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Carbapenem Resistance in Gram-negative Pathogens: Emerging Non-metallo-carbapenemases

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Abstract: Non-metallo-carbapenemases involved in acquired resistance are of Ambler molecular class A, C and D. Class A, clavulanic acid-inhibited carbapenemases are either chromosome-encoded (SME-1 to SME-3, IMI-1 to IMI-2, NMC-A) in *Serratia marcescens* and *Enterobacter cloacae*, or plasmid-encoded, such as KPC-1 to KPC-4 in *Klebsiella* and *Enterobacter* spp. and GES-2 (*Pseudomonas aeruginosa*), -4 (*Klebsiella pneumoniae*), -5 (*Escherichia coli*) and -6 (*Klebsiella pneumoniae*). In class C, imipenem resistance has been associated with porin loss and class C carbapenemases in *Pseudomonas aeruginosa*, *Enterobacter* spp. and *Klebsiella pneumoniae*. CMY-10 is a plasmid-encoded class C β-lactamase with a wide spectrum of substrates. Unlike the well-studied class C extended-spectrum β-lactamase from *Enterobacter cloacae* GC1, the Ω-loop does not affect the active site conformation and the catalytic activity of CMY-10. Instead, three amino acid deletion in the R2 active site appears to be responsible for the extended-spectrum activity of CMY-10. This observation demonstrates the hydrolyzing activity of CMY-10 toward imipenem with a long R2 substituent. CMY-10 is the only Ambler class C β-lactamase with carbapenemase activity without reduced uptake (porin loss) so far. Finally, class D carbapenemases are increasingly reported in *Acinetobacter baumannii* but compromise carbapenem susceptibility only marginally. The sources of the acquired non-metallo-carbapenemase genes remain unknown and these enzymes may remain underestimated as a consequence of the lack of their detection. The more three-dimensional structural information of these enzymes is necessary for the development of new inhibitors for non-metallo-carbapenemases or new β-lactam antibiotics.

Key words: Biochemical characteristics, discovery chronology, β-lactam antibiotic, β-lactamase inhibitor, non-metallo-carbapenemases resistance

Introduction

β-Lactamases (EC 3.5.2.6) hydrolytically inactivate β-lactam antibiotics and thus the expression of β-lactamases is a prevalent resistance mechanism of pathogenic bacteria to β-lactam antibiotics (penicillins, cephalosporins, monobactams and carbapenems). Based on the sequence homology, β-lactamases are grouped into four molecular classes, A, B, C and D (Ambler, 1980). Carbapenems (imipenem and meropenem) have the broadest activity spectra of any β-lactam antibiotic and are often the most appropriate agents for use in the treatment of infections caused by multiresistant

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Table 1: Chronology of discovery of class A carbapenemases

Enzyme	Clinical strains	Origin (Year)	Encodement site	pI	GenBank No.	Reference(s)
SME-1	*Serratia marcescens* S6 and S8	UK (1982)	Chromosome	9.7	Z28968	Naas *et al.*, 1994b; Yang *et al.*, 1990;
SME-2	*Serratia marcescens* 4126	USA (1992)	Chromosome	9.5	AF275256	Queenan *et al.*, 2000
SME-3	*Serratia marcescens*	USA (2004)	Chromosome		AY584237	UP[b]
IMI-1	*Enterobacter cloacae* 1413B	USA (1984)	Chromosome	7.05	U50278	Majiduddin and Palzkill, 2003
NMC-A	*Enterobacter cloacae* NOR-1	France (1990)	Chromosome	6.9	Z21956	Naas *et al.*, 1994a; Nordmann *et al.*, 1993
KPC-1	*Klebsiella pneumoniae* 1534	USA (1997)	Nonconjugative plasmid	6.7	AF297554	Yigit *et al.*, 2001
KPC-2	*Klebsiella oxytoca* 3127	USA (1998)	Conjugative plasmid	6.9[a]	AY210886	Yigit *et al.*, 2003
KPC-3	*Klebsiella pneumoniae* CL 5761	USA (2001)	Conjugative plasmid	5.97[a]	AF395881	Woodford *et al.*, 2004
KPC-4	*Enterobacter* sp. E624	UK (2004)			AY700571	UP
GES-2	*Pseudomonas aeruginosa* GW-1	South Africa (2000)	Integron in plasmid	5.8	AF326355	Poirel *et al.*, 2001
GES-4	*Klebsiella pneumoniae* KG502	Japan (2002)	Integron in plasmid	6.9	AB116260	Wachino *et al.*, 2004b
GES-5	*Escherichia coli* 365-02	Greece (2003)	Integron in plasmid	5.8	AY494717	Vourli *et al.*, 2004
GES-6	*Klebsiella pneumoniae* 78-01	Greece (2003)	Integron in plasmid	6.9	AY494718	Vourli *et al.*, 2004

[a]The pI value was 6.7 in the report by Bratu *et al.* (2005c), [b]UP, unpublished, Blank, not determined

Gram-negative aerobic bacteria. The emergence and spread of acquired carbapenem resistance due to carbapenemases are therefore a major concern and have been dubbed a global sentinel event (Richet *et al.*, 2001). Based on the requirement of divalent cations for enzyme activation, carbapenemases can be divided two groups: I) metallo-carbapenemases (Zinc-dependent class B) and ii) non-metallo-carbapenemases (Zinc-independent class A, C and D). Metallo-carbapenemases have been well described and updated (http://www.lahey.org/studies/webt.asp) (Livermore and Woodford, 2000; Nordmann and Poirel, 2002) but new reports of non-metallo-carbapenemases have been increasing over the last few years. Thus, this study considers only non-metallo-carbapenemases but not metallo-carbapenemases.

The Molecular Class A Carbapenemases

Class A carbapenemases have been discovered most frequently in isolates of *Enterobacteriaceae* and also in species such as *Pseudomonas aeruginosa*. The emergence and dissemination of transferable and plasmid-encoded class A carbapenemase-producing strains represent an additional threat in the evolving world of β-lactamase-mediated resistance in Gram-negative aerobic bacteria (Table 1).

SME-type Carbapenemases

SME-1 was the first class A carbapenemase that was identified from two *S. marcescens* strains collected in London 1982 (Table 1) (Yang *et al.*, 1990). SME-1-producing *S. marcescens* strains were also discovered in Minnesota 1985 and in Chicago 1999 (Gales *et al.*, 2001). SME-2-producing *S. marcescens* strains were collected from Los Angeles in 1992 and Boston in 1997

Table 2: Catalytic constants (k_{cat}) of class A carbapenemases

Antimicrobial agents	k_{cat} (s^{-1})[a]							
	SME-1	SME-2	IMI-1	NMC-A	KPC-1	KPC-2	GES-2	GES-4
Penicillin G	19.3	21.3	36	260	32	51	0.4	130
Cloxacillin					25	35		
Ampicillin	181	204	190		110	210		19
Amoxicillin				816			0.7	
Ticarcillin				81			0.06	
Piperacillin			6.1				0.3	
Cephaloridine	980	1081	2000		340	530	0.5	490
Cephalothin			200	2820	75	69	0.3	
Cefminox								7.7
Cefoxitin	<0.15	<0.17	0.3	5.0	0.26	0.31	NH[b]	85
Cefotetan				5.3				
Cefotaxime	<0.98	<1.0	3.4	286	14	22	2.2	17
Ceftazidime	<0.07	<0.09	0.0068	4.7	0.10	≤0.12	ND[c]	2.5
Moxalactam				8.6				
Cefepime							1.1	
Cefpirome								3.6
Imipenem	104	136	89	1040	12	15	0.004	0.38
Meropenem	8.9	7.3	10	12	3.0	4.0	NH	
Aztreonam	108	140	51	707	20	30	NH	NC[d]

