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MECHANISMS OF RESISTANCE

In Vivo Evolution of CTX-M-215, a Novel Narrow-Spectrum β-Lactamase in an *Escherichia coli* Clinical Isolate Conferring Resistance to Mecillinam

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ABSTRACT Here, we report a novel narrow-spectrum β-lactamase CTX-M-215 identified in an *Escherichia coli* clinical isolate in China and conferring high-level resistance to mecillinam but not to cefotaxime. CTX-M-215 differed from CTX-M-125, a CTX-M extended-spectrum β-lactamase (ESBL), by an N132D substitution, which decreased hydrolytic activities toward penicillins and cephalosporins except for mecillinam. High similarity was observed between CTX-M-215- and CTX-M-125-bearing plasmids, carried by different isolates in the same patient, indicating *in vivo* evolution of CTX-M-215 from CTX-M-125.

KEYWORDS narrow-spectrum β-lactamase, CTX-M-type enzyme, mecillinam, cefotaxime, N132D

CTX-M-type extended-spectrum β-lactamases (ESBLs) are the most prevalent type of ESBLs in *Enterobacterales* (1). According to genetic relatedness, CTX-M ESBLs have been grouped into the following six clusters: the CTX-M-1, -2, -8, -9, -25, and KLUC groups (1). Recently, several hybrid enzymes have been identified, including CTX-M-64, CTX-M-116, CTX-M-123, CTX-M-132, and CTX-M-137, which have been generated by recombination between two bla_{CTX-M} genes (2).

CTX-M-type ESBLs were so termed for their excellent activity against cefotaxime. S237 and R276 residues are conserved in the CTX-M family and have been associated with the expansion of catalytic activity through accommodation of the bulky oxyimino side chains of cefotaxime (3). The D240G substitution in CTX-M enzymes (such as in CTX-M-15, CTX-M-27, and CTX-M-55) improves hydrolytic activity against ceftazidime. In addition, an L169Q mutant of CTX-M-27, named CTX-M-93, possesses higher activity against ceftazidime at the expense of penicillinase activity (4). Most recently, CTX-M-127, an N132D mutant of CTX-M-15, was identified to confer resistance to mecillinam (5). Finally, CTX-M-33, a CTX-M-15 derivative with N106S, was reported to possess low hydrolytic activity against carbapenem (6).

Unlike TEM- and SHV-type β-lactamases, which consist of narrow-spectrum, extended-spectrum, and inhibitor-resistant enzymes (groups 2b, 2be, and 2br according to Bush-Jacoby-Medeiros classification [7]), CTX-M enzymes discovered in clinical isolates have been exclusively ESBLs belonging to group 2be. Here, we present data on

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TABLE 1 MICs for the clinical isolate *E. coli* EC96 and transformants carrying bla_{CTX-M}

Antibiotic(s)	MIC (μg/ml) for:					
	E. coli EC96	*E. coli* DH5α(pEC96-CTX-M-215)[a]	*E. coli* DH5α(pCTX-M-14)[b]	*E. coli* DH5α(pCTX-M-125)[b]	*E. coli* DH5α(pCTX-M-215)[b]	*E. coli* DH5α(pHSG396)
Ampicillin	>2,048	32	>2,048	2,048	32	8
Ampicillin-clavulanate	64/32	8/4	8/4	8/4	8/4	8/4
Piperacillin	2,048	8	512	512	8	4
Piperacillin-tazobactam	128/4	4/4	4/4	4/4	4/4	4/4
Mecillinam	>128	>128	1	1	>128	0.25
Mecillinam-clavulanate	16/8	4/2	0.5/0.25	0.5/0.25	4/2	0.25/0.125
Cefazolin	512	4	256	256	2	1
Cefuroxime	>2,048	8	2,048	1,024	8	8
Cefotaxime	>2,048	0.125	128	128	0.125	0.125
Cefotaxime-clavulanate	16/8	0.125/0.06	1/0.5	2/1	≤0.06/0.03	≤0.06/0.03
Ceftazidime	>128	0.5	4	4	0.25	0.5
Ceftazidime-clavulanate	8/4	0.25/0.125	1/0.5	1/0.5	0.25/0.125	0.5/0.25
Cefoperazone	>2,048	2	128	64	4	0.5
Cefoperazone-sulbactam	128/64	1/0.5	32/16	32/16	1/0.5	0.25/0.125
Cefepime	>128	≤0.06	8	16	≤0.06	≤0.06
Cefmetazole	>128	2	2	1	1	1
Cefoxitin	>128	8	8	4	4	4
Aztreonam	>128	0.125	8	16	0.125	0.25
Imipenem	16	0.125	0.125	0.125	0.125	0.125
Meropenem	16	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06
Ertapenem	128	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06
Polymyxin B	≤0.06	0.125	0.125	0.125	0.125	0.125
Amikacin	>128	1	1	1	2	1
Ciprofloxacin	>64	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06
Fosfomycin[c]	>1,024	0.125	ND[d]	ND	0.125	ND
Tigecycline	1	0.25	0.25	0.25	0.25	0.25

