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Characterization of CTX-M-140, a Variant of CTX-M-14 Extended-Spectrum β-Lactamase with Decreased Cephalosporin Hydrolytic Activity, from Cephalosporin-Resistant *Proteus mirabilis*

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CTX-M-140, a novel CTX-M-type extended-spectrum β-lactamase (ESBL), was identified in cephalosporin-resistant clinical isolates of *Proteus mirabilis*. CTX-M-140 contained an alanine-to-threonine substitution at position 109 compared to its putative progenitor, CTX-M-14. When it was expressed in an *Escherichia coli* isogenic background, CTX-M-140 conferred 4- to 32-fold lower MICs of cephalosporins than those with CTX-M-14, indicating that the phenotype was attributable to this single substitution. For four mutants of CTX-M-14 that were constructed by site-directed mutagenesis (A109E, A109D, A109K, and A109R mutants), MICs of cephalosporins were similar to those for the *E. coli* host strain, which suggested that the alanine at position 109 was essential for cephalosporin hydrolysis. The kinetic properties of native CTX-M-14 and CTX-M-140 were consistent with the MICs for the *E. coli* clones. Compared with that of CTX-M-14, a lower hydrolytic activity against cephalosporins was observed for CTX-M-140. $bla_{\text{CTX-M-140}}$ is located on the chromosome as determined by I-CeuI pulsed-field gel electrophoresis (I-CeuI-PFGE) and Southern hybridization. The genetic environment surrounding $bla_{\text{CTX-M-140}}$ is identical to the sequence found in different plasmids with $bla_{\text{CTX-M-9-group}}$ genes among the *Enterobacteriaceae*. Genome sequencing and analysis showed that *P. mirabilis* strains with $bla_{\text{CTX-M-140}}$ have a genome size of ~4 Mbp, with a GC content of 38.7% and 23 putative antibiotic resistance genes. Our results indicate that alanine at position 109 is critical for the hydrolytic activity of CTX-M-14 against oxyimino-cephalosporins.

Proteus mirabilis is a common cause of health care-associated urinary tract infections. Cephalosporins have been used as the drugs of choice to treat infections caused by ampicillin-resistant *P. mirabilis*. However, *P. mirabilis* clinical isolates exhibiting resistance to expanded-spectrum β-lactam agents have been reported widely in many parts of the world (1–3).

Due to the lack of an intrinsic chromosomally encoded β-lactamase, cephalosporin resistance in *P. mirabilis* is entirely dependent upon acquired β-lactamases, primarily extended-spectrum β-lactamases (ESBLs) and, in some instances, acquired AmpC β-lactamases (3–8). The prevalence of ESBL-producing *P. mirabilis* has increased in several geographical locations; among the ESBLs, the most predominant are CTX-M enzymes, with other β-lactamases, such as TEM, VEB, and PER types, being identified at lower frequencies (1).

CTX-M-type ESBLs, with 179 variants (as of March 2016), can be grouped into five main clusters according to genetic relatedness (the CTX-M-1, -2, -8, -9, and -25 groups) and into several chimeras, including CTX-M-64, CTX-M-116, CTX-M-123, CTX-M-132, and CTX-M-137. Among them, members of the CTX-M-1 group (CTX-M-3, -12, -15, -32, and -55/57), the CTX-M-2 group (CTX-M-2), the CTX-M-9 group (CTX-M-9, -14, and -90), and the CTX-M-25 group (CTX-M-25 and -41) have been reported in *P. mirabilis* (1, 9–11).

Here we present data on CTX-M-140, a naturally occurring variant of CTX-M-14 found in *P. mirabilis* clinical isolates and possessing an amino acid substitution at position 109 (Ala109Thr) and a decreased cephalosporin hydrolytic activity.

MATERIALS AND METHODS

Patients, isolates, and plasmids. Four nonduplicate *P. mirabilis* clinical isolates resistant to third-generation cephalosporins were obtained as part of an antimicrobial resistance monitoring project at a 3,000-bed hospital in Guangdong Province in southern China in July 2012. All four isolates were recovered from urine or other urogenital specimens from inpatients in the urology ward of the hospital. *P. mirabilis* GB03 was identified from an indwelling catheter urine specimen from an 88-year-old male patient with bladder cancer. *P. mirabilis* GB08 was cultured from midstream urine of an 80-year-old male patient with bladder cancer. *P. mirabilis* GB11 was identified from a catheter urine specimen from a 71-year-old

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TABLE 1 Primers used for PCR cloning of $bla_{CTX\text{-}M\text{-}14}$ and $bla_{CTX\text{-}M\text{-}140}$ and for site-directed mutagenesis

