM and IMP enzymes and represented a new family of MBLs. SPM-1 contained the classic MBL zinc-binding motifs and showed the highest identity (35.5%) to IMP-1. The predicted molecular weight of the protein was 27,515 kDa, larger than that of IMP (25,041 kDa) or VIM (25,322 kDa) MBLs [501]. *bla*$_{SPM-1}$ was carried on a plasmid that could be transformed into both *Escherichia coli* and *P. aeruginosa* [501]. Upstream of *bla*$_{SPM-1}$, a novel common region (CR4) was identified, comprising an open reading frame, *orf495*, whose product shares significant identity with putative recombinases, such as Orf513 [435]. The emergence and dissemination of an epidemic SPM-1-producing *P. aeruginosa* clone among unrelated Brazilian hospitals has contributed to the high carbapenem resistance rates observed in Brazil [437]. The first nosocomial outbreak of *P. aeruginosa* producing SPM-1 was reported in 2004 [502]. The hydrolytic profile of SPM-1 bears similarity to that of IMP-1. Zinc chelator studies established that none of the chelators completely inhibited SPM-1 ([503] and Table **27**).

GIM-1 (**G**erman **IM**ipenemase) was reported in 2004 from *P. aeruginosa* isolated in Germany, as part of the SENTRY Antimicrobial Surveillance Program in 2002 [504]. The isolates showed resistance to imipenem, meropenem, ceftazidime, cefepime, and piperacillin-tazobactam; they were only susceptible to polymyxin B. GIM-1 differed in its primary sequence from that described for IMP, VIM, and SPM-1 enzymes by 39 to 43%, 28 to 31%, and 28%, respectively. *bla*$_{GIM-1}$ was found on a 22-kb nontransferable plasmid and located in the first position of a 6-kb class 1 integron, In77, followed by *aacA4*, *aadA1*, and *bla*$_{OXA-2}$. Kinetic analyses revealed that GIM-1 has no clear preference for any substrate and did not hydrolyze aztreonam. The comparison of GIM-1 kinetic values with the parameters reported for other clinically relevant