[a]The corresponding *E. coli* DH5α transformant of *E. coli* EC96, with plasmid pEC96-CTX-M-215.
[b]Transformant harboring a PCR-generated recombinant plasmid encoding CTX-M-14 or CTX-M-125 or CTX-M-215.
[c]MICs were determined by Etest.
[d]ND, not determined.

CTX-M-215, a naturally occurring narrow-spectrum CTX-M-9 group enzyme that was found in an *Escherichia coli* clinical isolate, and reconstruct its evolutionary path from CTX-M-125, its ESBL precursor.

Characterization of CTX-M-215-producing clinical isolate. *E. coli* EC96 was isolated from a patient with recurrent biliary tract infection (BTI) in 2015. MICs were determined by Etest (bioMérieux) or broth microdilution method as recommended by the Clinical and Laboratory Standards Institute (CLSI) (8). EC96 was resistant to all of the tested β-lactams, ciprofloxacin, amikacin, and fosfomycin (Table 1). A new bla_{CTX-M}, named $bla_{CTX-M-215}$, was identified together with $bla_{CTX-M-55}$ by PCR and sequencing using primers listed in Table S1 in the supplemental material (9). The $bla_{CTX-M-215}$ contained a 1-bp mutation compared to $bla_{CTX-M-125}$, generating an asparagine to aspartic acid substitution at position 132 of the CTX-M enzyme (N132D). Further investigation showed persistence of the CTX-M-215-producing *E. coli*, which was isolated on multiple occasions from the bile of the patient from March to December, 2015. Notably, a CTX-M-125-producing *Klebsiella pneumoniae* isolate, KP120, had been recovered from the bile of the same patient 4 years earlier. Piperacillin-tazobactam, cefoperazone-sulbactam, amikacin, fosfomycin, imipenem, and meropenem were used to treat recurrent BTI episodes of this patient during these years.

Whole-genome sequencing was performed on EC96 and KP120 using the Illumina HiSeq and PacBio platforms. Extracted plasmid DNA of EC96 was transformed into the recipient strain *E. coli* DH5α and selected on LB agar plates supplemented with ampicillin. Two kinds of transformants were identified carrying $bla_{CTX-M-215}$ and $bla_{CTX-M-55}$, respectively, by PCR and sequencing using specific primers for the $bla_{CTX-M-9}$ and $bla_{CTX-M-1}$ groups. EC96 carried five plasmids approximately 153, 130, 94, 68, and 32 kb in sizes (see Fig. S1 in the supplemental material). The 68,439-bp plasmid, named pEC96-CTX-M-215, carried $bla_{CTX-M-215}$ both in EC96 and its transformant as

shown by S1-PFGE (Fig. S1). In addition, EC96 also carried $bla_{CTX\text{-}M\text{-}55}$, *rmtB*, and *fosA3*, which were located on the 153,051-bp plasmid, conferring resistance to the third generation cephalosporins (including ceftazidime), amikacin, and fosfomycin (Table 1). While the isolate was resistant to carbapenems, no known carbapenemases or plasmid-mediated AmpC β-lactamase genes were identified by ResFinder (https://cge.cbs.dtu.dk/services/), and no carbapenemase activity was detected by modified carbapenem inactivation method (mCIM) test (8). *ompC* and *ompF* were disrupted by insertion sequences, indicating that porin deficiency may have contributed in the carbapenem resistance of EC96 as reported previously (10). However, further investigations are still needed to decipher the mechanism of carbapenem resistance in EC96.