[a]Adapted from data presented by Queenan *et al.* (2000) for SME-1 and -2, Rasmussen *et al.* (1996) for IMI-1, Rasmussen *et al.* (1996) for IMI-1, Mariotte-Boyer *et al.* (1996) for NMC-A, Yigit *et al.* (2003) for KPC-1 and -2, Poirel *et al.* (2000) for GES-2 and Wachino *et al.* (2004b) for GES-4, [b]NH, not hydrolyzed since the initial rate of hydrolysis was lower than 0.001 μM^{-1} s^{-1}, [c]ND, not determinable due to very high K_m values, [d]NC, not calculated, Blank, not provided in each reference

(Gales *et al*., 2001; Queenan *et al*., 2000). In 2004, SME-3 has been identified from *S. marcescens* strains isolated in USA (K. Bush, personal data). Genomic comparison of worldwide-encountered *S. marcescens* strains that had SME-type enzymes indicated a global dissemination of a distinct *S. marcescens* subtype (Gales *et al*., 2001). The *sme-1* gene was chromosome-encoded. Expression of *sme-1* was inducible (Naas *et al*., 1995). The *sme-1* gene was preceded by a Lys-type regulatory gene similar to *ampR* genes found upstream of the naturally encoded cephalosporinase (*ampC*-type genes). The LysR-type regulator named *smeR* increased biosynthesis of the enzyme at a basal state and further increased its biosynthesis when β-lactam-mediated induction occurs (Naas *et al*., 1995). The SME-2 differs from SME-1 by only a single amino acid substitution at position 207, where valine is replaced by glutamic acid. The SME-3 differs from SME-2 by only a single amino acid substitution at position 108, where histidine is replaced by tyrosine. The cloned sme-1 and sme-2 genes conferred resistance to carbapenems, penicillins, cephalothin and aztreonam; on the other hand, it conferred no significant resistance to cefoxitin, cefotaxime, or ceftazidime (Naas *et al*., 1994b). K_m and k_{cat} values for each substrate were similar for both enzymes (Table 2 and 3), indicating that the single amino acid change in SME-2 did not affect the substrate binding or hydrolysis rates. The sme-1 and SME-2 had the highest affinity (the lowest K_m) for penicillin and meropenem. Cefoxitin, cefotaxime and ceftazidime were poor substrates, with hydrolysis rates too slow to obtain accurate K_m values (Table 2 and 3). These enzymes significantly hydrolyze penicillins, cephalothin, aztreonam and carbapenems (e.g., imipenem and meropenem). These enzymes were inhibited by clavulanate and tazobactam and hydrolyzed imipenem more than meropenem (Gales *et al*., 2001; Queenan *et al*., 2000). Biochemical characterization of SME-3 has not been reported yet. Three-dimensional model of SME-1 showed that

Table 3: Affinity constants (K_m) of class A carbapenemases

Antimicrobial agents	K_m (μM)[a]							
	SME-1	SME-2	IMI-1	NMC-A	KPC-1	KPC-2	GES-2	GES-4
Penicillin G	16.7	17.7	64	28	23	27	4	160
Cloxacillin					100	79		
Ampicillin	488	609	780		130	230		62
Amoxicillin				90			25.8	
Ticarcillin				152			13.3	
Piperacillin			13				22.8	
Cephaloridine	770	859	1070		560	500	7.7	2200
Cephalothin			130	185	53	82	3	
Cefminox								370
Cefoxitin	ND[b]	ND	45	93[c]	120	180	NH[d]	810
Cefotetan				80[c]				
Cefotaxime	ND	ND	190		160	220	890	700
Ceftazidime	ND	ND	270	90[c]	94	ND	>3000	1500
Moxalactam				300[c]				
Cefepime							1900	
Cefpirome								340
Imipenem	202	313	170	92	81	51	0.45	4.7
Meropenem	13.4	9.6	26	4.35	12	15	NH	
Aztreonam	259	277	93	125	310	360	NH	NH

[a]Adapted from data presented by Queenan *et al.* (2000) for SME-1 and -2, Rasmussen *et al.* (1996) for IMI-1, Rasmussen *et al.* (1996) for IMI-1, Mariotte-Boyer *et al.* (1996) for NMC-A, Yigit *et al.* (2003) for KPC-1 and -2, Poirel *et al.* (2000) for GES-2 and Wachino *et al.* (2004b) for GES-4, [b]ND, not determinable since hydrolysis was too slow, [c]K_m was measured as K_i with penicillin G as competitor substrate, [d]NH, not hydrolyzed since the initial rate of hydrolysis was lower than 0.001 $\mu M^{-1} s^{-1}$, Blank, not provided in each reference

five active site residues (Cys69, His105, Arg220, Ser237 and Cys238) were characteristic of carbapenemases when compared to most other class A β-lactamases such as TEM-1 (Raquet *et al*., 1997). A disulfide bridge (C69-C238) drew the β-3 strand further into the active site when compared to the TEM-1 structure (Raquet *et al*., 1997). Therefore, the disulfide bridge allowed a better interaction of β-lactam carbonyl of imipenem with the oxyanion hole of the SME-1 enzyme than with that of TEM-1 (Majiduddin and Palzkill, 2003; Raquet *et al*., 1997).

IMI-type Carbapenemases

IMI-1 was the second type of chromosome-encoded class A carbapenemase that was identified from the *Enterobacter cloacae* strain isolated from a groin wound of a patient hospitalized in Southern California (Table 1) (Rasmussen *et al*., 1996). IMI-2-producing *E. asburiae* environmental strains were discovered in Arkansas River (Wichita, KS, USA) 1999, in Kansas River (Topeka, KS, USA) 2000, in Des Moines River (Des Moines, IA, USA) 2001 and in Mississippi River (Minneapolis, MN, USA) 2001 (Aubron *et al*., 2005). Expression of *imi-1* and *imi-2* genes was inducible, due to an upstream-located LysR-type regulatory gene such as *imiR* (Aubron *et al*., 2005; Rasmussen *et al*., 1996). The *imi-2* gene was encoded by a 66-kb transferable plasmid and differed from *imi-1* by 11 nucleotide substitutions (Aubron *et al*., 2005). Thus, the discovery of *imi-2* gene represents an additional threat in evolving world of carbapenemase-mediated resistance in bacteria. The IMI-2 has two amino acid substitutions (asparagine to aspartic acid at position Ambler 35 and tyrosine to histidine at position Ambler 105) compared to IMI-1. Compared to the cloned *sme*-type genes, the cloned *imi-1* and *imi-2* genes conferred similar MIC patterns (Rasmussen *et al*., 1996). Although IMI-1 shared 71% amino acid identity with SME-1, it had an overall similar hydrolysis profiles (Table 2 and 3) since cysteines at amino acids residues 69 and 238 were conserved in SME-1, IMI-1,

NMC-A and KPC-1 and formed a disulfide bond (Majiduddin and Palzkill, 2003; Raquet *et al*., 1997). IMI-1 was inhibited by all the three β-lactamase inhibitors. Tazobactam was the strongest inhibitor, with an IC_{50} of 30 nM; this was followed by clavulanic acid, with an IC_{50} of 280 nM. Sulbactam was the least inhibitory, with an IC_{50} of 1,800 nM (Rasmussen *et al*., 1996).