Primer use	Direction of primer	Primer sequence (5′–3′)	Annealing temp (°C)
PCR cloning of $bla_{CTX\text{-}M\text{-}14}$/$bla_{CTX\text{-}M\text{-}140}$[a]	Forward	GCGTCTAGAATGGTGACAAAGAGAGTGCA	50
	Reverse	GCGAAGCTTTTACAGCCCTTCGGCGATGA	50
Introduction of A109E change[b,c]	Forward	TTAACTACAATCCGATTGAGGAAAAACACGTCAACGGC	64
	Reverse	GCCGTTGACGTGTTTTTCCTCAATCGGATTGTAGTTAA	64
Introduction of A109D change[b,c]	Forward	GTTAACTACAATCCGATTGACGAAAAACACGTCAACGGC	66
	Reverse	GCCGTTGACGTGTTTTTCGTCAATCGGATTGTAGTTAAC	66
Introduction of A109K change[b,c]	Forward	GGTTAACTACAATCCGATTAAGGAAAAACACGTCAACGGCAC	66
	Reverse	GTGCCGTTGACGTGTTTTTCCTTAATCGGATTGTAGTTAACC	66
Introduction of A109R change[b,c]	Forward	GTTAACTACAATCCGATTCGCGAAAAACACGTCAACGG	66
	Reverse	CCGTTGACGTGTTTTTCGCGAATCGGATTGTAGTTAAC	66

[a] XbaI and HindIII sites in the forward and reverse primers, respectively, are underlined.
[b] Modified base pairs are underlined.
[c] A, alanine; D, aspartic acid; E, glutamic acid; K, lysine; R, arginine.

male patient who was in renal failure secondary to diabetes mellitus. *P. mirabilis* GB12 was isolated from midstream urine of a 68-year-old male patient with bladder cancer. All the isolates were identified using the API 20E system (bioMérieux, Marcy l'Etoile, France). This study was approved by the ethics committee at Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, China.

Escherichia coli DH10B was used as the host strain for cloning experiments. pBC-SK(−) (Agilent Technologies, Santa Clara, CA) was used as the vector for genomic and PCR cloning.

Antimicrobial susceptibility testing. MICs of β-lactam agents for the four clinical isolates, for *E. coli* clones carrying the $bla_{CTX\text{-}M\text{-}14}$ or $bla_{CTX\text{-}M\text{-}140}$ gene, and for mutants of these clones were determined by the agar dilution method and were interpreted by the breakpoints defined by the Clinical and Laboratory Standards Institute (CLSI) (12).

Molecular detection of resistance genes. PCR analyses were performed to identify various resistance genes as described in our previous study (13), which included the ESBL genes bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$, the plasmid-mediated AmpC β-lactamase genes $bla_{CMY\text{-}1}$, $bla_{CMY\text{-}2}$, bla_{DHA}, bla_{ACC}, bla_{ACT}, and bla_{FOX}, the plasmid-mediated quinolone resistance (PMQR) genes *qnrA*, *-B*, *-C*, and *-S* and *qepA*, and the aminoglycoside resistance genes *armA*, *rmtA*, *-B*, *-C*, and *-D*, and *aac*(6′)*-Ib-cr*. Sequencing was performed on both strands by use of an ABI13700 instrument (Applied Biosystems, Foster City, CA) for all PCR products.

PFGE analysis. Molecular strain typing of the four isolates was performed by pulsed-field gel electrophoresis (PFGE) using the restriction enzyme XbaI (MBI Fermentas, Vilnius, Lithuania). The DNA fragments were separated in 1.0% agarose gels in 0.5× Tris-borate-EDTA buffer with a CHEF-Mapper XA apparatus (Bio-Rad, Hercules, CA) for 20 h at 6 V/cm and 14°C.

Plasmid conjugation and transformation. Azide-resistant *E. coli* J53 was used as the recipient for the conjugation and transformation experiments. The transconjugants or transformants were selected on lysogenic agar containing sodium azide (100 μg/ml) plus ampicillin (100 μg/ml).

PCR-based replicon typing of plasmids. PCR-based replicon typing was performed for the transconjugants according to a previously described method (14).

PCR and genomic cloning and sequencing of $bla_{CTX\text{-}M\text{-}14}$ and $bla_{CTX\text{-}M\text{-}140}$. The $bla_{CTX\text{-}M\text{-}14}$ and $bla_{CTX\text{-}M\text{-}140}$ genes were amplified by PCR using the primers CTX-M-14-F (with an XbaI restriction site) and CTX-M-14-R (with an HindIII restriction site) (Table 1). The genomic DNAs of the four clinical isolates were purified, digested with HindIII (MBI Fermentas), and used for cloning to investigate the genetic environments of both $bla_{CTX\text{-}M\text{-}14}$ and $bla_{CTX\text{-}M\text{-}140}$. Both the PCR products and genomic DNAs were then ligated into the cloning vector pBC-SK(−), which had been cut by the appropriate restriction endonuclease. The ligation products were transferred to *E. coli* DH10B by electroporation, and the cells were cultured on lysogenic agar supplemented with ampicillin (100 μg/ml) and chloramphenicol (30 μg/ml). The cloned DNA fragment was then sequenced in full by using several sequencing primers (see Table S1 in the supplemental material).

Purification of CTX-M-14 and CTX-M-140. Purification of CTX-M-14 and CTX-M-140 was carried out as described previously (15), with the modification that the supernatant of the *E. coli* DH10B clone harboring PCR-generated $bla_{CTX\text{-}M\text{-}14}$ or $bla_{CTX\text{-}M\text{-}140}$ was subjected to ultrasonication and ultracentrifugation and then loaded onto a HiTrap SP HP column (GE Healthcare, Piscataway, NJ) and eluted with sodium chloride. The protein was concentrated and desalinated by ultrafiltration (Millipore, MA).