Comparison of CTX-M-215- and CTX-M-125-encoding plasmids. CTX-M-encoding plasmids in EC96 and KP120 were annotated *in silico* using RAST 2.0 combined with BLASTP/BLASTN, ResFinder, PlasmidFinder, plasmid multilocus sequence typing (pMLST) (11), and ISfinder (https://www-is.biotoul.fr/index.php). Both pEC96-CTX-M-215 and pKP120-CTX-M-125 were IncFII plasmids and genetically closely related to each other with an overall identity of more than 99.9% (see Fig. S2 in the supplemental material). Subtyping of FII plasmid showed that pKP120-CTX-M-125 belonged to type F14:A−:B−, while pEC96-CTX-M-215 belonged to type F14v:A−:B−, with one point mutation compared to the FII_14 allele. The $bla_{CTX\text{-}M\text{-}215}$ gene is putatively derived from $bla_{CTX\text{-}M\text{-}125}$, with single nucleotide replacement of A403G. The $bla_{CTX\text{-}M\text{-}125}$ gene in pKP120-CTX-M-125 was located in a classic IS*Ecp1* transposition unit, which was mobilized into Tn*1722* at the *mcp* gene (alias *orfI*). Taken together, $bla_{CTX\text{-}M\text{-}215}$ was likely a descendant of $bla_{CTX\text{-}M\text{-}125}$, which was mobilized via the Tn*1722* transposition unit and IncFII plasmid between *E. coli* and *K. pneumoniae*.

Cloning of $bla_{CTX\text{-}M\text{-}14/125/215}$ and antimicrobial susceptibility testing. Isolates EC96, EC62, and KP120 carrying $bla_{CTX\text{-}M\text{-}215/14/125}$ were used as templates for PCR. The full-length $bla_{CTX\text{-}M}$ genes were cloned into the pHSG396 vector (TaKaRa, Japan) and transformed into *E. coli* DH5α. *E. coli* DH5α(pCTX-M-14) and *E. coli* DH5α(pCTX-M-125) were susceptible to mecillinam and exhibited a typical ESBL phenotype as do most CTX-M enzymes. Notably, *E. coli* DH5α(pEC96-CTX-M-215) was highly resistant to mecillinam but lost the ESBL phenotype (Table 1). CTX-M-215 conferred low-level resistance to ampicillin and was susceptible to cephalosporins (first to fourth generations), although it contributed to a modest 8-fold elevation of MICs of cefoperazone compared to the recipient strain. These results indicated that CTX-M-215 was a narrow-spectrum β-lactamase belonging to group 2b. Since there is only a single amino acid difference between CTX-M-125 and CTX-M-215, the substitution of N132D was considered to be responsible for these phenotypic differences.

Kinetic properties of CTX-M-14, CTX-M-125, and CTX-M-215. The $bla_{mCTX\text{-}M}$ genes (mCTX-M represents the mature form of CTX-M without signal peptides) were cloned into the pET28a(+) vector (Novagen, Germany) and transformed into *E. coli* BL21(DE3). The purities of CTX-M proteins were more than 95% as estimated by SDS-PAGE. Hydrolysis of β-lactams by CTX-M enzymes was measured using a UV-2700 spectrophotometer (Shimadzu, Kyoto, Japan) (2, 12). The overall catalytic efficiency (k_{cat}/K_m) of CTX-M-14 and CTX-M-125 was similar to that in a previous report (Table 2) (13). Compared to CTX-M-14 and CTX-M-125, CTX-M-215 exhibited significantly increased hydrolytic activity of mecillinam in contrast to the decreased affinity and diminished turnover for the other β-lactams. These results were consistent with the MICs in the *E. coli* DH5α clones (Tables 1 and 2). The hydrolytic activities of the three CTX-M enzymes were undetectable against imipenem, meropenem, and ertapenem. The 50% enzyme inhibition (IC_{50}) was measured for inhibitors with nitrocefin as the substrate at a concentration of 100 μM (6). Both CTX-M-125 and CTX-M-215 could be inhibited at nanomolar levels by clavulanic acid, tazobactam, and sulbactam, although the IC_{50}s for CTX-M-215 were 7-, 52-, and 7-fold higher, respectively, than those for CTX-M-125 (see Table S2 in the supplemental material).

TABLE 2 Steady-state kinetic parameters of CTX-M-14, CTX-M-125, and CTX-M-215[a]