NMC-A Carbapenemase

NMC-A was the third type of chromosome-encoded class A carbapenemase that was identified from the *E. cloacae* strain isolated from the pus of a fistulized subcutaneous abscess of a patient hospitalized in Paris (Table 1) (Nordmann *et al*., 1993). Expression of *nmcA* gene was also inducible, due to an upstream-located LysR-type regulatory gene such as *nmcR* (Naas *et al*., 1994a). AmpD (cytosolic *N*-acetyl-anhydromuramyl-L-alanine amidase; a negative regulator in *ampC* expression) was involved in the regulation of expression of *nmcA* and *ampC*, suggesting that structurally unrelated genes might be under the control of an identical genetic system (Naas *et al*., 2001). The cloned *nmcA* gene conferred resistance to ampicillin, ticarcillin, cephalothin, cefamandole, aztreonam and imipenem, whereas it remained susceptible to cefotaxime and ceftazidime (Naas *et al*., 1994a). These patterns are also similar to the cloned *sme*-type genes. NMC-A shares 71 and 97% amino acid identity with SME-1 and IMI-1, respectively. The IC_{50} values of clavulanate and tazobactam for NMC-A were about 6 ~ 9-fold higher than those obtained for SME-1, thus indicating that NMC-A was inhibited less as compared to SME-1 (Mariotte-Boyer *et al*., 1996). Compared to SME-1, NMC-A had similar hydrolytic properties towards carbapenems and cephamycins but catalytic efficiencies (k_{cat}/K_m) 10 ~ 100-fold greater than those of SME-1 (Table 2). In addition to Cys69 and Cys238 residues forming a disulfide bridge, the position of Asn132 in NMC-A provided critical additional space in the region of the protein where carbapenems and cephamycins need to be accommodated (Swarén *et al*., 1998).

KPC-type Carbapenemases

KPC-1 was the fourth and more recently reported type of plasmid-encoded class A carbapenemase that was identified from the *Klebsiella pneumoniae* strain isolated from a patient hospitalized in North Carolina (Table 1) (Yigit *et al*., 2001). KPC-2 was produced by a *K. oxytoca* strain collected in New York 1998 (Yigit *et al*., 2003), by *K. pneumoniae* strains collected in Baltimore 1999 (Moland *et al*., 2003), by a *Salmonella* serotype Cubana strain collected in Maryland 1998 (Miriagou *et al*., 2003), by nineteen *Klebsiella* isolates collected in New York between 1997 and 2002 (Bradford *et al*., 2004), by an *Enterobacter* sp. strain collected in Boston 2001 (Hossain *et al*., 2004), by an *E. cloacae* strain collected in New York 2001 (Bratu *et al*., 2005a), by sixteen *K. pneumoniae* strains collected in New York between 2003 and 2004 (Bratu *et al*., 2005b) and by fifty-nine *K. pneumoniae* strains collected in New York 2004 (Bratu *et al*., 2005c). Thus, the KPC-2 is likely being disseminated among species of *Enterobacteriaceae* through both conjugal plasmid transfer and transposition (Table 1). KPC-3 was produced by the outbreak strain of *K. pneumoniae* collected in New York between 2000 and 2001 (Woodford *et al*., 2004), by an *E. cloacae* strain collected in New York 2003 (Bratu *et al*., 2005a) and by three *K. pneumoniae* strains collected in New York 2004 (Bratu *et al*., 2005c). KPC-4-producing *Enterobacter* sp. strain was discovered in Scotland 2004 (D. M. Livermore, personal data). Expression of *kpc*-type genes was not inducible (Woodford *et al*., 2004; Yigit *et al*., 2001; Yigit *et al*., 2003). Compared to the amino acid sequence of KPC-1, KPC-2 has the Ser174→Gly substitution, KPC-3 has the Ser174→Gly and His272→Tyr substitutions and KPC-4 has the Pro103→Arg, Ser174→Gly and Val239→Gly substitutions. The cloned *kpc-1* and *kpc-2* genes conferred resistance to carbapenems, extended-

Table 4: Amino acid substitutions of GES-type extended-spectrum β-lactamases (ESBLs)

	Residue (coding triplet) at amino acid[a]:						
β-lactamase	62	104	125	170	243	Reference	Gen Bank No.
GES-1	Met(ATG)	Glu(GAA)	Ala(GCT)	Gly(GGC)	Gly(GGC)	Poirel *et al.*, 2000	AF156486
GES-2				Asn(AAC)		Poirel *et al.*, 2001	AF326355
GES-3	Thr(ACG)	Lys(AAA)				Wachino *et al.*, 2004a	AB113580
GES-4	Thr(ACG)	Lys(AAA)		Ser(AGC)		Wachino *et al.*, 2004b	AB116260
GES-5[b]				Ser(AGC)		Vourli *et al.*, 2004	AY494717
GES-6[b]		Lys(AAA)		Ser(AGC)		Vourli *et al.*, 2004	AY494718
GES-7[b]		Lys(AAA)				Giakkoupi *et al.*, 2000	AF208529
GES-8[b]			Leu(CTG)			Mavroidi *et al.*, 2001	AF329699
GES-9					Ser(AGC)	Poirel *et al.*, 2005a	AY920928

[a] Abmbler's position, [b]Names correspond to the recent update of GES nomenclature by Lee *et al.* (2005a): renamed GES-5, GES-6, GES-7 and GES-8 for GES-3 (Vourli *et al.*, 2004), GES-4 (Vourli *et al.*, 2004), IBC-1 (Giakkoupi *et al.*, 2000) and IBC-2 (Mavroidi *et al.*, 2001), respectively. The sequence of GES-7 (IBC-1) was changed in April 18 2003: Ala(GCT) in stead of Leu(CTG), Four GES ESBLs (GES-2, GES-4, GES-5 and GES-6) are class A carbapenemases

spectrum cephalosporins (ceftazidime, cefotaxime and so on) and aztreonam (Yigit *et al*., 2001; Yigit *et al*., 2003). MICs for KPC-3-producing transformants tended to be lower than those for KPC-1-producing transformants (Woodford *et al*., 2004; Yigit *et al*., 2001). KPC-1 has weak amino acid identity with the most closely related enzymes SME-1 (45%), IMI-1 (43%) and NMC-A (44%) which are chromosome-encoded class A carbapenemases. Compared to these enzymes, KPC-1 and KPC-2 activities were more inhibited by clavulanic acid and tazobactam, whereas their catalytic efficiencies for cephaloridine, imipenem, meropenem and aztreonam were lower (Table 2 and 3). The highest k_{cat} values of KPC-1 were obtained with cephaloridine and cefoxitin and ceftazidime had the lowest hydrolysis rates (k_{cat} values). KPC-1 showed hydrolytic activity against the carbapenems (Table 2). KPC-1 had the highest affinity for meropenem (Table 3). Since in addition to K_i values (Yigit *et al*., 2003), k_{cat} and K_m values for β-lactam substrates were similar for KPC-1 and KPC-2, Ser174Gly substitution did not cause changes in the hydrolytic profile of KPC-2. Biochemical characterization of KPC-3 and KPC-4 has not been reported yet. In addition to Cys69 and Cys238 residues forming a disulfide bridge, the position of Asn132 of NMC-A was conserved in KPC-1 and provided critical additional space in the region of the protein where carbapenems and cephamycins need to be accommodated in KPC-1 (Yigit *et al*., 2001).

