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Mutational Events in Cefotaximase Extended-Spectrum β-Lactamases of the CTX-M-1 Cluster Involved in Ceftazidime Resistance▽

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CTX-M β-lactamases, which show a high cefotaxime hydrolytic activity, constitute the most prevalent extended-spectrum β-lactamase (ESBL) type found among clinical isolates. The recent explosive diversification of CTX-M enzymes seems to have taken place due to the appearance of more efficient enzymes which are capable of hydrolyzing both cefotaxime and ceftazidime, especially among the CTX-M-1 cluster. A combined strategy of in vitro stepwise evolution experiments using *bla*$_{\text{CTX-M-1}}$, *bla*$_{\text{CTX-M-3}}$, and *bla*$_{\text{CTX-M-10}}$ genes and site-directed mutagenesis has been used to evaluate the role of ceftazidime and other β-lactam antibiotics in triggering the diversity found among enzymes belonging to this cluster. Two types of mutants, P167S and D240G, displaying high ceftazidime MICs but reduced resistance to cefotaxime and/or cefepime, respectively, were identified. Such an antagonistic pleiotropic effect was particularly evident with P167S/T mutations. The incompatibility between P167S and D240G changes was demonstrated, since double mutants reduced susceptibility to both ceftazidime and cefotaxime-cefepime; this may explain the absence of strains containing both mutations in the clinical environment. The role of A77V and N106S mutations, which are frequently associated with P167S/T and/or D240G, respectively, in natural strains, was investigated. The presence of A77V and N106S contributes to restore a high-level cefotaxime resistance phenotype, but only when associated with mutations P167S and D240G, respectively. However, A77V mutation increases resistance to both cefotaxime and ceftazidime when associated with CTX-M-10. This suggests that in this context this mutation might be considered a primary site involved in resistance to broad-spectrum cephalosporins.

β-Lactam agents constitute not only the most widely and frequently used group of antibacterial drugs but also the one that includes the largest variety of molecules. Continuous exposure to a diverse array of β-lactams seems to have been driving the explosive diversification found among all β-lactamase groups (18, 29). A number of investigations have identified the amino acid positions among classic TEM, SHV, or OXA enzymes that evolved under these multiple and coincidental selective events. Such changes are responsible for increasing affinity and hydrolytic activity against the newest β-lactams, leading to extended-spectrum β-lactamases (ESBLs) (11, 21, 34).

CTX-M enzymes probably originated in environmental organisms, where they confer a negligible level of resistance to cefotaxime (12, 27, 32). However, when present as acquired β-lactamases in *Enterobacteriaceae* (known as cefotaximases) they confer a high cefotaxime-hydrolytic activity (4, 19). Since the original description in 1989 (4), a rapid and extensive radiation of different CTX-M clusters and variants has occurred, with more than 60 molecular forms described to date and classified into five clusters based on amino acid sequence homology (http://www.lahey.org/studies/webt.htm). Such accelerated variability might result from genetic drift, favored not only by its widespread dispersal but also by its frequent location in mobile and promiscuous genetic platforms (10, 26). We examine here to what extent such genetic variability might derive from successive adaptive responses driven by the coincidental exposure of CTX-M-harboring bacteria to a diversity of β-lactam molecules, including cefotaxime-ceftriaxone, cefepime, and ceftazidime.

Acquisition of ceftazidime resistance among members of the CTX-M family is frequently associated with mutations at positions 167 (P167S/T/Q) and 240 (D240G), according to the Ambler et al. numbering scheme for β-lactamases (1). Enzymes putatively encoding ceftazidime resistance have evolved among various phylogenetic branches of the CTX-M family, i.e., the CTX-M-1 (CTX-M-15, CTX-M-23, CTX-M-28, CTX-M-29, CTXM-32, CTX-M-33, CTX-M-42, CTX-M-52, CTX-M-53, CTX-M-54, CTX-M-55, and CTX-M-58), CTX-M-2 (CTX-M-35 and CTX-M-43), CTX-M-9 (CTX-M-16, CTX-M-19, and CTX-M-27), and CTX-M-25 (CTX-M-25 and CTX-M-41) lineages.

In the present study, the effect of ceftazidime and other β-lactam antibiotics in triggering the natural CTX-M-1 cluster mutational diversity is explored by using a combination of in vitro stepwise evolution experiments with hypermutagenic bacteria and site-directed mutagenesis on particular positions to assess phenotypic changes in the same isogenic genetic context (3, 15, 16, 20, 28).

