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MECHANISMS OF RESISTANCE

Genetic and Functional Characterization of $bla_{CTX\text{-}M\text{-}199}$, a Novel Tazobactam and Sulbactam Resistance-Encoding Gene Located in a Conjugative *mcr-1*-Bearing IncI2 Plasmid

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ABSTRACT We report the genetic and functional characterization of a novel CTX-M-199 β-lactamase, which was encoded by a $bla_{CTX\text{-}M\text{-}64}$ variant gene found in a conjugative *mcr-1*-bearing IncI2 plasmid and exhibited resistance to β-lactamase inhibitors, tazobactam, and sulbactam.

KEYWORDS CTX-M-199, inhibitor resistance, *mcr-1*, conjugative IncI2 plasmid, function, enzyme kinetics

Plasmid evolution plays an important role in bacterial antimicrobial resistance development. This process is usually slow but may accelerate when building blocks of various plasmid backbones and mobile elements harboring different antimicrobial resistance genes become readily available in various ecological niches. Since the discovery of the colistin resistance gene *mcr-1*, an increasing number of plasmids carrying this resistance determinant have been reported, among which the ~60-kb IncI2 plasmid was one of the three common types of conjugative *mcr-1*-bearing plasmids (1, 2). This IncI2 plasmid has been reported to carry an additional $bla_{CTX\text{-}M\text{-}55}$ or $bla_{CTX\text{-}M\text{-}64}$ gene (2, 3). In this study, we report the recovery of a similar IncI2-type *mcr-1*-bearing conjugative plasmid that harbors a novel variant of $bla_{CTX\text{-}M\text{-}64}$, which encodes resistance to class A β-lactamase inhibitors. The widespread presence of this plasmid in clinical bacterial pathogens is expected to further limit the choices of antimicrobial treatment.

One hundred thirty-eight *mcr-1*-positive *Enterobacteriaceae* isolates, including 128 *Escherichia coli*, 5 *Citrobacter freundii*, 3 *Klebsiella pneumoniae*, 1 *Enterobacter cloacae*, and 1 *Enterobacter aerogenes* strains, were recovered from stool samples from hospital patients in Zhejiang Province, China, during the period October 2015 to May 2016. These isolates were subjected to screening for the presence of extended-spectrum β-lactamase (ESBL) genes, as described previously (4). Four isolates were found to harbor $bla_{CTX\text{-}M}$ genes, which were subsequently confirmed to be $bla_{CTX\text{-}M\text{-}64}$ in one *E. coli* isolate (strain JH89) and its variants in the other three isolates (from strains ZE36, ZE722, and EB70). These isolates were recovered from stool samples collected from patients whose age ranged from 2 to 53 years. Antimicrobial susceptibility was deter-

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mined for these four *E. coli* isolates by the broth microdilution method, following recommendations of the Clinical and Laboratory Standards Institute (CLSI) (5). These strains were found to exhibit resistance to colistin and all cephalosporins, except for strains JH89, ZE722, and EB70, which remained susceptible to cefmetazole (Table 1). However, all isolates were susceptible to carbapenem and tigecycline (MICs, ≤0.25 mg/liter). Surprisingly, three of these *E. coli* isolates carrying a variant of $bla_{CTX\text{-}M\text{-}64}$ were resistant to a combination of cephalosporins and their inhibitors tazobactam and sulbactam but not clavulanic acid, with the exception of ZE36, which was also resistant to cephalosporin-clavulanic acid (Table 1). Whole-genome sequencing of this strain identified an additional β-lactamase, $bla_{OXA\text{-}10}$, in ZE36, which might be responsible for its resistance to cephalosporin-clavulanic acid (data not shown). Multilocus sequence typing (MLST) of these four *E. coli* isolates showed that they belonged to different sequence types (STs), namely, JH89 (ST648), ZE36 (ST156), ZE722 (ST117), and EB70 (ST1193) (6). Conjugation experiments performed on these isolates as previously described (7) showed that plasmids carrying the $bla_{CTX\text{-}M}$ gene in these four strains could be conjugated to EC600 (Rif^r). S1 nuclease digestion followed by pulsed-field gel electrophoresis and Southern hybridization were also performed on these four strains and their transconjugants, with results showing that both the $bla_{CTX\text{-}M}$ and *mcr-1* genes were located in the same conjugative plasmid, with a size of around ~60 kb (1). Transconjugants that contained a plasmid which harbored a $bla_{CTX\text{-}M\text{-}64}$ variant were resistant to various combinations of cephalosporins and their inhibitors, including tazobactam and sulbactam, but not to clavulanic acid (Table 1). The ~60-kb plasmids from the transconjugant of ZE36 were sequenced using the Illumina HiSeq 2500 platform (BioNova Biotech Co. Ltd., Beijing, China) and the PacBio platform. The complete sequence of this plasmid, designated pZE36 (KY802014), was found to be 65,846 bp in size. The plasmid sequences were submitted to the RAST tool for annotations and modified manually by BLAST (8). A BLASTN search against the nr database identified four similar IncI2 plasmids, pBA76-*MCR-1* (KX013540), pE15017 (KX772778), pA31-12 (KX034083), and pSCS23 (KU934209), which harbored the *mcr-1* and $bla_{CTX\text{-}M\text{-}55}$-$bla_{CTX\text{-}M\text{-}64}$ genes. Comparison of all five similar plasmids showed that they exhibited a high degree of similarity except (i) IS*Apl1* was located upstream of *mcr-1* in two plasmids but absent in the others, and (ii) the plasmid pBA76-*MCR-1* carried $bla_{CTX\text{-}M\text{-}64}$ and pZE36 carried a variant of $bla_{CTX\text{-}M\text{-}64}$, whereas the other three carried $bla_{CTX\text{-}M\text{-}55}$ (see Fig. S1 in the supplemental material).