GES-type Carbapenemases

GES-2 was the fifth and most recently reported type of plasmid-encoded class A carbapenemase that was identified from the *Pseudomonas aeruginosa* strain isolated from a patient hospitalized in Zimbabwe (Table 1) (Poirel *et al*., 2001). According to the new nomenclature system (Lee *et al*., 2005a), GES-type extended-spectrum β-lactamases (ESBLs) included nine variants from GES-1 to GES-9 (Giakkoupi *et al*., 2000; Mavroidi *et al*., 2001; Poirel *et al*., 2000, 2001 and 2005a; Vourli *et al*., 2004; Wachino *et al*., 2004a and 2004b). These enzymes are point-mutant derivatives of the GES-1 ESBL (Table 4). Of nine GES-type ESBLs, GES-2, GES-4, GES-5 and GES-6 are class A carbapenemases (Table 1 and 4). GES-5-producing *K. pneumoniae* strains were disseminated in Republic of Korea (Jeong *et al*., 2005). Like *kpc*-type genes, expression of *ges*-type genes was also not inducible (Poirel *et al*., 2000; Vourli *et al*., 2004; Wachino *et al*., 2004b). GES-2 has weak amino acid identity with SME-1 (38%), IMI-1 (37%), NMC-A (37%) and KPC-1 (40%). GES-2- and GES-4-producing clinical strains exhibited resistance to extended-spectrum cephalosporins and

intermediate susceptibility to carbapenems (Poirel *et al*., 2001; Wachino *et al*., 2004b). GES-4-producing clinical strain was also resistant to cephamycins (Wachino *et al*., 2004b). GES-5-producing clinical strain was resistant to ceftazidime and susceptible to cefotaxime, carbapenems and aztreonam (Vourli *et al*., 2004). However, GES-6-producing clinical strain was resistant to all these β-lactams (Vourli *et al*., 2004). The cloned *ges-6* genes conferred low-level resistance to carbapenems (Vourli *et al*., 2004). The same has been observed also for GES-2, GES-4 and GES-6 (Poirel *et al*., 2000; Vourli *et al*., 2004; Wachino *et al*., 2004b). It is likely that the high level of carbapenem resistance observed in the GES-6-producing clinical strain is due to the simultaneous operation of additional resistance mechanisms (e.g., decreased outer membrane permeability). GES-2 had significant catalytic efficiency (k_{cat}/K_{m}) against imipenem (Table 2 and 3). Like other class A carbapenemases, GES-2 contained two cysteine residues in position 69 and 238 that may form a disulfide bridge. The GES-2 catalytic efficiency against imipenem was 100-fold higher than that of GES-1 (Poirel *et al*., 2000 and 2001). However, GES-2 activity against imipenem remained 1000-fold less than that of SME-1 and NMC-A (Table 2 and 3). Thus, this cysteine bridge may enable the catalytic site to bind imipenem, but other amino acid residues are likely to be involved in the significant catalytic efficiency of the carbapenem-hydrolyzing enzymes against imipenem. A glycine-to-asparagine substitution in GES-2 (Table 4) extends its activity to imipenem. This substitution occurred in an amino acid position of the Ω-loop of class A β-lactamases that is of primary importance in the catalytic activity of these enzymes (Banerjee *et al*., 1998; Matagne *et al*., 1998). This substitution may enlarge the pocket that houses the hydroxyethyl moiety of imipenem on the alpha face of the acyl enzyme for GES-2 (Maveyraud *et al*., 1998). The obvious increase in the catalytic efficiency against cephamycin and imipenem seen for GES-4 were not detected for GES-2 and GES-3 (Table 2 and 3) (Wachino *et al*., 2004a). IC_{50} values of clavulanic acid and tazobactam were 5.0 and 2.5 μM for GES-1, 1.0 and 0.5 μM for GES-2, 1.5 and 0.19 μM for GES-3 and 15 and 1.4 μM for GES-4 (Poirel *et al*., 2000 and 2001; Wachino *et al*., 2004a and 2004b). Clavulanic acid and tazobactam are active against GES-2 and GES-3 but less so against GES-1 and GES-4. GES-4 had a single glycine-to-serine substitution within the Ω-loop region of class A β-lactamases compared with the sequence of GES-3 (Table 4). These findings suggest that the single amino acid substitution (not glycine-to-asparagine but glycine-to-serine) at position 170 may affect not only cephamycin and carbapenems but also inhibitor resistance. To elucidate this nature of GES-4, molecular modeling analysis as well as X-ray crystallographic analysis should be performed in the future.

The Molecular Class C Carbapenemases

Clinically, class C β-lactamases, together with class A enzymes, are the most commonly encountered (Waley, 1992) and pose therapeutic problems because they can confer resistance to cephamycins (cefoxitin and cefotetan), penicillins, cephalosporins and β-lactam-β-lactamase inhibitor combinations and are not significantly inhibited by clinically used β-lactamase inhibitors like clavulanic acid. Class C β-lactamases are typically synthesized by Gram-negative organisms. They are mainly chromosomal class C β-lactamases in several potential pathogens, such as *Acinetobacter* spp., *Aeromonas* spp., *Chromobacterium violaceum*, *Citrobacter* spp., *Enterobacter* spp., *Escherichia coli*, *Morganella* spp., *Proteus rettgeri*, *Pseudomonas aeruginosa, Serratia* spp. and *Yersinia enterocolitica* (Lee *et al*., 2002; Philippon *et al*., 2002). After the first report of a plasmidic cephamycin-hydrolyzing β-lactamase (CMY-1) from Republic of Korea (Bauernfeind *et al*., 1989), recently plasmid-encoded class C β-lactamases have been reported in *Klebsiella pneumoniae* (CMY-2, CMY-8, CMY-12, MOX-1, MOX-2, FOX-1, FOX-5, LAT-1, LAT-2, LAT-2b, ACT-1, MIR-1 and

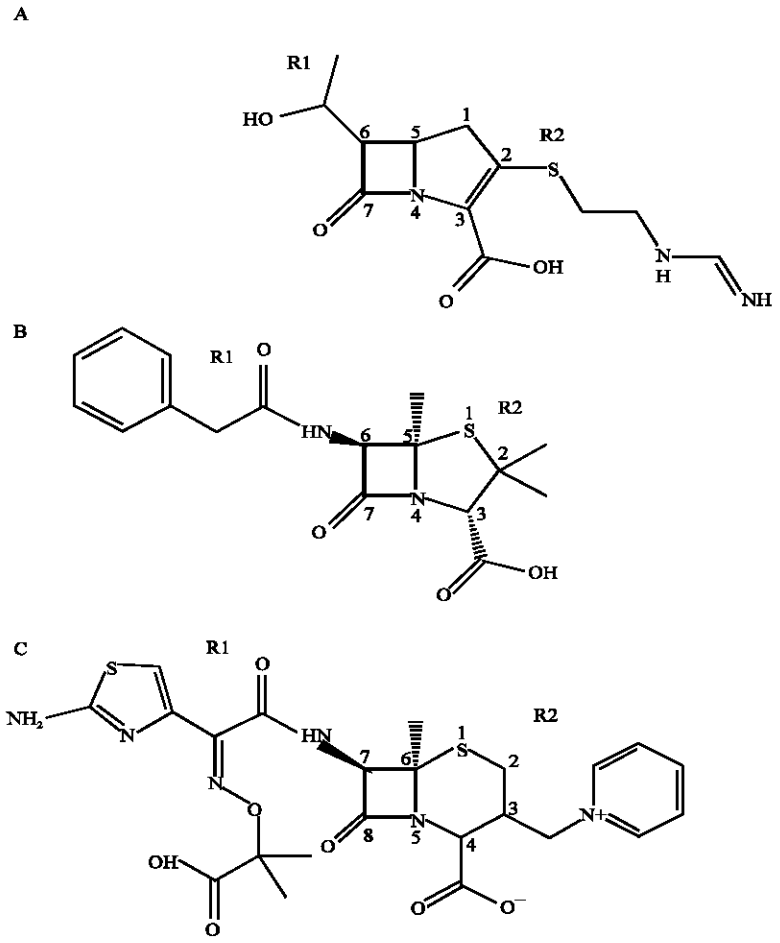

Fig. 1: The schematic drawing of imipenem (A), benzylpenicillin (B) and ceftazidime (C). The β-lactam nucleus is numbered and the lactam ring is presented by bold lines. The R1 and R2 side chains located at C7 (C6) and C3 (C2) position of the β-lactam nucleus are labeled, respectively

ACC-1), *Klebsiella oxytoca* (CMY-5 and FOX-3), *E. coli* (CMY-4, CMY-6, CMY-7, CMY-9, CMY-11, CMY-13, FOX-2, FOX-4, BIL-1, LAT-3 and LAT-4), *Salmonella enteritidis* (DHA-1), *P. mirabilis* (CMY-3, CMY-12, CMY-14 and CMY-15), *S. senftenberg* (CMY-2b) and *E. aerogenes* K9911729 (CMY-10) (Decré *et al*., 2002; Kim *et al*., 2004; Lee *et al*., 2002, 2003 and 2004a; Literacka *et al*., 2004; Miriagou *et al*., 2004; Philippon *et al*., 2002). Compared with chromosomal enzymes, plasmidic class C β-lactamases are more problematic since they are transmissible to other bacterial species and are often expressed in large amounts (Marchese *et al*., 1998).