	CTX-M-14			CTX-M-125			CTX-M-215		
Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})
Ampicillin	41.07 ± 9.10	32.72 ± 2.89	0.80	24.55 ± 4.60	19.29 ± 0.88	0.79	440.20 ± 54.76	2.98 ± 0.17	0.0068
Piperacillin	11.55 ± 5.05	3.87 ± 0.62	0.33	9.27 ± 2.11	1.82 ± 0.13	0.20	24.31 ± 2.74	0.42 ± 0.02	0.0173
Mecillinam	377.4 ± 149.7	1.02 ± 0.31	0.0027	559 ± 218.5	2.09 ± 0.67	0.0037	132.1 ± 15.61	72.51 ± 4.71	0.55
Nitrocefin	20.13 ± 7.15	560.5 ± 103.6	27.84	8.04 ± 1.02	201.78 ± 7.21	25.09	64.75 ± 19.54	42.72 ± 5.22	0.6598
Cefuroxime	25.01 ± 3.52	55.77 ± 3.44	2.23	34.96 ± 3.23	45.69 ± 2.38	1.31	64.52 ± 10.69	0.10 ± 0.01	0.0015
Cefotaxime	16.33 ± 3.02	16.54 ± 0.87	1.01	23.82 ± 6.71	19.74 ± 1.33	0.83	65.68 ± 14.47	0.05 ± 0.01	0.0007
Ceftazidime	>8,000	ND[b]	ND	>8,000	ND	ND	>8,000	ND	ND
Imipenem	nd[c]	<0.01	nd	nd	<0.01	nd	nd	<0.01	nd
Meropenem	nd	<0.01	nd	nd	<0.01	nd	nd	<0.01	nd
Ertapenem	nd	<0.01	nd	nd	<0.01	nd	nd	<0.01	nd

[a]Data are the averages of the results obtained from three independent experiments.
[b]ND, not determined due to the low activity of the enzymes toward ceftazidime.
[c]nd, not determined due to a low initial rate of hydrolysis.

Docking models of cefotaxime and mecillinam with CTX-M enzymes. Structures of CTX-M-215 were generated from the cocrystal structures of CTX-M-14 S70G and CTX-M-9 S70G, which are in complex with cefotaxime and piperacillin, respectively (PDB accession numbers 4PM5 and 3Q07). Standard precision docking was conducted using Glide (Schrödinger). In PDB 4PM5 (3), the side chain amide of N132 could form hydrogen bond networks with N104 and K73 (Fig. 1A). The amide groups of both N132 and N104 formed hydrogen bonds with the carbonyl of cefotaxime, thus contributing to the binding affinity of the ligand. However, the N132D substitution in CTX-M-215 disrupted the hydrogen bond network. The increased distance between D132 and cefotaxime (about 3.5 Å) and N104 (about 2.9 Å) suggested that there existed electrostatic repulsion (Fig. 1B).

The positively charged amidine moiety of mecillinam could form electrostatic repulsion with the amide NH on both N132 and N104, which was not favorable for the binding to CTX-M-14 (Fig. 1C). Conversely, tight binding was observed between mecillinam and CTX-M-215 since a salt bridge was formed between the protonated amidine N and D132 as well as a hydrogen bond (Fig. 1D). Therefore, the N132D substitution appeared to increase hydrolytic activity against mecillinam but decrease activity against other penicillins and cephalosporins.

By the end of 2019, 213 CTX-M enzymes were released in GenBank. Beside CTX-M-215, 8 CTX-M enzymes possess substitutions in the SDN loop. CTX-M-191 and CTX-M-127 (5) contain the N132D substitution as well (accession numbers WP_102607451.1 and WP_088245211.1). Substitutions of N132H and S130T were found in CTX-M-81/181 and CTX-M-190/199/218, respectively (accession numbers WP_063860080.1/WP_063860035.1 and WP_069280706.1/WP_087587947.1/WP_109791214.1). CTX-M-81 was reported to confer similar resistance profiles to those of CTX-M-14 (14), whereas CTX-M-190 and CTX-M-199 confer resistance to both cefotaxime and β-lactamase inhibitors sulbactam and tazobactam (15, 16). More recently, mecillinam-resistant *E. coli* was identified in Denmark during pivmecillinam treatment of urinary tract infection (UTI) due to a single mutation (A403G) in $bla_{\text{CTX-M-15}}$ to $bla_{\text{CTX-M-127}}$ (5).

Mecillinam is a unique penicillin displaying activities against multidrug-resistant *Enterobacterales*, including many ESBL, AmpC, and some carbapenemase producers (IMP/NDM) (17–19). Pivmecillinam (amdinocillin pivoxil) is an oral prodrug of mecillinam that has been widely used in northern Europe for the treatment of uncomplicated UTI (20). Pivmecillinam was recently released in the United Kingdom (2010) and Germany (2016) but is not yet available/licensed in China or North America (17, 20). The selective pressure that contributed to the emergence of CTX-M-215, therefore, remains unknown.