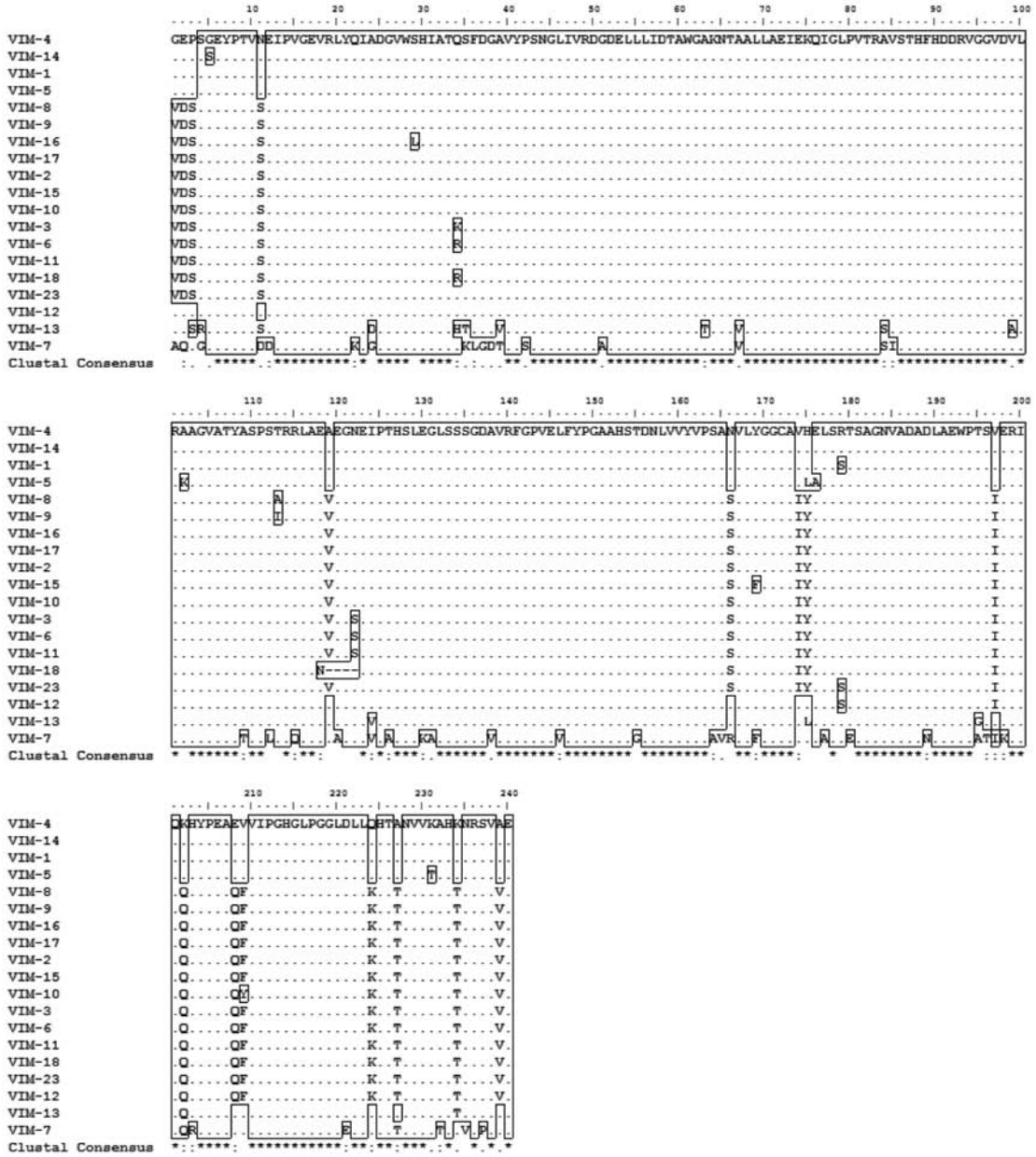

Fig. 7. Amino acid alignment of VIM-MBLs (mature protein).

MBLs showed that, in general, the *k*cat/*Km* ratios obtained for GIM-1 were lower than those of other MBLs, specifically IMP-1, VIM-1, and SPM-1 (Table **27**).

NDM-1 was reported in 2007 from *E. coli* isolates collected in New Delhi, India [505]**.** The isolate was resistant to ampicillin, piperacillin, cephalothin, cefoxitin, cefotaxime, cefuroxime, ceftazidime, aztreonam, cefepime, ertapenem, imipenem, meropenem and ciprofloxacin. NDM-1 shares very little identity with other MBLs, being the most similar to VIM- 1/VIM-2 with only 32.4% identity. The gene encoding NDM-1 was found on a 140 kb plasmid in *E. coli* and was flanked by PAI and IS*26*/Tn*3*. This 392 bp region was downstream to the 4.8 kb complex class 1 integron containing Int, *arr*-2, *ere*2A, *aad*A1 and *cml*A7 as gene cassettes and *qacΔ1*. Downstream of the integron is an intact copy of IS*CR1*. NDM-1 is a monomeric enzyme of 28kDa, and can hydrolyse all β-lactams except for aztreonam. Compared to VIM-2, NDM-1 displays tighter binding to most cephalosporins and in particular cefuroxime, cefotaxime and cephalothin and also to the penicillins. NDM-1 does not bind the carbapenems as tightly as IMP-1 or VIM-2 and turns over the carbapenems at a similar rate to VIM-2 (Table **27**).

NDM-1 has recently been identified within *P. aeruginosa* and *A. baumannii.* The location of the resistance mechanisms in promiscuous plasmids and their association with flexible genetic platforms together with the poorly controlled use of antibiotics are most likely factors that have contributed to the explosive emergence of NDM-1-producing bacteria. In addition, the lack of surveillance and control policies also contribute to the spread of NDM-producing organisms. Given that NDM-1 bacteria are mainly transmitted via oral - fecal, inadequate water sanitation and other basic hygiene facilities have exacerbated this problem.