Sequence analysis was performed on both the wild-type and variant $bla_{CTX\text{-}M\text{-}64}$ genes, and the data showed that the CTX-M-64 variant enzyme contained two amino acid substitutions at position 109 and 130, resulting in $A^{109}T$ and $S^{130}T$ changes, respectively. We then designated this mutated $bla_{CTX\text{-}M\text{-}64}$ gene $bla_{CTX\text{-}M\text{-}199}$ (KY786032). DNA fragments harboring $bla_{CTX\text{-}M\text{-}64}$ and $bla_{CTX\text{-}M\text{-}199}$, together with the 225-bp upstream and 113-bp downstream regions, were amplified by PCR using primers (as shown in Table S1 in the supplemental material), which contained the BamHI and HindIII restriction sites at the 5′ end of the primers. The amplified fragments were cloned to vector pHSG396 (TaKaRa, Dalian, China) to obtain pHSG396-$bla_{CTX\text{-}M\text{-}64}$ and pHSG396-$bla_{CTX\text{-}M\text{-}199}$. Subsequent antimicrobial susceptibility tests showed that *E. coli* carrying pHSG396-$bla_{CTX\text{-}M\text{-}64}$ displayed resistance only to cephalosporins, whereas strains carrying pHSG396-$bla_{CTX\text{-}M\text{-}199}$ exhibited resistance to cephalosporins and various combinations of cephalosporins and tazobactam-sulbactam but not clavulanate-avibactam, thereby confirming that the $bla_{CTX\text{-}M\text{-}199}$ gene was responsible for mediating the inhibitor resistance phenotype exhibited by the test strains.

Site-directed mutagenesis was then performed on $bla_{CTX\text{-}M\text{-}64}$ and $bla_{CTX\text{-}M\text{-}199}$ to investigate the degree of contribution of various amino acid substitutions to the change in enzymatic activity of both CTX-M-64 and CTX-M-199, using primers listed in Table S1 and as described previously (9, 10). In CTX-M-199, the $T^{130}S$ mutation, but not $T^{109}A$, resulted in susceptibility to piperacillin-tazobactam, cefotaxime-tazobactam, and cefotaxime-sulbactam, suggesting that T^{130} contributed directly to phenotypic resistance to tazobactam and sulbactam (Table 1). Similarly, in CTX-M-64, $S^{130}T$, but not

TABLE 1 Antimicrobial susceptibility profiles of clinical *E. coli* isolates and *E. coli* TG1 strains carrying various bla_{CTX-M} genes