MATERIALS AND METHODS

Cloning *bla*$_{\text{CTX-M-1}}$ genes in an isogenic context. Genes encoding five of the most representative ESBLs belonging to the CTX-M-1 β-lactamase cluster considered to be associated (i) with resistance only to cefotaxime (the cefotaximases

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TABLE 1. Primers used in this study

Primer	Nucleotide sequence (5′–3′)[a]	Position (bp) in $bla_{CTX-M-3}$ sequence (DQ328958[b])
A77V-F	TGATGGCCGTGGCC GCGGTGCTGAA	229–254
A77V-R	TTCAGCACCGCGGC CACGGCCATCA	254–229
N106S-F	CCTTGTTAACTATA GTCCGATT	311–333
N106S-R	CCGCAATCGGACTA TAGTT	337–318
P167S-F	CGTACCGAGTCGAC GTTAAACA	498–520
P167S-R	TGTTTAACGTCGAC TCGGTACG	520–498
D240G-F	AAACCGGCAGCGG TGGCTAT	709–728
D240G-R	ATAGCCACCGCTGC CGGTTT	729–709
CTX-MEco-FW	GGAATTCGACTATT CATGTTGTTGT TATT	−47–26 upstream bla
CTX-MPst-RV	AACTGCAGTTCCGC TA*TTA*CAAACCGT	Including codon stop

[a] The EcoRI and PstI restriction sites are underlined in primers CTX-M-Eco and CTX-M-Pst, respectively. The italic letters in primer CTX-M-Pst represent the codon stop in $bla_{CTX-M-3}$.
[b] GenBank accession number.

CTX-M-1, CTX-M-3, and CTX-M-10) or (ii) with resistance to cefotaxime and ceftazidime (CTX-M-15 and CTX-M-32) were cloned in an isogenic context. Primers CTX-MEco-FW and CTX-MPst-RV recognizing a homologous sequence 29 bp upstream of the bla_{CTX-M} gene initiation codon and the stop codon, respectively, and containing the EcoRI and PstI restriction sites were used to amplify the complete *bla* genes ($bla_{CTX-M-1}$, $bla_{CTX-M-3}$, $bla_{CTX-M-10}$, $bla_{CTX-M-15}$, and $bla_{CTX-M-32}$) (Table 1). The PCR conditions were as follows: 12 min at 94°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min, and extension at 72°C for 1 min, with a final extension step at 72°C for 10 min. Wild and subsequent variants of $bla_{CTX-M-1-like}$ genes were cloned by using pBGS18 (Kan^r) as a vector (33). Purified PCR products and pBGS18$^-$ plasmid were digested with EcoRI and PstI enzymes (Takara Bio, Inc., Shiga, Japan) and further ligated with T4 DNA ligase (Roche Diagnostics S.L., Barcelona, Spain). Recombinant plasmids containing $bla_{CTX-M-1}$, $bla_{CTX-M-3}$, $bla_{CTX-M-10}$, $bla_{CTX-M-15}$, and $bla_{CTX-M-32}$ were designated by pBX1, pBX3, pBX10, pBX15, and pBX32, respectively, and transformed in two isogenic (plasmid-free) *Escherichia coli* strains: MI1443, a Δ*ampC* normomutable strain (9), and GB20, a hypermutable derivative of MI1443, which harbors a defective *mutS* gene (Δ*ampC*, *mutS*::Tn*10*) (16). Transformants in MI1443 were used as the baseline control (nonevolved) strains.

Daily serial passages. GB20 transformants carrying pBX1, pBX3, and pBX10 were inoculated in six independent tubes containing 5 ml of Luria-Bertani broth with tetracycline (20 μg/ml), kanamycin (50 μg/ml), and increasing concentrations of ceftazidime, starting from twofold less than the corresponding MIC to 128 μg/ml (MICs of 0.5 to 256 μg/ml), as previously described (16). Several clones from each bacterial pool at different ceftazidime concentrations were selected and transformed into *E. coli* strain MI1443, with ceftazidime (fourfold the MIC of wild-type MI1443 control strains carrying the corresponding nonevolved bla_{CTX-M} genes) and kanamycin (50 μg/ml) used as selector agents. Recombinant plasmid DNA extracted from several clones was further transformed using only kanamycin as the selector agent. bla_{CTX-M} genes corresponding to clones displaying significant reduction in susceptibility to ceftazidime (≥2-fold) in comparison with control strains were sequenced by using an ABI Prism 377 device (PE Applied Biosystems, Foster City, CA).