To escape the inactivation by β-lactamases, extended-spectrum (third-generation) antibiotics such as ceftazidime and cefotaxime have been developed. Extended-spectrum β-lactam antibiotics are characterized mainly by bulky oxyimino groups such as a 2-(2-aminothiazole-4-yl)-2-oxyimino substituent (R1) at position C7 of the β-lactam nucleus (Fig. 1). The new antibiotics had been

poor substrates for β-lactamases but the clinical usage of them gave rise to extended-spectrum (ES) β-lactamases that can hydrolyze the oxyimino β-lactams as well as usual β-lactams. For example, a natural mutant of class C β-lactamase that hydrolyzes the oxyimino β-lactam antibiotics has been isolated from the *Enterobacter cloacae* strain GC1 (Nukaga *et al*., 1995) and *E. cloacae* strain CHE (Barnaud *et al*., 2001).

In previous studies, it has been demonstrated that CMY-10 from a clinical isolate of *Enterobacter aerogenes* is a plasmid-encoded class C ES β-lactamase conferring resistance to cefoxitin and cefotetan as well as to penicillins and extended-spectrum cephalosporins (Bauernfeind *et al*., 1998; Lee *et al*., 2003). The previous analyses of the nucleotide sequence surrounding *cmy-10* gene have implied that integration of an unknown chromosomal class C β-lactamase gene into a *sul1*-type complex integron of a large resident plasmid pYMG-1, followed by transconjugation, is involved in the evolution of *cmy-10* gene (Lee *et al*., 2003 and 2004a). The high sequence identity between plasmidic and chromosomal β-lactamases clearly defines the origin of the plasmidic enzymes: MIR-1, a plasmidic β-lactamase, shows over 90% sequence identity to a chromosomal enzyme, AmpC, from *E. cloacae* (Papanicolaou *et al*., 1990). In case of CMY-10, however, the root is obscure since there is no closely related chromosomal class C β-lactamase.

Although the need to develop new antibiotics targeting β-lactamases has been driving extensive structural studies, the structural information on class C β-lactamase is still restricted. So far, four kinds of class C apo-enzymes from *Citrobacter freundii*, *Escherichia coli*, *E. cloacae* P99 and *E. cloacae* GC1 have been structurally studied (Crichlow *et al*., 1999; Lobkovsky *et al*., 1993; Oefner *et al*., 1990; Usher *et al*., 1998). However, all of them are chromosomal enzymes and furthermore the GC1 β-lactamase, a natural mutant derived from P99 β-lactamase, is the only ES β-lactamase with the structural information. GC1 β-lactamase has a wider R1 site than P99 β-lactamase, which is entirely attributed to the unique insertion of three amino acids in the Ω-loop forming one boundary of the R1 site (Crichlow *et al*., 1999).

In order to investigate the structural basis for the extended substrate spectrum of CMY-10, the 1.55 Å data of CMY-10 was used for the crystallographic structure determination (Lee *et al*., 2004b and 2005b). Based on complex structures between chromosomal class C β-lactamases and inhibitors/β-lactam antibiotics (Beadle *et al*., 2002; Nukaga *et al*., 2004; Usher *et al*., 1998; Wouters *et al*., 2003), the active site of CMY-10 can be divided into two subsites: R1 site and R2 site. The R1 site refers to the region that accommodates the R1 side chain at C7 of the β-lactam nucleus in β-lactam antibiotics and the R2 site represents the opposite region interacting with the right part of the β-lactam ring including the R2 side chain at C3 (Fig. 1). The Ω-loop of the R1 site of CMY-10 without such an insertion mutation is more rigid than that of GC1 β-lactamase. Taking the extent of structural alterations into account, the R2 site rather than the R1 site appears to contribute predominantly to the ES activity of CMY-10.

Imipenem is resistant to the hydrolysis by both P99 and GC1 β-lactamases. The structural comparison between imipenem and benzylpenicillin, a good substrate of class C β-lactamases, sheds lights on the origin of the resistance. Imipenem and benzylpenicillin have the very similar core structure, but the size of the R1 and R2 side chains are different from each other. The R1 side chain of imipenem, the hydroxyethyl group at C6, is much smaller than that of benzylpenicillin (Fig. 1). Thus it is not likely that the small R1 side chain impedes the hydrolysis since there should be no problem in the accommodation of the small side chain by class C enzymes. Instead, it is highly probable that the long R2 side chain of imipenem is the main cause of the catalytic failure.

CMY-10 contains a widely-opened R2 site (due to three amino acids deletion in the α-region) into which the long R2 side chain of imipenem could fit snugly. Based on this perspective, it was tested whether CMY-10 would hydrolyze imipenem. As expected, CMY-10 hydrolyzed imipenem with considerable catalytic efficiency. Especially, the k_{cat} value of CMY-10 toward imipenem is ~2-fold higher than that of CMY-10 toward ceftazidime. Since the k_{cat} value reflects the deacylation rate, CMY-10 and imipenem appear to form a stable acyl-enzyme intermediate. CMY-10 is the only Ambler class C β-lactamase with carbapenemase activity without reduced uptake (porin loss) so far. Imipenem resistance has been associated with porin loss and class C β-lactamases in *P. aeruginosa* (Livermore, 2004), *Enterobacter* spp. (Lee *et al*., 1991) and *K. pneumoniae* (Bradford *et al*., 1997).

The emergence of class C ES β-lactamases (Barnaud *et al*., 2001; Nukaga *et al*., 1995) and class C ES β-lactamase (ESBL) (CMY-10) with carbapenemase activity should change the present treatment options using extended-spectrum cephalosporins for cefoxitin-resistant non-class C β-lactamase and non-ESBL producers and carbapenems for cefoxitin-resistant class C β-lactamase producers. Phenotypic susceptibility testing to distinguish the difference between organisms producing ESBLs or plasmid-encoded class C β-lactamases is also difficult. Proper surveillance will require the implementation of molecular testing such as the multiplex PCR (Perez-Perez and Hanson, 2002) in clinical laboratories to identify these new types of β-lactamases and to stop the emergence of them. The physician should prescribe the most appropriate antibiotic to decrease the selection pressure driving the propagation of class C ES β-lactamase with carbapenemase activity.