Kinetic measurements. The kinetic parameters of the purified CTX-M-14 and CTX-M-140 proteins were determined in 50 mM phosphate buffer (pH 7.0) by use of a UV-2550 spectrophotometer (Shimadzu Corp., Tokyo, Japan). The absorption maxima of the substrates used were as follows: ampicillin, 235 nm; cephalothin, 262 nm; ceftazidime, 274 nm; cefotaxime, 264 nm; cefepime, 275 nm; and aztreonam, 293 nm. The 50% inhibitory concentration (IC_{50}) was determined as the clavulanic acid concentration that reduced the hydrolysis rate of 100 μM ampicillin by 50% under conditions in which the enzyme was preincubated with various concentrations of the inhibitor for 5 min before addition of the substrate.

Site-directed mutagenesis. To further delineate the role of the amino acid at position 109, site-directed mutagenesis was performed to generate mutants of $bla_{CTX\text{-}M\text{-}14}$. PCR was performed with Phusion high-fidelity DNA polymerase (New England BioLabs, Ipswich, MA) by using mutagenic primers (Table 1) and the PCR clone carrying $bla_{CTX\text{-}M\text{-}14}$ as the template. Mutagenesis was performed using a QuikChange II site-directed mutagenesis kit (Agilent Technologies). Mutations in the recombinant plasmids were confirmed by DNA sequencing.

Genome sequencing and analysis. Libraries of *P. mirabilis* GB08 and *P. mirabilis* GB11 were constructed with 500-bp paired-end fragments. Using an Illumina HiSeq 2000 platform, 6,646,514 reads were produced for *P. mirabilis* GB08, and 11,931,104 reads for *P. mirabilis* GB11, with a read length of 125 bp. Reads were filtered to exclude those containing adaptor sequences, PCR duplicates, and those of low sequencing quality, in accordance with the criterion that over 50% of bases of the read have a Phred score of <20. The reads were assembled with SOAPdenovo (16).

Gene prediction was executed with Glimmer (17). The predicted open reading frames (ORFs) were annotated using the GO, KEGG, Swiss-Prot, NR, and COG databases.

Genome comparison. Assembly results were aligned with the reference sequences of strains HI4320 (AM942759) and BB2000 (CP004022) by using BLASTp and BLASTn. The criteria used to evaluate homology were an expectation value of <1e−5, an identity of >80%, and a homology length of >150 nucleotides (nt).

Identification of antibiotic resistance-related genes. After prediction of protein-encoding regions, the Antibiotic Resistance Genes Database (ARDB; http://ardb.cbcb.umd.edu), which contains 13,293 genes, 377 types, 257 antibiotics, 124 phyla, and 3,369 species, was used to detect possible resistance genes. We aligned the two isolates with sequences in the ARDB database by using BLASTp. Annotation results were filtered to have identities of >60% and E values of <1e−5.

Accession number(s). The data from this whole-genome shotgun project have been deposited at DDBJ/EMBL/GenBank under accession number LQNN00000000 for *P. mirabilis* GB08 and LQNO00000000 for *P. mirabilis* GB11. The versions described in this paper are LQNN01000000 and LQNO01000000, respectively.

RESULTS AND DISCUSSION

Antimicrobial susceptibility of clinical isolates. All four *P. mirabilis* isolates were resistant to ampicillin, piperacillin, ceftriaxone, cefotaxime, gentamicin, ciprofloxacin, tetracycline, nitrofurantoin, and trimethoprim-sulfamethoxazole but not to cefoxitin, ceftazidime, aztreonam, imipenem, meropenem, amikacin, ticarcillin-clavulanic acid, piperacillin-tazobactam, amoxicillin-clavulanic acid, and ampicillin-sulbactam. *P. mirabilis* GB03 and GB08 were also resistant to levofloxacin and tobramycin (Table 2).

PCR identification of β-lactamase genes. All four isolates were positive for a $bla_{\text{CTX-M-9-group}}$ gene. *P. mirabilis* GB03 and *P. mirabilis* GB08 carried $bla_{\text{CTX-M-14}}$, which is a variant of $bla_{\text{CTX-M-9}}$ with an alanine-to-valine substitution at position 231 in the deduced amino acid sequence according to the Ambler numbering scheme (18). *P. mirabilis* GB11 and GB12 carried $bla_{\text{CTX-M-140}}$, a novel variant of $bla_{\text{CTX-M-14}}$ with an alanine-to-threonine substitution at position 109. $bla_{\text{TEM-1}}$ was detected in all isolates, and bla_{ACC} was also carried by *P. mirabilis* GB11 and *P. mirabilis* GB12. The strains were negative for all other resistance genes tested by PCR.

PFGE. Isolate pairs *P. mirabilis* GB03 plus *P. mirabilis* GB08 and *P. mirabilis* GB11 plus *P. mirabilis* GB12 were clonally related as determined by PFGE, in accordance with the criteria of Tenover et al. (19) (see Fig. S1 in the supplemental material).