Site-directed mutagenesis. In order to analyze the role of A77V and N106S mutations in the evolution and adaptation of CTX-M-1-like β-lactamases and to investigate the effect of the presence of both P167S and D240G mutations in different CTX-M contexts, these point nucleotide mutations were introduced in the $bla_{CTX-M-1-like}$ genes. The set of primers used are listed in Table 1. In a first step, two fragments of the bla_{CTX-M} gene desired were amplified by using the corresponding forward (A77V-F, N106S-F, P167S-F, and D240G-F) and reverse (A77V-R, N106S-R, P167S-R, and D240G-R) primers, in combination with the primers CTX-MPst-Rv and CTX-MEco-Fw, respectively, under the PCR conditions described above. PCR products were purified from an agarose gel by using a QIAquick extraction gel kit (QIAquick gel extraction kit; Qiagen, GmbH, Hilden, Germany) and used as templates in a final PCR assay carried out with the CTX-MEco-Fw and CTX-MPst-Rv primers. PCR was performed with a mix (50-μl final volume) containing 1.5 mM $MgCl_2$, 1× buffer, 0.2 mM concentrations of deoxynucleoside triphosphates, 1 pmol of each primer, and finally 1.5 U of Taq-Gold DNA polymerase (Applied Biosystems, Foster City, CA) under the following conditions: 94°C for 12 min, followed by 15 amplification cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min, followed in turn by 20 amplification cycles of denaturation at 94°C for 1 min and annealing and extension at 72°C for 1.5 min, with a final extension step at 72°C for 10 min. Each final PCR product and the plasmid vector (pBGS18$^-$) were purified (QIAquick purification kit), digested with the enzymes EcoRI and PstI, ligated, and transformed into MI1443 cells as previously described (16), using kanamycin (50 μg/ml), ceftazidime (1 μg/ml), kanamycin (50 μg/ml), and cefotaxime (1 μg/ml) as selector agents. Plasmid DNA from a single clone per plate was extracted and transformed into MI1443 cells, selected only with kanamycin (50 μg/ml). Recombinant plasmids were extracted, and the corresponding *bla* gene was sequenced in order to confirm the presence of the desired mutation.

Susceptibility testing. The MICs for *E. coli* MI1443 carrying natural and artificial bla_{CTX-M} gene derivatives were determined by broth microdilution methods according to CLSI guidelines (8) or by the E-test, respectively. The antibiotics tested included cefotaxime, ceftazidime, cefepime, cefuroxime, and amoxicillin-clavulanate (Sigma-Aldrich, Inc., St. Louis, MO).

RESULTS

In vitro evolution of *E. coli* GB20 carrying $bla_{CTX-M-3}$ or $bla_{CTX-M-1}$. In vitro evolution of *E. coli* GB20 carrying the plasmid pBX3 under ceftazidime selection yielded a mutant, CTX-M-3.1, which carried the substitution on position 167 (located in the omega loop) for serine (P167S), as the result of the nucleotide change $C^{508}\rightarrow T$ in the $bla_{CTX-M-3.1}$ gene (Table 2). *E. coli* MI1443 carrying this $bla_{CTX-M-3.1}$ mutant was associated with a high increase in ceftazidime MIC (from 1 to 32 μg/ml), as well as a large decrease in cefotaxime and cefepime resistance (from 256 to 1 μg/ml and from 32 to 0.5 μg/ml, respectively). The cefuroxime MIC was slightly reduced from ≥256 to 128 μg/ml. A natural CTX-M-3 variant carrying a change in the same position, P167T, has already been detected (CTX-M-42; GenBank accession number DQ061159).