Currently, there have been a few variants of NDM-1 reported ; the first was NDM-2, in a clonal dissemination of *A. baumannii.* This variant had a C82G substitution resulting in an amino acid change (Pro28Ala). $bla_{\text{NDM-2}}$ was surrounded by two copies of

Table 27. Kinetic Paramerers of New MBLs

Antibiotics	VIM-1			IMP-1			SPM-1			GIM-1			NDM-1		
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)
Benzylpenicillin	841	29	0.034	520	320	0.62	38	108	2.8	46	6.6	0.14	16	11	0.68
Ampicillin	917	37	0.04	200	950	4.8	72	117	1.6	20	3.3	0.16	22	15	0.66
Carbenicillin	75	167	2.2	ND	ND	0.02	814	74	0.09	170	4.1	0.02			
Piperacillin	3,500	1,860	0.53	ND	ND	0.72	59	117	2	69	6.9	0.10	12	14	1.17
Mezlocillin	346	255	0.74												
Ticarcillin	1,117	452	0.41	740	1.1	0.0015	0.35	*a*	*a*	57	2.3	0.04			
Cephalothin	53	281	5.3	21	48	2.4	4	43	11.7	22	16	0.72	10	4	0.40
Cephaloridine	30	313	10	22	53	2.4	18	14	0.8						
Cefoxitin	131	26	0.2	8	16	2	2	8	4	206	8.3	0.04	49	1	0.02
Cefuroxime	42	324	7.7	37	8	0.22	4	37	8.8	7	5.9	0.80	8	5	0.61
Cefotaxime	247	169	0.68	4[a]	1.3	0.35	9	16	1.9	4	1.1	0.24	10	6	0.58
Ceftazidime	794	60	0.076	44	8	0.18	46	28	0.6	31	18	0.58	181	5	0.03
Cefepime	145	549	3.8	11	7	0.66	18	18	1	431	17	0.04	77	13	0.17
Cefpirome	287	707	2.5	14[a]	9	0.64									
Imipenem	1.5	2.0	1.3	39	46	1.2	37	33	1	287	27	0.09	94	20	0.21
Meropenem	48	13	0.27	10	50	5	281	63	0.22	25	2.7	0.11	49	12	0.25
Biapenem	7.5	8.5	1.1	28	160	6									
Aztreonam	>1,000	<0.01	<0.0001	> 1,000	> 0.01	<0.0001	0.3	*a*	*a*	ND	ND	ND	ND		

References: VIM-1 [497]; IMP-1 [421]; SPM-1 [503]; GIM-1 [504]; NDM-1 [505]
a K_m was obtained as K_i
ND: not determinable
Buffers and experimental conditions as indicated in the corresponding references

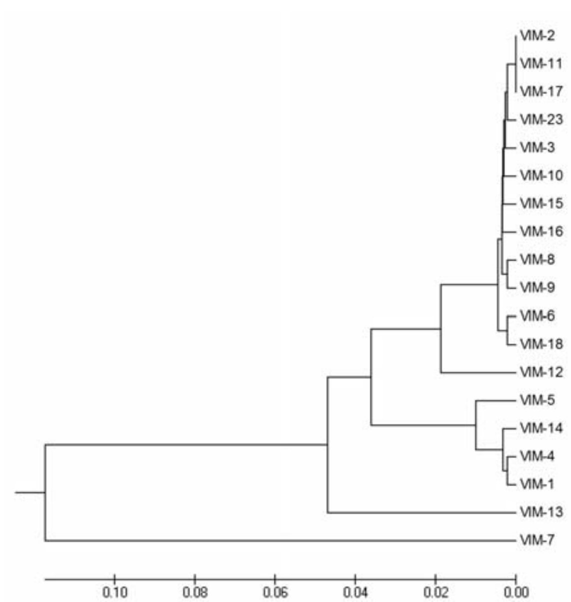

Fig. 8. Phylogenic relationship among VIM-MBLs.

IS*Aba125*. The strain lacked detectable plasmids and bla_{NDM-2} could not be transferred by conjugation [505a].