The Molecular Class D Carbapenemases

As shown in Table 5, class D (OXA-type) carbapenemases have been discovered most frequently in isolates of *Acinetobacter baumannii* and also in species such as *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Shewanella* spp. and *Ralstonia pickettii*. 80 OXA-type β-lactamases (oxacillinases) have been found thus far (http://www.lahey.org/studies/webt.asp). Among 80 oxacillinases, 25 OXA-type carbapenemases have been discovered (Fig. 2). Sequenced OXA-type carbapenemases include five subgroup (eight distinct clusters) as follows: (I) OXA-23-like cluster (OXA-23, -27 and -49) (Afzal-Shah *et al*., 2001; Donald *et al*., 2000; Paton *et al*., 1993), (II) OXA-24-like cluster (OXA-24, -25, -26, -40 and -72) (Afzal-Shah *et al*., 2001; Bou *et al*., 2000a; Héritier *et al*., 2003), (III) OXA-51-like cluster (OXA-51, -64, -65, -66, -68, -69, -70, -71, -75, -76 and -77) (Brown *et al*., 2005; Brown and Amyes, 2005), (IV) OXA-48-like cluster (OXA-48 and -54) (Poirel *et al*., 2004a and 2004b; Venkateswaran *et al*., 1999) and (V) a subgroup that includes four distinct clusters showing 32 ~ 46% identity each other (OXA-50, -55, -58 and -60) (Girlich *et al*., 2004a and 2004b; Héritier *et al*., 2004; Holloway, 1955; Nordmann *et al*., 2000; Poirel *et al*., 2005b). OXA-23 and OXA-27 have 99% amino acid identity, whereas they have only 58 ~ 59% identity with those of OXA-24-like cluster, 54 ~ 57% identity with those of OXA-51-like cluster, 36% identity with those of OXA-48-like cluster and 33 ~ 48% identity with those of subgroup V. OXA-48 shared 92% amino acid identity with OXA-54 but was weakly related to other class D β-lactamase, with less than 46% amino acid identity with any other OXA-type β-lactamase. Compared to other carbapenemases, these OXA-type enzymes have weak carbapenemase activity (Table 2, 3, 6 and 7). These enzymes might contribute to carbapenem resistance if other carbapenem mechanisms were present, such as the absence of outer membrane protein(s), reduced expression of penicillin-binding protein(s) and/or overexpression of efflux pump (Fernández-Cuenca *et al*., 2003; Héritier *et al*., 2005).

Table 5: Chronology of discovery of class D carbapenemases

Enzyme	Clinical strains	Origin (Year)	Encodement site	pI	GenBank No.	Reference No.[c]
OXA-23	*Acinetobacter baumannii* 6B92	UK (1985)	Conjugative plasmid	6.65	AJ132105	1, 2
OXA-27	*Acinetobacter baumannii* I-16	Singapore (1995)	(Not transmissible)	6.8	AF201828	3
OXA-49	*Acinetobacter baumannii*	China			AY288523	UP[b]
OXA-24	*Acinetobacter baumannii* RYC 52763/97	Spain (1997)	(Not transmissible)	9.0	AJ239129	4
OXA-25	*Acinetobacter baumannii* 327009	Spain (1998)	(Not transmissible)	8.0	AF201826	3
OXA-26	*Acinetobacter baumannii* 04737	Belgium (1998)	(Not transmissible)	7.9	AF201827	3
OXA-40	*Acinetobacter baumannii* CLA-1	France	Chromosome	8.6	AF509241	5
OXA-72	*Acinetobacter baumannii*	Thailand			AY739646	UP
OXA-51	*Acinetobacter baumannii* 788	Argentina (1994)	(Not transmissible)	7.0	AJ309734	6
OXA-64	*Acinetobacter baumannii* 889	South Africa			AY750907	7
OXA-65	*Acinetobacter baumannii* 790	Argentina			AY750908	7
OXA-66	*Acinetobacter baumannii* 806	Spain				
	Acinetobacter baumannii 811	Hong Kong			AY750909	7
	Acinetobacter baumannii 864	Singapore				
OXA-68	*Acinetobacter baumannii* 809	Spain			AY750910	7
OXA-69	*Acinetobacter baumannii* 877	Singapore				
	Acinetobacter baumannii 823	Turkey			AY859527	7
OXA-70	*Acinetobacter baumannii* 812	Hong Kong			AY750912	7
OXA-71	*Acinetobacter baumannii* 888	South Africa			AY859528	7
OXA-75	*Acinetobacter baumannii*	France			AY859529	UP
OXA-76	*Acinetobacter baumannii*	France			AY949203	UP
OXA-77	*Acinetobacter baumannii*	France			AY949202	UP
OXA-48	*Klebsiella pneumoniae* 11978	Turkey (2001)	Plasmid	7.2	AY236073	8
OXA-54	*Shewanella oneidensis* MR-1[a]	USA (1987)	Chromosome	6.8	AY500137	9, 10
OXA-50	*Pseudomonas aeruginosa* PAO1	Australia (1955)	Chromosome	8.6	AY306130	11, 12
OXA-55	*Shewanella algae* KB-1	France (2001)	Chromosome	8.6	AY343493	13
OXA-58	*Acinetobacter baumannii* MAD	France (2003)	Plasmid	7.2	AY570763	14
OXA-60	*Ralstonia pickettii* PIC-1	France (1998)	Chromosome	5.1	AF525303	15, 16

[a]Environmental isolate collected from a lake sediment and implicated as opportunistic pathogens of humans and aquatic animals. [b]UP, unpublished. Blank, not provided in each reference. [c]Reference number 1: Paton *et al.*, 1993; number 2: Donald *et al.*, 2000; number 3: Afzal-Shah *et al.*, 2001; number 4: Bou *et al.*, 2000a; number 5: Héritier *et al.*, 2003; number 6: Brown *et al.*, 2005; number 7: Brown and Amyes *et al.*, 2005; number 8: Poirel *et al.*, 2004a; number 9: Poirel *et al.*, 2004b; number 10: Venkateswaran *et al.*, 1999; number 11: Girlich *et al.*, 2004a; number 12: Holloway, 1955; number 13: Héritier *et al.*, 2004; number 14: Poirel *et al.*, 2005b; number 15: Girlich *et al.*, 2004b; number 16: Nordmann *et al.*, 2000

OXA-23-like Cluster (Subgroup I)

OXA-23 was the first class D carbapenemase identified from *A. baumannii* strain collected in Edinburgh 1985 (Table 5) (Paton *et al*., 1993). OXA-23-producing *A. baumannii* strains were also discovered in Brazil 1999 (Dalla-Costa *et al*., 2003). OXA-23-producing *Proteus mirabilis* CFO239 was discovered among clinical strains isolated in France between 1996 and 1999 (Bonnet *et al*., 2002). OXA-23-producing *A. baumannii* strains appeared as an outbreak in Republic of Korea 2003 (Jeon *et al*., 2005). The chronology of discovery of OXA-27 and -49 is shown in Table 5. OXA-23 has 99% amino acid identity with OXA-27 and -49 (Fig. 2). From the limited kinetic data available, OXA-23 appeared more active than OXA-27 against cephaloridine (V_{max}, 35.5% of that for penicillin

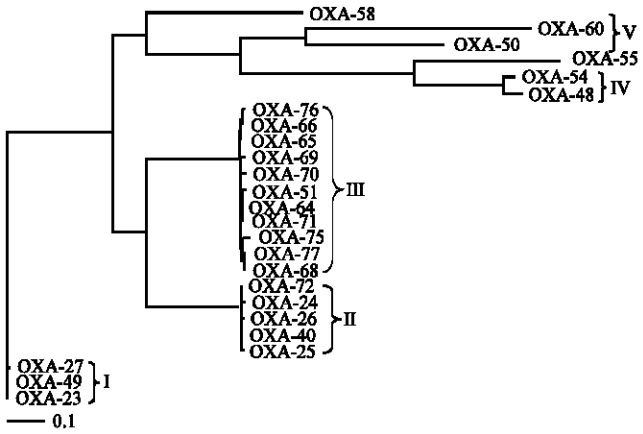

Fig. 2: Dendrogram of 25 class D carbapenemases (calculated by CLUSTAL W [Thompson *et al*., 1994] using PRODIST [Protein Distance Matrix] and FITCH [Fitch-Margoliash and Least-Squares Distance Methods] from the PHYLIP package of Joseph Felsenstein [Department of Genetics at the University of Washington]). Branch length values represent relative phylogenetic distance. According to the identity of their amino acid sequences with OXA-23, 25 class D carbapenemases can be classified into five subgroups (eight clusters): I, OXA-23-like cluster (99% amino acid identity each other); II, OXA-24-like cluster (99% identity each other); III, OXA-51-like cluster (95~98% identity each other); IV, OXA-48-like cluster (92% identity each other); V, subgroup that includes four distinct clusters showing 32~46% identity each other

G compared with 6% for OXA-27), whereas imipenem-hydrolyzing activity was relatively weaker for OXA-23 (Table 5) (Afzal-Shah *et al*., 2001). Curiously, OXA-23 hydrolyzed oxacillin rapidly, relative to ampicillin, whereas OXA-27 had only weak activity against both these compounds compared with penicillin G (Afzal-Shah *et al*., 2001). OXA-27 was inhibited, albeit weakly, by clavulanic acid and tazobactam (Afzal-Shah *et al*., 2001).