The same type of mutant (P167S) was also found in *E. coli* GB20 carrying the plasmid pBX1, harboring $bla_{CTX-M-1}$. This mutant, CTX-M-1.1, was associated with the highest ceftazidime MIC observed (256 μg/ml) among all of the mutants studied (Table 2). In addition, in vitro evolution of $bla_{CTX-M-1}$ also yielded the mutant CTX-M-1.2, containing the P167T change, which corresponds to the natural CTX-M variant CTX-M-58 (GenBank accession number EF210159). The antibiotic resistance phenotype of *E. coli* MI1443 carrying this $bla_{CTX-M-1}$ mutant was identical to that of the former CTX-M-1.1 variant. Surprisingly, under our experimental conditions we were unable to detect the expected $bla_{CTX-M-1}$ or $bla_{CTX-M-3}$ derivatives ($bla_{CTX-M-15}$ or $bla_{CTX-M-32}$, respectively) carrying the D240G change, which have been associated with increased ceftazidime resistance (6, 30). Comparison of these variants in an isogenic context allowed us to observe that the resulting MIC of ceftazidime was not as high as expected (2 μg/ml; Table 2). These variants probably did not emerge under our experimental conditions because the MICs for the correspondent transformants (control strains) were lower than the con-

TABLE 2. MICs of different β-lactams among the CTX-M-1 variants obtained in nature or via in vitro selection experiments

CTX-M natural variant[a]	Mutant[b]	Amino acid change[c]								MICs (μg/ml) of the β-lactam antibiotics tested[d]			
		V27A	Q38R	A77V	N114D	A140S	P167S	D240G	D288N	CTX	CAZ	FEP	CXM
CTX-M-10										256	1	32	≥256
	CTX-M-10.1						+			1	32	1	32
	CTX-M-10.2							+		≥256	16	4	≥256
CTX-M-3		+	+							256	1	32	≥256
	CTX-M-3.1	+	+				+			1	32	0.5	128
	CTX-M-3.2[f]	+	+	+			+			4	32	0.25	64
CTX-M-1		+	+	+	+	+			+	≥256	4	128	≥256
	CTX-M-1.1	+	+	+	+	+	+		+	16	256	4	≥256
	CTX-M-1.2[g]	+	+	+	+	+	(+)[e]		+	16	256	4	≥256
CTX-M-15		+	+					+		128	2	1	≥256
CTX-M-32		+	+	+	+	+		+	+	256	2	4	≥256

[a] Corresponding CTX-M ESBL variants isolated in nature.
[b] CTX-M-1-like mutants obtained by in vitro evolution experiments.
[c] Amino acid changes present in each CTX-M-allele compared to CTX-M-10 are represented by "+".
[d] The MICs obtained for each antibiotic tested were determined by broth microdilution. CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; CXM, cefuroxime.
[e] The mutation in this position corresponds to the change P167T.
[f] CTX-M-3.2 = CTX-M-52.
[g] CTX-M-1.2 = CTX-M-58.

centration used to select transformant cells in the in vitro evolution experiments (fourfold MIC to ceftazidime) (see Materials and Methods).

In the case of the $bla_{\text{CTX-M-3}}$ in vitro evolution experiment, a second mutant was found, CTX-M-3.2. This variant consists of a double mutant, P167S+A77V, and corresponds to the natural enzyme CTX-M-52 (GenBank accession number DQ223685). This double mutation causes the *E. coli* MI1443 strain to be resistant to ceftazidime (MIC of 32 μg/ml, which is equivalent to that mediated by the single P167S mutant CTX-M-3.1 [see above]). However, it partially restored the original cefotaxime resistance phenotype (from 1 to 4 μg/ml) (Table 2). This effect appears to be mediated by the presence of the A77V change, which has frequently been observed in natural CTX-M β-lactamases of different phylogenetic clusters (CTX-M-1, CTX-M-9, or CTX-M-25).

Site-directed mutagenesis of CTX-M-3. The change $C^{230}\rightarrow T$ (A77V), which partially restores cefotaxime resistance in the P167S evolved mutant CTX-M-3.2 (from 1 to 4 μg/ml), was introduced by site-directed mutagenesis in the wild $bla_{\text{CTX-M-3}}$ gene. Interestingly, in the absence of the P167S mutation (CTX-M-3.3), cefotaxime resistance slightly decreases (from 256 to 128 μg/ml), and there was also a decrease in cefepime resistance (from 32 to 1.5 μg/ml) (Table 3). Furthermore, change A77V was also observed with the D240G mutation in the CTX-M-57 natural derivative (GenBank accession number DQ810789). This variant (D240+A77V) was constructed by introducing the change A77V into CTX-M-15 (CTX-M-3.4).