Location and genetic environment of $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-140}}$. Cephalosporin-resistant transconjugants were successfully obtained for *P. mirabilis* GB03 and *P. mirabilis* GB08, both including $bla_{\text{CTX-M-14}}$. They were positive for $bla_{\text{CTX-M-14}}$ as well as $bla_{\text{TEM-1}}$ and were typed as having IncFIC plasmids. However, $bla_{\text{CTX-M-140}}$ failed to transfer to *E. coli* by either plasmid conjugation or transformation of electrocompetent cells with extracted plasmids for *P. mirabilis* GB11 and *P. mirabilis* GB12. On the other hand, bla_{TEM}-carrying transconjugants were obtained for *P. mirabilis* GB11 and *P. mirabilis* GB12, indicating that bla_{TEM} was located on the plasmid and was not associated with $bla_{\text{CTX-M-140}}$, which was located on the chromosome in the two isolates. Results of I-CeuI-PFGE and Southern hybridization with $bla_{\text{CTX-M-9-group}}$ and 16S rRNA genes as probes confirmed that $bla_{\text{CTX-M-140}}$ was located on the chromosome in *P. mirabilis* GB11 and *P. mirabilis* GB12, which is the likely reason that transformation of $bla_{\text{CTX-M-140}}$ was unsuccessful (Fig. 1). Chromo-

TABLE 2 Antimicrobial MICs for CTX-M-14- and CTX-M-140-producing *P. mirabilis* strains and their clones and variants

Antimicrobial	MIC (μg/ml)												
	P. mirabilis strains				*E. coli* strains								
	GB03 (producing CTX-M-14)	GB08 (producing CTX-M-14)	GB11 (producing CTX-M-140)	GB12 (producing CTX-M-140)	J53 (pGB03)[a]	J53 (pGB08)[a]	DH10B (pCTX-M-14)[b]	DH10B (pCTX-M-140)[b]	DH10B (pCTX-M-14 A109E)[c,d]	DH10B (pCTX-M-14 A109D)[c,d]	DH10B (pCTX-M-14 A109K)[c,d]	DH10B (pCTX-M-14 A109R)[c,d]	J53	DH10B [pBC-SK(−)]
Ampicillin	>256	>256	>256	>256	>256	>256	>256	>256	32	32	32	32	8	8
Cefoxitin	4	4	4	4	8	8	8	8	8	8	8	8	8	8
Cefotaxime	32	16	8	4	128	128	128	4	0.063	0.063	0.063	0.063	0.125	0.063
Cefotaxime-clavulanic acid	<0.032	<0.032	<0.032	<0.032	0.063	0.063	0.063	<0.032	0.063	0.063	0.063	0.063	0.032	0.063
Ceftazidime	2	2	1	0.5	16	8	16	1	0.5	1	0.5	1	0.25	0.5
Ceftazidime-clavulanic acid	0.125	0.125	0.125	0.063	0.25	0.25	0.5	0.5	0.5	0.5	0.5	0.5	0.125	0.5
Ceftriaxone	64	32	4	4	128	64	128	32	0.125	0.125	0.125	0.125	0.125	0.063
Aztreonam	1	0.25	0.063	0.063	8	8	32	4	0.25	0.25	0.25	0.25	0.063	0.125
Cefepime	4	4	2	2	4	2	2	0.25	0.063	0.063	0.063	0.063	0.016	<0.032

[a] Transconjugant harboring a CTX-M-encoding plasmid from a clinical isolate.
[b] Transformant harboring a PCR-generated recombinant plasmid encoding CTX-M-14 or CTX-M-140.
[c] Transformant harboring a plasmid encoding a CTX-M-14 mutant construct.
[d] A, alanine; D, aspartic acid; E, glutamic acid; K, lysine; R, arginine.

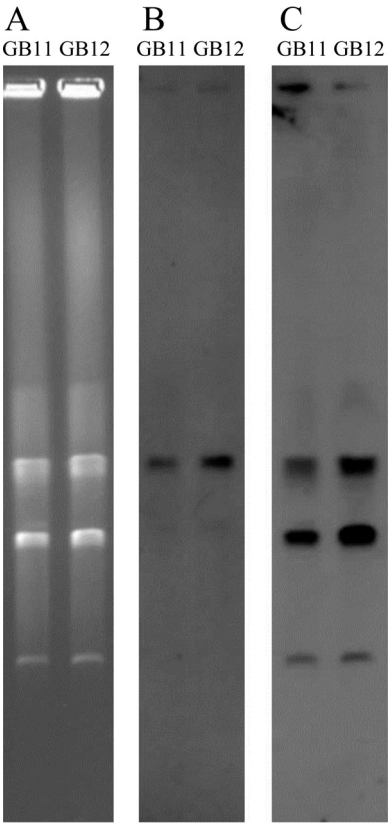

FIG 1 Chromosomal location of $bla_{CTX\text{-}M\text{-}140}$ in *P. mirabilis* GB11 and *P. mirabilis* GB12. (A) PFGE separation of I-CeuI-digested DNAs from *P. mirabilis* GB11 and *P. mirabilis* GB12. (B and C) Southern hybridization with a $bla_{CTX\text{-}M\text{-}9\text{-}group}$ gene probe (B) and a 16S rRNA gene probe (C).

somally located $bla_{CTX\text{-}M}$ genes, including $bla_{CTX\text{-}M\text{-}2}$, $bla_{CTX\text{-}M\text{-}12}$, $bla_{CTX\text{-}M\text{-}14}$, $bla_{CTX\text{-}M\text{-}15}$, $bla_{CTX\text{-}M\text{-}41}$, and $bla_{CTX\text{-}M\text{-}90}$, have been found in several countries (9, 11, 20).