In conclusion, substitutions in CTX-M enzymes incur diverse alterations to the catalytic activity of CTX-M enzymes, generating ESBLs, inhibitor-resistant β-lactamases,

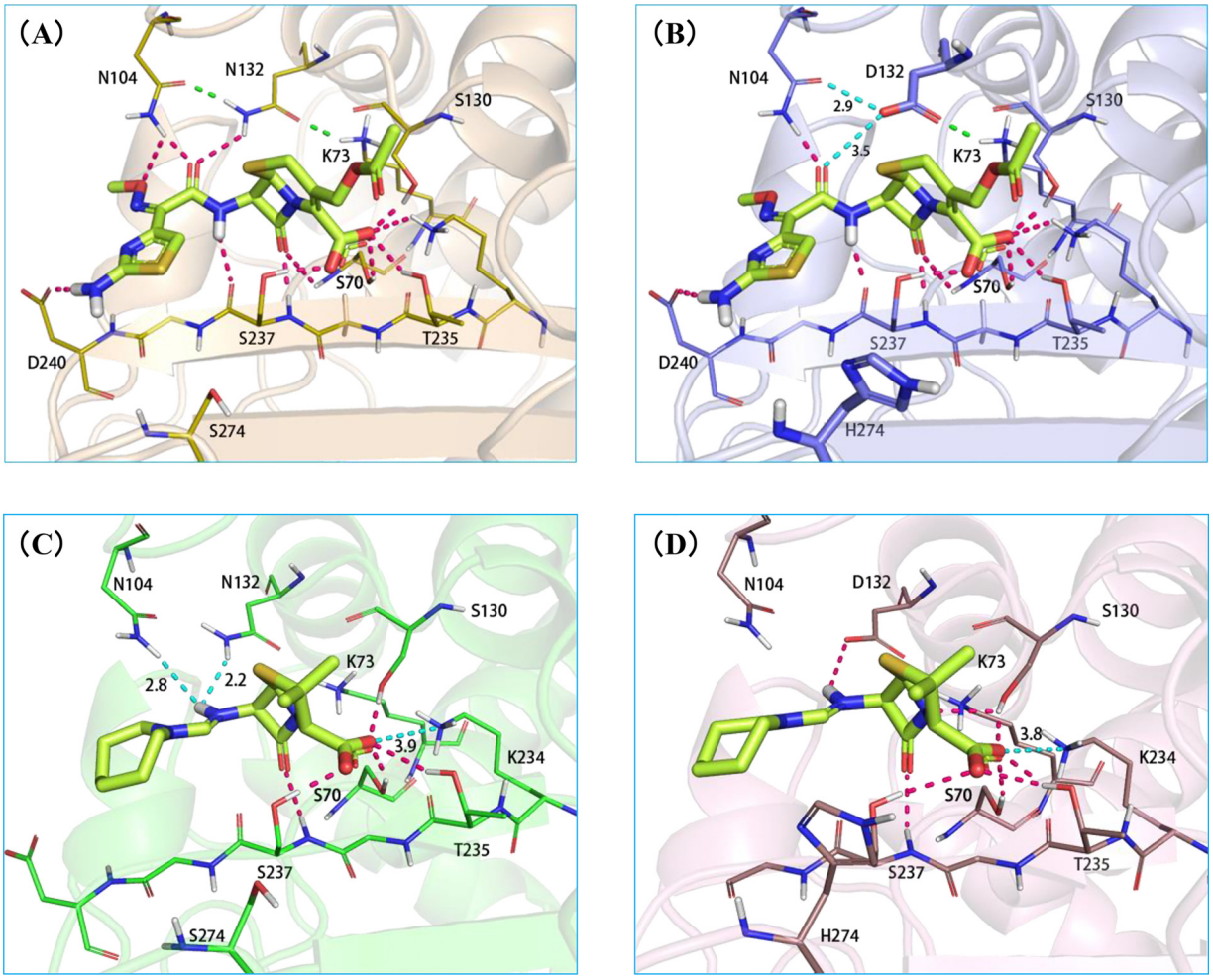

FIG 1 Key polar interactions between enzyme CTX-M-14 (A) or CTX-M-215 (B) and cefotaxime and between CTX-M-14 (C) or CTX-M-215 (D) and mecillinam. The green broken lines represent intramolecular hydrogen bonds, the pink broken lines represent intermolecular hydrogen bonds, and the blue broken lines represent distance.

carbapenemases, or narrow-spectrum β-lactamases. Discovery of narrow-spectrum CTX-M enzymes has implications on β-lactam resistance prediction based on the presence of $bla_{\text{CTX-M}}$ genes.

Accession number(s). The complete sequences of the chromosome and plasmids of isolates EC96 and KP120 have been deposited in GenBank under BioSample accession numbers SAMN15903653 and SAMN15903654.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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We declare no conflicts of interest.

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