OXA-24-like Cluster (Subgroup II)

OXA-24-producing *A. baumannii* strains were first discovered and appeared as a nosocomial outbreak in Madrid, Spain 1999 (Bou *et al*., 2000a; Jeon *et al*., 2005). The chronology of discovery of OXA-25, -26, -40 and -72 is shown in Table 5. OXA-40-producing *A. baumannii* strains were also discovered in Spain between 1998 and 1999 (Bou *et al*., 2000b) and in Portugal between 1998 and 2003 (Lopez-Otsoa *et al*., 2002). OXA-24 has 99% amino acid identity with OXA-25, -26, -40 and -72 (Fig. 2). OXA-24 hydrolyzed penicillin G and cephaloridine, but did not hydrolyzed oxacillin, in contrast to OXA-25, -26 and -40 (Table 6 and 7). With regard to carbapenems, the OXA-24 showed a moderate of hydrolysis, which indicated well with the increase in the carbapenem MICs obtained for the transformant harboring *oxa-24* gene. According to ClustalW (1.83) multiple sequence alignment analysis of 25 OXA-type carbapenemases (Fig. 2), all OXA-type carbapenemases except for OXA-50 (STYK, positions DBL-Ambler class D β-lactamase numbering -70 to 73) retain the STFK tetrad, which is an active site found in class D β-lactamases (Donald *et al*., 2000). Among five structural elements, a KTG (positions 216 to 218) is observed in all OXA-type carbapenemases except for

Table 6: Catalytic constants (k_{cat}) of class D (OXA-type) carbapenemases

Antimicro-	k_{cat} (s^{-1})[f]											
bial agents	OXA-27[a]	-24b	-25[a]	-26a	-40	-51b	-48	-54	-50	-55	-58	-60
Penicillin G	100	370	100	100	5		245	120	110	4	5.5	420
Cloxacillin					NH	32			NH	0.5		70
Ampicillin	6		21	55	5	1499	340	540	>3[d]	8	1	
Amoxicillin												60
Ticarcillin					1		45	30	>0.1	1	1	200
Carbenicillin	<0.0005		76	25								
Piperacillin	4		22	53	1			20	>2	3	2.5	>300[e]
Oxacillin	0.4	NH[c]	440	500	2	484	25	35	>0.2	5	1.5	>130
Cephaloridine	6	200	33	27	5	149	2	1	>2	10		>0.5
Cephalothin	0.3		3	8	3		3	3	0.2	0.6	0.1	NH
Cefoxitin												NH
Cefuroxime	1.0		0.4	0.04		NH						NH
Cefotaxime	0.2		0.2	<0.003	NH	NH	10	15		NH	NH	NH
Ceftazidime	0.0005		0.01	0.1	20	NH	4	NH		2	NH	2
Moxalactam									>0.01			
Cefepime					NH		1	1		NH	NH	
Cefpirome					NH		8	4		0.1	0.1	
Imipenem	0.1	15	3	2.4	0.1	47	2	1	0.1	0.1	0.1	0.5
Meropenem	0.04	280	0.4	0.4	NH	NH	0.1	0.1	>0.02	0.05	<0.01	NH
Aztreonam	<0.0005			<0.003	NH		NH	NH		NH	NH	NH

[a]Relative (%) values of V_{max} for penicillin G, [b]V_{max} (μmo min^{-1} μL^{-1}) values, [c]NH, not hydrolyzed, [d]Since the K_m values were very high, k_{cat} values could not be determined precisely. The k_{cat} value was determined for K_m value of 1 mM (corresponding to a minimal value), [e]Data were determined for the corresponding K_m value (2 or 1 mM), [f]Adapted from data presented by Afzal-Shah *et al.* (2001) for OXA-27, -25 and -26, Bou *et al.* (2000a) for OXA-24, Héritier *et al.* (2003) for OXA-40, Brown *et al.* (2005) for OXA-51, Poirel *et al.* (2004a) for OXA-48, Poirel *et al.* (2004b) (2001) for OXA-54, Girlich *et al.* (2004a) for OXA-50, Héritier *et al.* (2004) for OXA-55, Poirel *et al.* (2005b) for OXA-58, Girlich *et al.* (2004b) for OXA-60, Blank, not provided in each reference

OXA-23, -27, -48, -49, -50, -54, -55 and -60, having a KSG element. Other YGN (positions 144 to 146) is conserved in OXA-23, -24, -25, -26, -27, -40, -49 and -72, whereas all OXA-type carbapenemases except for these eight enzymes have a FGN element. OXA-type carbapenemases exhibit many different structural features and little is known about the relevance of these differences for explaining the variabilities of the hydrolysis spectra. OXA-25 and OXA-26 was higher inhibited, albeit weakly, by clavulanic acid and tazobactam than OXA-24 did (Afzal-Shah *et al*., 2001).

OXA-51-like Cluster (Subgroup III)

The chronology of discovery of this cluster is shown in Table 5. OXA-51 has 95 ~ 98% amino acid identity with OXA-64, -65, -66, -68, -69, -70, -71, -75, -76 and -77 (Fig. 2). OXA-51 showed slow hydrolysis of oxacillin and cloxacillin, whereas OXA-17 demonstrated weak activity against them (Table 6 and 7). OXA-24 failed completely to hydrolyze oxacillin (Bou *et al*., 2000a). None of the cephalosporins was hydrolyzed by OXA-51, with the exception of cephaloridine, which was a weaker substrate than ampicillin (Brown *et al*., 2005). Slow hydrolysis of imipenem by OXA-51 was detected, but not meropenem and this was also demonstrated with OXA-23 and -40 (Table 6 and 7). Inhibition of OXA-51 activity was detected with clavulanic acid (Brown *et al*., 2005). With the exception of OXA-23 and -40, the IC_{50} values detected for OXA-51 with this inhibitor were higher than values observed for the other class D carbapenemases (Brown *et al*., 2005).