Sequence analysis of the ~6.5-kb genetic environment surrounding $bla_{CTX\text{-}M\text{-}14}$ and $bla_{CTX\text{-}M\text{-}140}$ revealed 100% identity for the four isolates. IS*Ecp1* was located upstream of $bla_{CTX\text{-}M\text{-}14}$/$bla_{CTX\text{-}M\text{-}140}$. A 42-bp spacer was identified between the right inverted repeat (IRR) of IS*Ecp1* and $bla_{CTX\text{-}M\text{-}14}$/$bla_{CTX\text{-}M\text{-}140}$. IS*903D* and *iroN* were located downstream of the $bla_{CTX\text{-}M\text{-}14}$/$bla_{CTX\text{-}M\text{-}140}$ termination codon. The genetic structure was identical to the sequence found in various $bla_{CTX\text{-}M\text{-}9\text{-}group}$-carrying plasmids in members of the *Enterobacteriaceae*, including pKP96 from *Klebsiella pneumoniae* (accession no. EU195449), pETN48 from *E. coli* O102-ST405 (accession no. FQ482074), pHeBE7 from *E. coli* HeB7 (accession no. KT002541), and p112298-KPC from *Citrobacter freundii* (accession no. KP987215) (21, 22). These results indicate that this genetic structure has likely already spread in $bla_{CTX\text{-}M\text{-}9\text{-}group}$-harboring *Enterobacteriaceae*.

PCR cloning of $bla_{CTX\text{-}M\text{-}14}$ and $bla_{CTX\text{-}M\text{-}140}$. β-Lactam MICs for *E. coli* DH10B clones harboring $bla_{CTX\text{-}M\text{-}14}$ and $bla_{CTX\text{-}M\text{-}140}$ are provided in Table 2. Notably, compared with that for *E. coli* DH10B(pCTX-M-14), the MIC of cefotaxime for *E. coli* DH10B(pCTX-M-140) was 32-fold lower (MICs, 4 versus 128 μg/ml). In addition, 8- to 16-fold decreases were observed for the ceftazidime, aztreonam, and cefepime MICs for *E. coli* DH10B(pCTX-M-140) compared to *E. coli* DH10B(pCTX-M-14). Since CTX-M-14 and CTX-M-140 differed by only a single mutation, resulting in the Ala109Thr change, these phenotypic changes were likely due to this single substitution.

Purification and kinetic properties of CTX-M-14 and CTX-M-140. The purity of the CTX-M-14 and CTX-M-140 enzymes was estimated to be over 90% by SDS-PAGE analysis (data not shown). The molecular mass of both mature enzymes was calculated to be 31.0 kDa. The kinetic parameters of CTX-M-14 and CTX-M-140 are shown in Table 3. In general, the kinetic properties of CTX-M-14 and CTX-M-140 were consistent with the MICs for *E. coli* clones, and the kinetic properties of CTX-M-14 were similar to those in a previous report (23). Reduced hydrolytic activities against ampicillin, cephalothin, and cefotaxime were observed for CTX-M-140 compared to CTX-M-14. This was due to both a lower K_m value and a higher k_{cat} value. Hydrolysis of ceftazidime and cefepime was measurable for CTX-M-14 but not for CTX-M-140. The IC_{50}s of clavulanic acid for CTX-M-14 and CTX-M-140 were 0.072 μM and 0.051 μM, respectively. Our results suggested that the Ala109Thr substitution indeed altered the kinetic properties of CTX-M-140, which would account for the lower MICs of cephalosporins than those with its putative progenitor, CTX-M-14.

CTX-M-14 mutants with substitutions at position 109. Four mutants (A109E, A109D, A109K, and A109R mutants) were successfully constructed. The MICs of detected antimicrobials for these mutants were similar to those for the *E. coli* DH10B[pBC-SK(−)] host strain and consistently lower than those for *E. coli* DH10B(pCTX-M-14) (Table 2). These results combined with the MIC findings and kinetic properties described above indicate that the amino acid alanine at position 109 is critical for CTX-M-14 in