NDM-4 (differs from NDM-1 by a single Met154Leu substitution) has increased hydrolytic activities for carbapenems and several cephalsosporins [505b]. NDM-5 conserves the same substitution and differs from NDM-4 by a Val88Leu substitution. In this case, TOP10 transformants displayed reduced susceptibility to cephalosporins and carbapenems [505c]. To date, no publications have described NDM-3 or NDM6.

SIM-1 (for **S**eoul **IM**ipenemase) was reported from seven multidrug resistant *A. baumannii* isolated in Korea [506], although they exhibited relatively low imipenem and meropenem MICs (8 to 16 μg/ml). SIM-1 is a new member of subclass B1, displaying 64 to 69% identity with IMP-type MBLs, its closest relatives. The bla_{SIM-1} gene was carried on a gene cassette inserted into a class 1 integron, which included three additional cassettes (*arr-3*, *catB3*, and *aadA1*).

KHM-1 was reported from *C. freundii* isolated at the Kyorin University Hospital (Tokyo) in 2008 [507]. This isolate, recovered in 1997, was resistant to most β-lactam antibiotics, except monobactams and showed reduced susceptibility for carbapenems. The coding gene for KHM-1was not detected within integrons and no sequence homologies for site-specific cointegration events, ORF or

transmissible elements were detected upstream of bla_{KHM-1}. Purified KHM-1 shows high catalytic efficiency towards cephalosporins (cephaloridine, cefoxitin, cefotaxime, ceftazidime) and low *kcat* / *Km* values of penicillin G, ampicillin, meropenem and imipenem (Table **27**).

Recently reported MBLs are **AIM-1** (D. Yong *et al.*, 48th ICAAC, C1-890, 2008) and DIM-1 [508], identified in *Pseudomonas* spp. in Australia and The Netherlands, respectively.

Some Concluding Remarks

An Urgent Need for Kinetic Evaluation Standardization

Evaluation of β-lactamases is a critical parameter in establishing the characteristics of these enzymes. However, as demonstrated in this review, there are strong discrepancies in data obtained for the same set of enzymes; some of these differences occur because of the different experimental approaches or conditions used. Guidelines need to be developed for reporting enzymatic analysis and evaluation of data for publication. Although these ideas have been discussed among β-lactamase researchers, no guidelines have been published so that hydrolysis data from different laboratories can be compared. There needs to be a set of drugs and conditions established for hydrolytic analysis of specific classes of β-lactamases. However, these guidelines need to take into consideration the actual interaction between organism and drug. Therefore, assay conditions need to reflect these types of interactions as closely as possible. Then kinetic comparisons between laboratories can be used to analize the correlation between activity and β-lactam MICs.

Requirements for a "Successful β-lactamase": The β-lactamases point of view

Good hydrolytic activity and concentration of enzyme are important requirements to a successful β-lactamase. The concentration of the enzyme is dependent on the level of gene expression encoding the enzyme. This can be controlled by the promoter sequence ofthe gene, the copy number of the gene, and in the case of integrons, the placement of the gene within the integron which relates back to promoter strength. Therefore, a successful β-lactamase requires more than the ability to simply hydrolyze a drug.

Requirements for a "Successful β-lactamase": The Human Factor

Lack of detection is an additional requirement for a successful β-lactamase when the definition of success is the spread of the resistance mechanisms to patient populations. The inability to detect certain β-lactamase phenotypes in the clinical laboratory allows the spread and mobilization of the gene to genetic platforms capable of increasing the efficiency of mobilization. Once susceptibility tests and detection methods are available, there is a chance to contain further spread, but this is not usually the case. We knew of the arrival of TEM β-lactmase into enterobacterial plasmids, but this did not help us identify or inhibit the spread of TEM and SHV derived-ESBLs, or the rise of CTX-Ms as the most prevalent ESBL, nor have we been able to prevent the dissemination of "mobile" metallo and (more recently) class A carbapenemases such as KPCs. The trend over the last 30 years is to evaluate a curiosity only to find that the curiosity has diseminated "undiagnosed". More communication, networking and cooperation from clinical microbiology laboratories, academic groups, diagnostic companies, health authorities and pharmaceutical companies is needed if we are ever going to curb the rapid emergence of various β-lactamases in the coming years.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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