OXA-48-like Cluster (Subgroup IV)

OXA-48 has 92% amino acid identity with OXA-54 (Fig. 2). OXA-48 had a narrow-spectrum hydrolysis profile that included penicillins, cephalothin and imipenem and to a lesser extent,

Table 7: Affinity constants (K_m) of class D (OXA-type) carbapenemases

Antimicrobial agents	K_m (μM)[d]											
	OXA-27	-24	-25	-26	-40	-51	-48	-54	-50	-55	-58	-60
Penicillin G	88	65	100	25	23		40	60	800	25	50	40
Cloxacillin					ND[b]	129			>1000	190		1500
Ampicillin	3		21	15	220	25	5200	4300	>1000	500	130	
Amoxicillin												300
Ticarcillin					60		55	240	>1000	20	240	400
Carbenicillin			300	210								
Piperacillin	10		55	10	23		410	240	>1000	110	50	>2000
Oxacillin	208	NH[a]	840	580	876	531	30	75	>1000	390	70	>2000
Cephaloridine	3	395	590	640	1000	77	27	45	>1000	750		>1000
Cephalothin	260		80	90	72		20	230	900	70	150	NH
Cefoxitin												NH
Cefuroxime						ND						NH
Cefotaxime	0.1		35		ND	ND	190	1600		ND	ND	NH
Ceftazidime					2500	ND	5100	ND		4700	ND	1000
Moxalactam									>1000			
Cefepime					ND		160	180		ND	ND	
Cefpirome					ND		390	110		670	200	
Imipenem	20	20	11	3	6.5	11	14	4	20	20	7.5	2
Meropenem	15	775	12	3	ND	ND	20	125	>1000	500	0.075[c]	NH
Aztreonam					ND		ND	ND		ND	ND	NH

[a]NH, not hydrolyzed, [b]ND, not determinable since hydrolysis was too slow, [c]The K_i was determined with penicillin G as the substrate, [d]Adapted from data presented by Afzal-Shah *et al.* (2001) for OXA-27, -25 and -26, Bou *et al.* (2000a) for OXA-24, Héritier *et al.* (2003) for OXA-40, Brown *et al.* (2005) for OXA-51, Poirel *et al.* (2004a) for OXA-48, Poirel *et al.* (2004b) (2001) or OXA-54, Girlich *et al.* (2004a) for OXA-50, Héritier *et al.* (2004) for OXA-55, Poirel *et al.* (2005b) for OXA-58, Girlich *et al.* (2004b) for OXA-60, Blank, not provided in each reference

cefotaxime, ceftazidime, cefepime and cefpirome (Table 6 and 7). OXA-54 had a broad-spectrum hydrolysis profile, including penicillins, cephalothin, cefepime and imipenem and, to a lesser extent, cefuroxime, cefotaxime and cefepime (Table 6 and 7). The catalytic efficiency (k_{cat}/K_m) of OXA-48 for imipenem was similar to that of OXA-54 and 10- and 2-fold higher than those reported for OXA-40 (Héritier *et al*., 2003) and KPC-1 (Yigit *et al*., 2003), respectively. Hydrolysis of meropenem was at a low level, as observed for the other carbapenem-hydrolyzing oxacillinases, including OXA-24, -25, -26 and -40. Oxacillin was significantly hydrolyzed by OXA-48 and -54, whereas it was slightly hydrolyzed by the other carbapenem-hydrolyzing oxacillinases, including OXA-40 (Table 6 and 7). OXA-48 and -54 were weakly inhibited by clavulanic acid (IC_{50} of 16 and 50 μM, respectively), tazobactam (IC_{50} of 1.7 and 2 μM, respectively) and sulbactam (IC_{50} of 50 and 150 μM, respectively) (Poirel *et al*., 2004a and 2004b). These IC_{50}s were lower than those reported for OXA-40 (Héritier *et al*., 2003).

Subgroup V (Four Distinct Clusters)

The chronology of discovery of these four clusters is shown in Table 5. OXA-58-producing *A. baumannii* strains appeared as a nosocomial outbreak in France between 2003 and 2004 (Silva *et al*., 2004). OXA-50 has 38 ~ 46% amino acid identity with OXA-55, -58 and -60.

OXA-50 had a narrow-spectrum hydrolysis profile that included ampicillin, penicillin G, cephaloridine, cephalothin, piperacillin and imipenem (Table 6 and 7). Hydrolysis of oxacillin or cloxacillin by OXA-50 was not detected, as in the case for OXA 23, -24, -25, -26, -27 and -40 (Table 6 and 7). Hydrolysis of oxacillin or cloxacillin by OXA-55 and -58 was detected, whereas that of OXA-60 at a low level (Héritier *et al*., 2004; Poirel *et al*., 2005b). The catalytic activity (k_{cat}) of

OXA-50 for imipenem was similar to those of OXA-27, -40, -55 and -58 and the affinity (K_m) of OXA-50 for imipenem was identical to those of OXA-24, -27 and -55. OXA-60 and -58 hydrolyzed imipenem twice and 50-fold, respectively as much as OXA-40, -50 and 55 (Table 6 and 7). Hydrolysis of meropenem was observed for OXA-24, -25, -26, -40, -50 and -55, whereas it was not detected in case of OXA-58 and -60. OXA-58 had some hydrolytic activity against cefpirome, whereas no activity against ceftazidime, cefotaxime and cefepime remained (Poirel *et al*., 2005b). No hydrolysis of cephalothin was observed for OXA-60, whereas it was the best cephalosporin substrate for OXA-22 (Girlich *et al*., 2004b; Nordmann *et al*., 2000). As measured by determination of IC_{50} values, OXA-50, -55, -58 and -60 were weakly inhibited by clavulanic acid (IC_{50} of 500, 700, 310 and 450 μM, respectively), tazobactam (IC_{50} of 350, 12, 60 and 73 μM, respectively) and sulbactam (IC_{50} of >2000, 300, 2500 and 320 μM, respectively) (Girlich *et al*., 2004b; Héritier *et al*., 2004; Holloway, 1955; Nordmann *et al*., 2000; Poirel *et al*., 2005b).

Conclusions

This study emphasizes that non-metallo-carbapenemases are increasingly reported worldwide among nosocomial and community-acquired Gram-negative aerobes. Biochemical and structural characteristics of these enzymes are also emphasized for the future development of new β-lactams or β-lactamase inhibitors. Although carbapenems are the most powerful β-lactams, carbapenemases has been emerged, especially in *Enterobacteriaceae*, *P. aeruginosa* and *A. baumannii*. Enzymes that may be clinically worrying are of: the GES and KPC series in *P. aeruginosa* and *Enterobacteriaceae*; the OXA-type in *A. baumannii* (most prevalence), *P. aeruginosa*, *Shewanella* spp. and *Ralstonia pickettii*. These enzymes confer resistance to carbapenems, once expressed from Gram-negative species that possess either a natural occurring or an acquired low level of outer membrane permeability and/or efflux. The exception is the recent report that has announced plasmid-encoded KPC- and GES-type from *K. pneumoniae*, *P. aeruginosa* and *E. coli* isolates that confer high-level resistance to carbapenems by itself.

The emergence and dissemination of non-metallo-carbapenemases are a consequence of the selection pressure created by antibiotic usage. The inability of many clinical microbiology laboratories to provide timely and accurate information about the occurrence of non-metallo-carbapenemases will facilitate their spread. However, clinical detection of non-metallo-carbapenemase producers remains difficult based on a simple phenotypic analysis of antibiotic susceptibility testing, since most of these enzymes confer reduced susceptibility to carbapenems. A PCR-based technique may be used for detection of known or unknown non-metallo-carbapenemases. Detection of non-metallo-carbapenemases may be based on the degenerated primers (Jeon *et al*., 2005; Jeong *et al*., 2004; Lee *et al*., 2001; Nordmann and Poirel, 2002).

One of the most interesting aspects of further studies will be to estimate precisely the prevalence of non-metallo-carbapenemases among clinically relevant Gram-negative isolates and to analyze the selection power of β-lactam and non-β-lactam antibiotics, since non-metallo-carbapenemase genes are often physically linked to at least aminoglycoside resistance genes in integron structures. The origin of these enzyme genes remains unknown. Determination of the natural reservoir of these enzymes may help to prevent dissemination of non-metallo-carbapenemase genes and may provide insights into the molecular mechanism of integron and gene cassette formation. When clinical isolates possess non-metallo-carbapenemases, overcoming resistance to carbapenems is not easy. These enzymes have a large accommodating active site and stability is difficult to engineer. Thus, the prospects for new inhibitors for non-metallo-carbapenemases are better.

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