TABLE 3 Kinetic parameters of CTX-M-14 and CTX-M-140

Substrate	Value of parameter[a]							
	$bla_{CTX\text{-}M\text{-}14}$				$bla_{CTX\text{-}M\text{-}140}$			
	K_m (μM)	V_{max} (μM s^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})	K_m (μM)	V_{max} (μM s^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})
Ampicillin	39.08 ± 14.43	2.62 ± 0.24	197.05 ± 17.98	5.35 ± 1.27	19.19 ± 10.26	2.55 ± 0.04	23.62 ± 0.32	1.44 ± 0.59
Cephalothin	185.14 ± 13.74	1.19 ± 0.27	1,116.63 ± 253.83	6.02 ± 1.19	123.61 ± 14.12	1.23 ± 0.32	115.39 ± 31.96	0.93 ± 0.24
Cefotaxime	58.45 ± 15.22	0.41 ± 0.08	761.49 ± 148.55	13.54 ± 4.08	125.30 ± 21.07	0.18 ± 0.02	343.90 ± 29.23	2.77 ± 0.26
Ceftazidime	223.78 ± 20.04	0.05 ± 0.008	0.90 ± 0.15	0.004 ± 0.0006	ND	ND	ND	ND
Cefepime	145.97 ± 6.97	0.02 ± 0.001	12.95 ± 0.90	0.09 ± 0.002	ND	ND	ND	ND
Aztreonam	202.27 ± 40.08	0.12 ± 0.01	11.23 ± 1.28	0.06 ± 0.008	187.74 ± 21.28	0.35 ± 0.05	3.23 ± 0.52	0.02 ± 0.001

[a] K_m values are the means for three different measurements ± standard deviations. ND, not determinable.

conferring high hydrolytic activity against oxyimino-cephalosporins (see Fig. S2 in the supplemental material).

Clinical isolates possessing a variant β-lactamase with reduced hydrolytic activities for all tested substrates compared to those of its putative progenitor would be expected to be eliminated quickly by selection. In order to clarify the possible reason for the origin of the chromosomally located $bla_{CTX\text{-}M\text{-}140}$ gene, we detected $bla_{CTX\text{-}M\text{-}14\text{-}group}$ genes in members of the *Enterobacteriaceae* isolated from the hospital half a year before and half a year after the dates when the $bla_{CTX\text{-}M\text{-}140}$-carrying isolates *P. mirabilis* GB11 and *P. mirabilis* GB12 were collected. As a result, we found more plasmid-encoded $bla_{CTX\text{-}M\text{-}14}$-producing isolates but not $bla_{CTX\text{-}M\text{-}140}$-producing isolates. We therefore assume that the amino acid substitution from alanine to threonine at position 109 of CTX-M-14 occurred by chance, after the transfer of the $bla_{CTX\text{-}M\text{-}14}$ gene from the plasmid to the chromosome.

As of March 2016, 179 variants of CTX-M have been reported (http://www.lahey.org/Studies/). The alanine residue at position 109 is conserved among CTX-M enzymes, except in three cases in which it is replaced by threonine, namely, CTX-M-8 (accession no. AF189721), CTX-M-121 (CTX-M-9 group; accession no. AFA 51699), and CTX-M-140 (CTX-M-9 group) (see Table S2 in the supplemental material) (24; this study). CTX-M-8 belongs to the CTX-M-8 group, which is the rarest CTX-M subgroup, with only two variants reported: CTX-M-40 and CTX-M-63. Contrarily, both of these CTX-M-8 variants have a Thr109Ala substitution (24). The MIC results for the oxyimino-cephalosporins cephalothin, cefotaxime, and ceftriaxone for CTX-M-8, CTX-M-121 (Jian-Hua Liu, personal communication), and CTX-M-140, with a threonine at position 109 (MICs, 4 to 16 μg/ml), were lower than those for most other CTX-M enzymes, with an alanine at position 109 which provided a high level of resistance to the oxyimino-cephalosporins (25). Thus, it is possible that enzymes with the alternative residue at position 109 are unstable and do not commonly persist.

Genome sequencing and analysis. The sequence assembly showed that *P. mirabilis* GB08 has a genome size of 4,048,083 bp, with a GC content of 38.9%. The scaffold number was 47, and the number of contigs was 59. *P. mirabilis* GB11 has a genome size of 4,152,585 bp, with a 38.7% GC content. The scaffold number was 47, and the number of contigs was 92. The coverages for *P. mirabilis* GB08 and *P. mirabilis* GB11 were 192-fold and 327-fold, respectively. Gene prediction showed that 3,715 genes were detected in *P. mirabilis* GB08 and 3,815 genes were detected in *P. mirabilis* GB11.

Genome comparison. We chose strains HI4320 and BB2000 for our reference genomes, as these are the only two complete genomes of *P. mirabilis* that have been published. The genome of strain HI4320 was the first published genome of *P. mirabilis* with complete annotation of the chromosome and plasmid pHI4320 (accession no. AM942760), and it has been used widely in *P. mirabilis*-related studies. The genome of strain BB2000 was published with complete annotation in 2013. When scaffolds of *P. mirabilis* GB08 including $bla_{CTX\text{-}M\text{-}14}$ and *P. mirabilis* GB11 including $bla_{CTX\text{-}M\text{-}140}$ were aligned to the reference sequences, several genomic insertion and deletion segments were identified in the two isolates (see Fig. S3 in the supplemental material).