Strain identification	β-Lactamase	MICs (μg/ml) for[a]:												
		PIP	CAZ	CTX	FEP	CMZ	PIP-TAZ	CFP-SUL	CTX-SUL	CTX-CLA	CAZ-CLA	CTX-AVI	FOS	PE
EC600		≤8	0.5	≤0.25	≤0.5	1	≤1/4	≤1/0.5	≤1/0.5	≤1/0.5	≤1/0.5	≤0.15/0.07	≤4	≤0.5
ZE36	CTX-M-199	>256	>32	1,024	>32	>32	128/4	128/64	128/64	16/8	64/32	2/1	>512	2
ZE36-T	CTX-M-199	256	16	1,024	32	1	64/4	64/32	64/32	≤1/0.5	≤1/0.5	≤0.15/0.07	≤4	1
ZE722	CTX-M-199	>256	8	1,024	>32	2	32/4	64/32	64/32	≤1/0.5	≤1/0.5	≤0.15/0.07	>512	4
ZE722-T	CTX-M-199	128	16	1,024	16	2	64/4	64/32	64/32	≤1/0.5	≤1/0.5	≤0.15/0.07	≤4	1
EB70	CTX-M-199	>256	8	1,024	>32	≤0.5	64/4	32/16	32/16	≤1/0.5	≤1/0.5	≤0.15/0.07	8	2
EB70-T	CTX-M-199	256	16	1,024	32	1	64/4	64/32	64/32	≤1/0.5	≤1/0.5	≤0.15/0.07	≤4	2
JH89	CTX-M-64	>256	32	1,024	>32	1	≤1/4	16/8	16/8	≤1/0.5	≤1/0.5	≤0.15/0.07	≤4	2
JH89-T	CTX-M-64	>256	>32	1,024	32	1	≤1/4	16/8	16/8	≤1/0.5	≤1/0.5	≤0.15/0.07	≤4	1
VC[b]		≤8	≤0.25	≤0.25	≤0.5	≤0.5	≤1/4	≤1/0.5	≤1/0.5	≤1/0.5	≤1/0.5	≤0.15/0.07	≤4	≤0.5
CTX-M-199[c]	CTX-M-199	256	16	1,024	>32	≤0.5	64/4	64/32	64/32	≤1/0.5	≤1/0.5	0.25/0.125	≤4	≤0.5
M199($T^{109}A$)	M199($T^{109}A$)	128	16	256	32	≤0.5	64/4	64/32	64/32	≤1/0.5	≤1/0.5	0.25/0.125	≤4	≤0.5
M199($T^{130}S$)	M199($T^{130}S$)	>256	>32	1,024	>32	≤0.5	≤1/4	4/2	4/2	≤1/0.5	2/1	≤0.15/0.07	≤4	≤0.5
CTX-M-64[d]	CTX-M-64	256	16	1,024	4	≤0.5	≤1/4	2/1	2/1	≤1/0.5	≤1/0.5	≤0.15/0.07	≤4	≤0.5
M64($A^{109}T$)	M64($A^{109}T$)	128	8	512	32	≤0.5	≤1/4	4/2	4/2	≤1/0.5	2/1	≤0.15/0.07	≤4	≤0.5
M64($S^{130}T$)	M64($S^{130}T$)	128	8	512	>32	≤0.5	64/4	64/32	64/32	≤1/0.5	≤1/0.5	0.25/0.125	≤4	≤0.5
M64($S^{130}D$)	M64($S^{130}D$)	≤8	≤0.25	≤0.25	≤0.5	≤0.5	≤1/4	≤1/0.5	≤1/0.5	≤1/0.5	≤1/0.5	≤0.15/0.07	≤4	≤0.5
M64($S^{130}G$)	M64($S^{130}G$)	≤8	≤0.25	≤0.25	≤0.5	≤0.5	≤1/4	≤1/0.5	≤1/0.5	≤1/0.5	≤1/0.5	≤0.15/0.07	≤4	≤0.5
M64($S^{130}N$)	M64($S^{130}N$)	≤8	≤0.25	≤0.25	≤0.5	≤0.5	≤1/4	≤1/0.5	≤1/0.5	≤1/0.5	≤1/0.5	≤0.15/0.07	≤4	≤0.5
M64($S^{130}V$)	M64($S^{130}V$)	≤8	≤0.25	≤0.25	≤0.5	≤0.5	≤1/4	≤1/0.5	≤1/0.5	≤1/0.5	≤1/0.5	≤0.15/0.07	≤4	≤0.5
M64($S^{130}C$)	M64($S^{130}C$)	≤8	≤0.25	≤0.25	≤0.5	≤0.5	≤1/4	≤1/0.5	≤1/0.5	≤1/0.5	≤1/0.5	≤0.15/0.07	≤4	≤0.5