TABLE 3. Significance of A77V and N106S substitutions among different CTX-M variants obtained by site-directed mutagenesis

CTX-M natural variant[a]	Mutant[b]	Amino acid changes[c]								MICs (μg/ml) of the β-lactam antibiotics tested[d]			
		V27A	Q38R	A77V	N106S	N114D	A140S	P167S	D240G	CTX	CAZ	FEP	CXM
CTX-M-10										256	1	32	≥256
	CTX-M-10.3			+						≥256	8	128	≥256
	CTX-M-10.4			+				+		4	64	1	64
	CTX-M-10.5			+					+	≥256	8	32	≥256
	CTX-M-10.6				+					2	0.5	1	≥256
	CTX-M-10.7				+			+		2	16	1	≥256
	CTX-M-10.8				+				+	64	2	4	≥256
	CTX-M-10.9							+	+	4	4	<0.25	≥256
CTX-M-3		+	+							256	1	32	≥256
	CTX-M-3.3	+	+	+						128	1	1.5	≥256
	CTX-M-3.4[e]	+	+	+					+	≥256	2	4	≥256
	CTX-M-3.5	+	+		+					3	0.5	1.5	≥256
	CTX-M-3.6	+	+		+			+		3	32	1.5	≥256
	CTX-M-3.7[f]	+	+		+				+	≥256	2	8	≥256
	CTX-M-3.8	+	+					+	+	1.5	8	0.5	≥256

[a] Corresponding CTX-M ESBL variants isolated in nature.
[b] CTX-M-1-like mutants obtained by directed mutagenesis.
[c] Amino acid changes present in each CTX-M allele compared to CTX-M-10.
[d] The MICs obtained for each antibiotic tested were determined by E-test. CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; CXM, cefuroxime.
[e] CTX-M-3.4 = CTX-M-54.
[f] CTX-M-3.7 = CTX-M-33.

This partially restored cefotaxime (from 128 to ≥256 μg/ml) and cefepime (from 1.5 to 4 μg/ml) resistance respect to CTX-M-15 (only the D240G mutation). Moreover, the D240G mutation was found to be associated with N106S in natural CTX-M-33, a CTX-M-15 (harboring only D240G) derivative of CTX-M-3 (17). This double mutant (D240G+N106S) was constructed by introducing the change N106S in CTX-M-15 (CTX-M-3.7) (Table 3). This change yielded an increase in cefotaxime (from 128 to ≥256 μg/ml) and cefepime (from 1 to 8 μg/ml) MICs while maintaining the original ceftazidime MIC (2 μg/ml) with respect to CTX-M-15 (Table 3). This pattern was also observed when the N106S change was associated with the P167S mutation: slight increases in cefotaxime and ceftazidime MIC values (from 1 to 3 μg/ml and from 0.5 to 1.5 μg/ml, respectively), while resistance to ceftazidime was unchanged (CTX-M-3.6). Similarly, as in the case of A77V, in the absence of D240G the N106S mutation decreased cefotaxime and cefepime resistance (from 256 to 3 and from 32 to 1.5 μg/ml, respectively) (CTX-M-3.5). These results indicate that the resistance phenotype resulting from A77V or N106S mutations depends on its association with P167S and/or D240G mutations, respectively.

To explain the absence of natural strains harboring simultaneous mutations in positions 167 and 240 of the $bla_{\text{CTX-M-3}}$ gene, the D240G mutation was introduced by site-directed mutagenesis into the P167S mutant CTX-M-3.1, giving rise to the double mutant CTX-M-3.8 (Table 3). This double mutant displayed a decrease in ceftazidime MIC (from 32 to 8 μg/ml), indicating an antagonistic effect of both mutations, each one able to increase ceftazidime resistance in the absence of the other.

In vitro evolution of *E. coli* GB20 carrying $bla_{\text{CTX-M-10}}$. Two different types of CTX-M-10 mutants with increased resistance to ceftazidime were obtained in our experimental conditions (Table 2). The CTX-M-10.1 mutant contains the P167S substitution. Such a change yielded a very high increase in ceftazidime MICs (from 1 to 32 μg/ml). Interestingly, this increase in resistance to ceftazidime was associated with a significant decrease in the cefotaxime (from 256 to 1 μg/ml), cefuroxime (from >256 to 32 μg/ml), and cefepime (from 32 to 1 μg/ml) MICs. The other type of mutant, CTX-M-10.2, was a $A^{725}\rightarrow G$ transition responsible for the amino acid change D240G and resulted in an MIC increase from 1 to 16 μg/ml. This substitution has been described in nature in the CTX-M-53 β-lactamase, which corresponds to a CTX-M-10 derivative (GenBank accession number DQ268764). In this case, the increase in ceftazidime resistance was not associated with any decrease in cefotaxime resistance, and only a reduction in cefepime MIC (from 32 to 4 μg/ml) was observed.