Features of antibiotic resistance-related genes. We identified 25 antibiotic resistance-related genes in *P. mirabilis* GB08 and 23 such genes in GB11 (see Fig. S4 in the supplemental material). Both *P. mirabilis* GB08 and *P. mirabilis* GB11 included bla_{TEM} and $bla_{CTX\text{-}M}$ β-lactamase genes, the aminoglycoside resistance genes *aac(3)-IIa*, *ant(3″)-Ia*, and *aph(3′)-Ia*, the tetracycline resistance genes *tet*(34) and *tet*(*J*), the chloramphenicol resistance gene *catA*, the trimethoprim resistance gene *dfrA*, the macrolide resistance gene *macB*, and putative multidrug resistance efflux pump genes, including *acrA* and *-B*, *emrD*, *mdtG*, *-H*, and *-K*, *mexB* and *-I*, and *tolC*. In addition, we detected another aminoglycoside resistance gene, *ant(2″)-Ia*, and the chloramphenicol efflux pump gene *cml* in *P. mirabilis* GB08.

Conclusions. We report here the unique properties of CTX-M-140, a novel CTX-M-14 variant which was found in *P. mirabilis* and had lost the ESBL properties of CTX-M-14 due to an Ala109Thr substitution. The findings indicate that alanine at position 109 is critical for the hydrolytic activity of CTX-M-14 against oxyimino-cephalosporins.

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REFERENCES

1. **Aragon LM, Mirelis B, Miro E, Mata C, Gomez L, Rivera A, Coll P, Navarro F.** 2008. Increase in beta-lactam-resistant *Proteus mirabilis* strains due to CTX-M- and CMY-type as well as new VEB- and inhibitor-resistant TEM-type beta-lactamases. J Antimicrob Chemother **61:**1029–1032. http://dx.doi.org/10.1093/jac/dkn056.
2. **Chanal C, Bonnet R, De Champs C, Sirot D, Labia R, Sirot J.** 2000. Prevalence of beta-lactamases among 1,072 clinical strains of *Proteus mirabilis*: a 2-year survey in a French hospital. Antimicrob Agents Chemother **44:**1930–1935. http://dx.doi.org/10.1128/AAC.44.7.1930-1935.2000.
3. **Park YJ, Lee S, Kim YR, Oh EJ, Woo GJ, Lee K.** 2006. Occurrence of extended-spectrum (beta)-lactamases and plasmid-mediated AmpC (beta)-lactamases among Korean isolates of *Proteus mirabilis*. J Antimicrob Chemother **57:**156–158.
4. **D'Andrea MM, Nucleo E, Luzzaro F, Giani T, Migliavacca R, Vailati F, Kroumova V, Pagani L, Rossolini GM.** 2006. CMY-16, a novel acquired AmpC-type beta-lactamase of the CMY/LAT lineage in multifocal monophyletic isolates of *Proteus mirabilis* from northern Italy. Antimicrob Agents Chemother **50:**618–624. http://dx.doi.org/10.1128/AAC.50.2.618-624.2006.
5. **Kim JY, Park YJ, Kim SI, Kang MW, Lee SO, Lee KY.** 2004. Nosocomial

outbreak by *Proteus mirabilis* producing extended-spectrum beta-lactamase VEB-1 in a Korean university hospital. J Antimicrob Chemother **54:**1144–1147. http://dx.doi.org/10.1093/jac/dkh486.