[a]For PIP-TAZ, the tazobactam was tested at a fixed concentration of 4 μg/ml. The CFP-SUL, CTX-SUL, CTX-CLA, CTX-AVI, and CAZ-CLA combinations were tested at a 2:1 ratio (antibiotic/inhibitor). PIP, piperacillin; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; CMZ, cefmetazole; PIP-TAZ, piperacillin-tazobactam; CFP-SUL, cefoperazone-sulbactam; CTX-SUL, cefotaxime-sulbactam; CAZ-CLA, ceftazidime-clavulanic acid; CTX-CLA, cefotaxime-clavulanic acid; CTX-AVI, cefotaxime-avibactam; FOS, fosfomycin; PE, polymyxin.

[b]*E. coli* TG1 carrying the cloning vector pHSG396.

[c]*E. coli* TG1 carrying pHSG396-$bla_{CTX-M-199}$ with different point mutations.

[d]*E. coli* TG1 carrying pHSG396-$bla_{CTX-M-64}$ with different point mutations.

A^{109}T, caused a dramatic increase in the MICs of piperacillin-tazobactam, cefotaxime-tazobactam, and cefotaxime-sulbactam (Table 1). These data therefore confirmed that the S^{130}T substitution in CTX-M-64 would result in phenotypic inhibitor resistance. Other substitutions, including S^{130}C, S^{130}D, S^{130}G, S^{130}N, and S^{130}V in CTX-M-64, exhibited dramatically reduced MICs for all cephalosporins yet remained susceptible to the inhibitors, suggesting that these amino acid substitutions caused impairment of the activity of CTX-M-64. S^{130}T was the only substitution that not only retained the CTX-M-64 activity but also resulted in resistance to the inhibitors tazobactam and sulbactam. This observation is consistent with that from a recent report on the discovery of a novel CTX-M-55 variant, CTX-M-190, which differed from CTX-M-55 by only the S^{130}T substitution (consensus nomenclature) and exhibited resistance to tazobactam and sulbactam (11).

To confirm the enzymatic activity of these enzymes, CTX-M-64, CTX-M-64 (S^{130}T), and CTX-M-199 without His tags were expressed and purified as previously described (9, 10). Briefly, DNA encoding $bla_{\text{CTX-M-64}}$, $bla_{\text{CTX-M-64}}$ (S^{130}T), and $bla_{\text{CTX-M-199}}$ lacking the N-terminal secretion peptide sequences was applied using primer as shown in Table S1 and cloned into the pET-28b vector. Successful clones were transformed into the Bl21(DE3) strain to express the proteins. These proteins were purified by running the cell lysate through Ni-NTA agarose, gel filtration, and DEAE columns. The His_6 tag of these proteins was removed by incubating the purified enzyme with thrombin and repurified by a gel filtration column to achieve protein with more than 95% purity. The pure proteins were subjected to determination of kinetic constants, as previously described (9, 10). Our data show that the kinetic constants on various cephalosporin antibiotics exhibited by CTX-M-199 and CTX-M-64(S^{130}T) were similar to that of CTX-M-64; however, their inhibition constants (K_i) and 50% inhibitory concentration (IC_{50}) to tazobactam and sulbactam were found to have increased by $\sim 1 \times 10^5$ to 1×10^7-fold, respectively, compared to those of CTX-M-64, further confirming the inhibitory effect of this mutation on inhibitor binding (Table 2). In addition, the K_i and IC_{50} of CTX-M-199 and CTX-M-64(S^{130}T) to clavulanate and avibactam did not change significantly compared to that of CTX-M-64. These data suggest that the S^{130}T mutation did not affect the binding of clavulanate and avibactam to CTX-M-64, which is very consistent with their resistance phenotypes. It should be noted that the IC_{50}s determined for different enzymes were significantly different from that of CTX-M-190, which is probably due to the use of nontagged protein and nitrocefin as the substrate in this study (11). Ser^{130} is one of the essential residues residing in the active site and involved in substrate catalysis in all class A β-lactamases. Mutation at residue Ser^{130} has also been reported in different types of class A β-lactamases, including TEM-59, TEM-76, and TEM-89, SHV-10, and CTX-M β-lactamases. However, all these class A β-lactamases carried the S^{130}G mutation, which led to resistance to inhibitors but also dramatically reduced their activity against cephalosporins (12–14). Consistent with this, our data show that the mutation of S^{130} to residues Gly, Ser, Val, Asp, and Asn dramatically reduced CTX-M-64 activity, while only S^{130}T retained catalytic activity and obtained inhibitor resistance, which was probably due to the high similarity of the side chain of these two residues, Ser and Thr.