Site-directed mutagenesis of $bla_{\text{CTX-M-10}}$. As in the case of $bla_{\text{CTX-M-3}}$, the presence of the A77V mutation in the P167S mutant (CTX-M-10.4) partially restored the cefotaxime resistance phenotype (the MIC changed from 1 to 4 μg/ml) (Table 3). When the A77V mutation was introduced in the variant carrying change D240G, resulting in mutant CTX-M-10.5, an increase in the cefepime MIC was observed (from 4 to 32 μg/ml). In contrast to what was expected from our previous results obtained with CTX-M-3 derivatives, the MICs of all β-lactam antibiotics, especially ceftazidime and cefepime were increased (from 1 to 8 and from 32 to 128 μg/ml, respectively) for the mutant carrying only the A77V substitution (CTX-M-10.3).

The presence of the N106S change in a P167S context (CTX-M-10.7) resulted in only a slight decrease in the ceftazidime MIC (from 32 to 16 μg/ml), without significant changes in cefotaxime or cefepime resistance. As in the case of CTX-M-3 containing only the A77V or N106S mutations, the presence of N106S in CTX-M-10 as a single mutation reduces the MICs of cefotaxime (from 256 to 2 μg/ml) and cefepime (from 32 to 1 μg/ml) (CTX-M-10.6), whereas in this genetic context, when associated with D240G change resulting in mutant CTX-M-10.8, the cefotaxime and ceftazidime MICs decreased (from ≥256 to 64 μg/ml and from 16 to 2 μg/ml, respectively) (Table 3). The antagonism observed in CTX-M-3 containing both P167S and D240G mutations was also explored here by introducing a P167S change into the mutant CTX-M-10.5, containing the D240G substitution. The variant obtained (CTX-M-10.9) yielded, as in the CTX-M-3 derivative, reduced activities against cefotaxime (from ≥256 to 4 μg/ml), ceftazidime (from 16 to 4 μg/ml), and cefepime (from 4 to <0.25 μg/ml) (Table 3).

DISCUSSION

CTX-M β-lactamases are currently the most prevalent group of ESBLs (5). The acronym for this particular group of enzymes (CTX) indicates its preferential ability to hydrolyze cefotaxime over ceftazidime. However, the selective landscape in the hospital setting frequently includes both cefotaxime-ceftriaxone (and more infrequently cefepime) as an anti-enterobacterial or anti-haemophilus/pneumococcal agent, and ceftazidime, as a broad-spectrum antipseudomonal drug. In the last few years new CTX-M variants, also able to efficiently hydrolyze ceftazidime, have evolved in this complex selective environment. In particular, the current CTX-Ms belonging to the CTX-M-1 cluster confer higher MICs of both cefotaxime and ceftazidime to the harboring bacteria than those of any other CTX-M cluster (23, 24). This advantage suggests that strains harboring CTX-M-1 cluster enzymes might emerge more easily in response to a ceftazidime challenge than those belonging to other CTX-M clusters, thereby facilitating further enzyme evolution, which could explain why CTX-M-1 has become the most diverse cluster (26 variants).

In the present study using the same isogenic hypermutagenic bacterial context, we explored by in vitro evolution the role of ceftazidime in triggering CTX-M-1 gene cluster diversity, in particular diversity of the CTX-M-1, CTX-M-3, and CTX-M-10 enzymes. The already-described P167S/T and D240G mutations involved in ceftazidime resistance phenotypes in natural isolates (2, 6, 30, 36) were consistently found in our in vitro evolution experiments under ceftazidime selective pressure, as observed previously (14, 22, 37).