6. **Karapavlidou P, Sofianou D, Manolis EN, Pournaras S, Tsakris A.** 2005. CTX-M-1 extended-spectrum beta-lactamase-producing *Proteus mirabilis* in Greece. Microb Drug Resist **11:**351–354. http://dx.doi.org/10.1089/mdr.2005.11.351.
7. **Miro E, Mirelis B, Navarro F, Rivera A, Mesa RJ, Roig MC, Gomez L, Coll P.** 2005. Surveillance of extended-spectrum beta-lactamases from clinical samples and faecal carriers in Barcelona, Spain. J Antimicrob Chemother **56:**1152–1155. http://dx.doi.org/10.1093/jac/dki395.
8. **Navarro F, Perez-Trallero E, Marimon JM, Aliaga R, Gomariz M, Mirelis B.** 2001. CMY-2-producing *Salmonella enterica*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Proteus mirabilis* and *Escherichia coli* strains isolated in Spain (October 1999-December 2000). J Antimicrob Chemother **48:**383–389. http://dx.doi.org/10.1093/jac/48.3.383.
9. **Song W, Kim J, Bae IK, Jeong SH, Seo YH, Shin JH, Jang SJ, Uh Y, Lee MK, Lee K.** 2011. Chromosome-encoded AmpC and CTX-M extended-spectrum beta-lactamases in clinical isolates of *Proteus mirabilis* from Korea. Antimicrob Agents Chemother **55:**1414–1419. http://dx.doi.org/10.1128/AAC.01835-09.
10. **Huang CW, Chien JH, Peng RY, Tsai DJ, Li MH, Lee HM, Lin CF, Lee MC, Liao CT.** 2015. Molecular epidemiology of CTX-M-type extended-spectrum beta-lactamase-producing *Proteus mirabilis* isolates in Taiwan. Int J Antimicrob Agents **45:**84–85. http://dx.doi.org/10.1016/j.ijantimicag.2014.09.004.
11. **Navon-Venezia S, Chmelnitsky I, Leavitt A, Carmeli Y.** 2008. Dissemination of the CTX-M-25 family beta-lactamases among *Klebsiella pneumoniae*, *Escherichia coli* and *Enterobacter cloacae* and identification of the novel enzyme CTX-M-41 in *Proteus mirabilis* in Israel. J Antimicrob Chemother **62:**289–295. http://dx.doi.org/10.1093/jac/dkn182.
12. **Clinical and Laboratory Standards Institute.** 2010. Performance standards for antimicrobial susceptibility testing; 20th informational supplement. CLSI, Wayne, PA.
13. **Tian GB, Huang YM, Fang ZL, Qing Y, Zhang XF, Huang X.** 2014. CTX-M-137, a hybrid of CTX-M-14-like and CTX-M-15-like beta-lactamases identified in an *Escherichia coli* clinical isolate. J Antimicrob Chemother **69:**2081–2085. http://dx.doi.org/10.1093/jac/dku126.
14. **Carattoli A, Bertini A, Villa L, Falbo V, Hopkins KL, Threlfall EJ.** 2005. Identification of plasmids by PCR-based replicon typing. J Microbiol Methods **63:**219–228. http://dx.doi.org/10.1016/j.mimet.2005.03.018.
15. **Tian GB, Adams-Haduch JM, Bogdanovich T, Wang HN, Doi Y.** 2011. PME-1, an extended-spectrum beta-lactamase identified in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother **55:**2710–2713. http://dx.doi.org/10.1128/AAC.01660-10.
16. **Luo R, Liu B, Xie Y, Li Z, Huang W, Yuan J, He G, Chen Y, Pan Q, Liu Y, Tang J, Wu G, Zhang H, Shi Y, Yu C, Wang B, Lu Y, Han C, Cheung DW, Yiu SM, Peng S, Xiaoqian Z, Liu G, Liao X, Li Y, Yang H, Wang J, Lam TW.** 2012. SOAPdenovo2: an empirically improved memory-efficient short-read de novo assembler. Giga Sci **1:**18. http://dx.doi.org/10.1186/2047-217X-1-18.
17. **Delcher AL, Bratke KA, Powers EC, Salzberg SL.** 2007. Identifying bacterial genes and endosymbiont DNA with Glimmer. Bioinformatics **23:**673–679. http://dx.doi.org/10.1093/bioinformatics/btm009.
18. **Ambler RP, Coulson AF, Frere JM, Ghuysen JM, Joris B, Forsman M, Levesque RC, Tiraby G, Waley SG.** 1991. A standard numbering scheme for the class A beta-lactamases. Biochem J **276:**269–270. http://dx.doi.org/10.1042/bj2760269.
19. **Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, Swaminathan B.** 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol **33:**2233–2239.
20. **Harada S, Ishii Y, Saga T, Kouyama Y, Tateda K, Yamaguchi K.** 2012. Chromosomal integration and location on IncT plasmids of the $bla_{CTX-M-2}$ gene in *Proteus mirabilis* clinical isolates. Antimicrob Agents Chemother **56:**1093–1096. http://dx.doi.org/10.1128/AAC.00258-11.
21. **Shen P, Jiang Y, Zhou Z, Zhang J, Yu Y, Li L.** 2008. Complete nucleotide sequence of pKP96, a 67 850 bp multiresistance plasmid encoding qnrA1, aac(6′)-Ib-cr and $bla_{CTX-M-24}$ from *Klebsiella pneumoniae*. J Antimicrob Chemother **62:**1252–1256. http://dx.doi.org/10.1093/jac/dkn397.
22. **Feng J, Qiu Y, Yin Z, Chen W, Yang H, Yang W, Wang J, Gao Y, Zhou D.** 2015. Coexistence of a novel KPC-2-encoding MDR plasmid and an NDM-1-encoding pNDM-HN380-like plasmid in a clinical isolate of *Citrobacter freundii*. J Antimicrob Chemother **70:**2987–2991. http://dx.doi.org/10.1093/jac/dkv232.
23. **Dutour C, Bonnet R, Marchandin H, Boyer M, Chanal C, Sirot D, Sirot J.** 2002. CTX-M-1, CTX-M-3, and CTX-M-14 beta-lactamases from *Enterobacteriaceae* isolated in France. Antimicrob Agents Chemother **46:**534–537. http://dx.doi.org/10.1128/AAC.46.2.534-537.2002.
24. **Zhao WH, Hu ZQ.** 2013. Epidemiology and genetics of CTX-M extended-spectrum beta-lactamases in Gram-negative bacteria. Crit Rev Microbiol **39:**79–101. http://dx.doi.org/10.3109/1040841X.2012.691460.
25. **Bonnet R, Sampaio JL, Labia R, De Champs C, Sirot D, Chanal C, Sirot J.** 2000. A novel CTX-M beta-lactamase (CTX-M-8) in cefotaxime-resistant *Enterobacteriaceae* isolated in Brazil. Antimicrob Agents Chemother **44:**1936–1942. http://dx.doi.org/10.1128/AAC.44.7.1936-1942.2000.