In conclusion, this study identified and characterized a novel CTX-M-199 β-lactamase that was resistant to tazobactam and sulbactam inhibitors. Data generated from this study depicted the evolutionary route of an antimicrobial resistance-encoding plasmid, in which the prototype *mcr-1*-bearing conjugative IncI2 plasmid acquired a mobile element carrying the $bla_{\text{CTX-M}}$ gene ($bla_{\text{CTX-M-64}}$ or $bla_{\text{CTX-M-55}}$), which then underwent further mutational changes that conferred the ability to become resistant to β-lactamase inhibitors. The evolution process could be mediated by frequent clinical usage of cephalosporin and cephalosporin plus inhibitor combinations.

Accession no(s). Plasmid sequencing data and the nucleotide sequence of the $bla_{\text{CTX-M-199}}$ gene from this study are available from GenBank database under accession numbers KY802014 and KY786032, respectively.

TABLE 2 Kinetic constants of CTX-M-64, CTX-M-64(S^{130}T), and CTX-M-199 and their IC_{50}s and K_is on β-lactamase inhibitors

CTX-M enzyme	Kinetic constant	Antibiotic substrate[a]							Inh	IC_{50} (nM)	K_i (nM)
		AMP	PIP	NCF	CEP	CFX	CRO	CTX			
CTX-M-64	K_m (μM)	22.04 ± 1.10	20.75 ± 2.90	20.3 ± 1.80	14.57 ± 1.6	15.31 ± 0.81	1.672 ± 0.15	23.89 ± 2.90	CLA	2.28	7.48×10^{-1}
	k_{cat} (s^{-1})	1,417.93	487.65	474.31	58.17	525.6	62.28	40.18	TAZ	7.52×10^{-1}	2.29×10^{-2}
	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	64.33	23.50	23.37	3.99	34.33	37.25	1.68	SUL	3.65×10^{-1}	1.48
									AVI	2.51×10^{5}	6.64×10^{1}
CTX-M-64 (S^{130}T)	K_m (μM)	20.17 ± 0.60	14.64 ± 1.40	3.07 ± 0.19	11.05 ± 1.00	11.59 ± 1.30	3.84 ± 0.47	6.34 ± 0.86	CLA	4.29×10^{1}	7.76×10^{-1}
	k_{cat} (s^{-1})	1,000.77	344.73	30.83	78.07	428.66	74.51	10.21	TAZ	8.47×10^{5}	3.22×10^{4}
	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	49.62	23.55	10.04	7.07	36.99	19.40	1.61	SUL	2.21×10^{6}	1.16×10^{5}
									AVI	4.50×10^{5}	4.86×10^{2}
CTX-M-199	K_m (μM)	19.37 ± 0.70	13.43 ± 1.20	2.98 ± 0.13	10.95 ± 1.20	12.04 ± 1.10	3.95 ± 0.42	6.11 ± 0.46	CLA	4.30×10^{1}	7.65×10^{-1}
	k_{cat} (s^{-1})	1,150.89	356.47	29.89	86.32	465.87	80.26	12.64	TAZ	8.52×10^{5}	3.20×10^{4}
	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	59.41	26.54	10.03	7.88	38.69	20.31	2.07	SUL	2.20×10^{6}	1.18×10^{5}
									AVI	4.84×10^{5}	4.43×10^{2}

[a]Each value is the average of the three independent experiments. AMP, ampicillin; PIP, piperacillin; NCF, nitrocefin; CEP, cephalothin; CFX, cefuroxime; CRO, ceftriaxone; CTX, cefotaxime; CLA, clavulanic acid; TAZ, tazobactam; SUL, sulbactam; AVI, avibactam; Inh, inhibitor.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00562-17.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

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We declare no conflict of interest.

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