P167S or P167T mutations were found in each of the in vitro evolution experiments. Such mutants corresponded to the highest ceftazidime MICs found in the present study: mutants CTX-M-10.4, CTX-M-3.2, and CTX-M-1.1 were associated with 32- to 256-fold increments in ceftazidime MICs (Table 2 and Table 3). However, a drastic mutational antagonistic pleiotropy was observed among all of these variants, as inferred from the association of high ceftazidime MICs with a concomitant loss of activity of other antibiotics, especially

cefotaxime and cefepime (2- to 256-fold and 8- to 64-fold reductions in the MICs, respectively). This effect has been described previously (23, 35) and has been associated with high instability in the omega loop (7, 31).

The incompatibility between the main mutations involved in ceftazidime resistance, P167S or D240G, was also assessed in the present study. Indeed, both mutations are never found together in natural strains, suggesting that they represent two independent ways to acquire a ceftazidime resistance phenotype in strains carrying a CTX-M-1 β-lactamase. Interestingly, D240G mutants, which are less efficient in ceftazidime hydrolysis, are found in the most prevalent CTX-M enzymes, as CTX-M-15, probably because they retain the activity against cefotaxime more than the P167S mutants. Nevertheless, in the presence of P167S or D240G, secondary site mutants might have played an important role seeking a balanced phenotype encompassing resistance to both cefotaxime and ceftazidime. Mutants containing the P167S change also display lower cefuroxime resistance, although this was not observed with the D240G mutation. Thus, exposure to cefuroxime, a widely used oral cephalosporin in the community, might be contributing to the high prevalence of CTX-M enzymes containing the D240G change.

The role of secondary sites in the evolution of CTX-M-1-like enzymes was ascertained in the present study either by using in vitro evolution assays or by site-directed mutagenesis. In particular, we detected and analyzed the effects of the A77V and N106S mutations alone or in combination with the P167S or D240G main mutations involved in ceftazidime resistance. Change A77V has a scarce and variable phenotypic effect when introduced by site-directed mutagenesis as a single mutation in different CTX-M contexts. Residue 77 belongs to the alfa-2 helix, being close to lysine 73 and also serine 70. Consequently, the A77V substitution alters the omega loop structure in relation with residue 167 (and Glu-166), since valine is much bigger than alanine (13). When associated with the P167S or D240G mutation, A77V directs the original strongly cefotaxime-ceftazidime-biased phenotype to a more balanced profile of inactivation of both ceftazidime and cefotaxime-cefepime. A similar effect occurs when mutation N106S was introduced in a CTX-M-3 carrying the D240G mutation, although the need for this balancing mutation is probably not as strong in the case of such enzymes. Curiously, residue 106 is not in the close vicinity of the active site but is located in the loop harboring residue 104, close to residue 132, which is implicated in the binding of β-lactams to the CTX-M catalytic cavity (14). These phenotype-equilibrating mutants have been frequently found in natural CTX-M enzymes (CTX-M-33, CTX-M-52, CTX-M-53, or CTX-M-57), suggesting that a more eclectic phenotype could has been subjected to positive selection in complex selective environments, involving exposure to ceftazidime, cefotaxime, and cefepime. We cannot rule out a possible recent role for cefepime in selecting variants such as CTX-M-33, where N106S is added to the D240G-containing CTX-M-15, leading to an MIC increase from 1 to 8 μg/ml. Finally, our results suggest that strains carrying CTX-M-10 enzymes could be better suited to evolving toward increased ceftazidime resistance than CTX-M-3. This prediction was not endorsed by current epidemiological scenarios, wherein strains with CTX-M-3 derivatives evolved more efficiently toward ceftazidime resistance and some of them, such as CTX-M-15, were much more widespread (10). Indeed, we were unable to obtain CTX-M-15 from a CTX-M-3 background. The antibiotic resistance phenotype provided by CTX-M-15 is possibly next to that of CTX-M-3 to be hooked by evolutionary forces. The general success of CTX-M-15 might be derived not from clear advantages in extended resistance phenotype but rather by its association with successful clones, plasmids, and genetic platforms, thereby revealing the difficulties of making predictions about the evolution of antibiotic resistance (25).

In summary, in addiction to the involvement of mutations in positions 167 and 240 in ceftazidime resistance phenotypes, we demonstrate here for the first time that A77V and N106S changes might have also played an important role in the evolution of CTX-M-1 cluster enzymes. On the other hand, the incompatibility between P167S and D240G changes suggests two different routes in the development of ceftazidime resistance.

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