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Short Communication

Characterization of CTX-M-22 and TEM-141 Encoded by a Single Plasmid from a Clinical Isolate of *Enterobacter cloacae* in China

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SUMMARY: We analyzed the resistance to expanded-spectrum cephalosporins of an *Enterobacter cloacae* clinical isolate, EC002, by transconjugation, isoelectric-focusing analysis, and cloning experiments. It produced two β-lactamases with isoelectric point values of 5.4 and 8.7, corresponding to TEM-141, a novel variant of TEM-1, and CTX-M-22, encoded by a transferable plasmid.

Resistance to expanded-spectrum cephalosporins commonly develops in *Enterobacter cloacae* during therapy due to the selection of mutants producing high levels of chromosomal AmpC (1,2). However, a high prevalence of plasmid-encoded expanded spectrum β-lactamase (ESBL) producers, in particular an unprecedented, rapid increase in the recognition of *E. cloacae* clinical isolates containing CTX-M-type β-lactamases, has been observed in China (3-7), which has been increasingly concerned about therapy for clinical infections. In an attempt to assess β-lactam resistance in *E. cloacae* isolated from our major teaching hospital in the northern region of Sichuan Province, China, we have initiated a characterization of the molecular mechanisms responsible for resistance to ESBL (5). Here we report the characterization of TEM-141, a novel variant of TEM-1, and CTX-M-22, encoded by a transferable plasmid from an *E. cloacae* clinical isolate EC002.

E. cloacae isolate EC002 was isolated from a sample obtained at the First Teaching Hospital of North Sichuan Medical College; the specimen was taken from the urine of an infected patient who was previously treated unsuccessfully with cefotaxime for 2 weeks in September 2004. The β-lactam resistance of strain EC002 was successfully transferred to strains of *Escherichia coli* JM109, by transconjugation (8). The analysis of the plasmid content of EC002 and its transconjugant revealed a plasmid, which was designated as pEC002. The clinical isolate EC002 and the transconjugants were resistant to all of the penicillins, as well as most of the cephalosporins tested (Table 1), which clearly demonstrated that pEC002 contributed to a β-lactam resistance phenotype similar to that observed in *E. cloacae* EC002. The pEC002 contained two *bla* genes, as determined by polymerase chain reaction (PCR) with previously described primers (5). Sequence analysis revealed that one of the nucleotide sequences (GenBank accession no. AY954529) was identical to that of the reported *Klebsiella pneumoniae* CTX-M-22 (AY080894), and the other was a novel TEM-type penicillinase, TEM-141 (AY956335). Compared to the $bla_{\text{CTX-M-1}}$ gene (X92506), the *bla* gene of CTX-M-22 had nine points nucleotide substitutions, while only three of these changes resulted in residue alteration (i.e., Val 80Ala, Asp 117Asn, and Ser143Ala). In comparison with TEM-1 (AY302260), the bla_{TEM} gene of pEC002 had three nucleotide substitutions, i.e., A → G at position 94, C → T at position 228, and G → T at position 396, yielding genetic code changes from AAA to GAA, GGC to GGT, and GCG to GCT, respectively. The first change led to a lycine-to-glutamic acid substitution at residue position 34 (on the basis of the standard β-lactamase numbering, as previously suggested [9]); the others were silent and resulted in no residue alteration. This pEC002-encoded TEM-type β-lactamase was submitted to the β-lactamase database at the Lahey Clinics (http://www.lahey.org/Studies), and was assigned as TEM-141, which has not been previously reported.

In order to determine the phenotype and activity of the pEC002-encoded β-lactamase, the PCR products for TEM-1 (a laboratory collection), TEM-141, and CTX-M-22 were cloned into vector pBK-cmv (kanamycin resistant; Stratagene, La Jolla, Calif., USA) after appropriate restriction digestion, yielding plasmids pBK-TEM-1, pBK-TEM-141, and pBK-CTX-M-22. All of the cloning and the *bla* genes were confirmed by nucleotide sequencing. Each of these three plasmids was used to transform *E. coli* JM109, and the transformants were selected with kanamycin and ampicillin (50 μg/ml each). EC002 and its transconjugants expressed two β-lactamases with isoelectric point (pI) values of 5.4 and 8.7 by isoelectric focusing (IEF) (10), corresponding to TEM-141 and CTX-M-22 for EC002 and its transconjugants. *E. coli* JM109 harboring recombinant plasmids pBK-TEM-1, pBK-TEM-141, and pBK-CTX-M-22 also expressed one β-lactamase with pIs of 5.4, 5.4, and 8.7, respectively. Both TEM-1 and TEM-141 showed similar substrate profiles that did not include oxyimino-cephalosporins (Table 2). Consistent with the MIC results (Table 1), CTX-M-22 hydrolyzed cefotaxime more efficiently than ceftazidime (Table 2).

Plasmid-mediated ESBLs are becoming increasingly frequent among clinical isolates of the family *Enterobacteriaceae* worldwide (11-15). Among the ESBLs, the CTX-M-type β-lactamases constitute a rapidly growing cluster of enzymes that have disseminated geographically, and these enzymes have been found predominantly in *Enterobacteriaceae* (11,12). In China, the emerging family of the CTX-M-type β-lactamases was the most frequently observed of the ESBLs, accounting for 30-40% ESBLs examined in *E. cloacae* isolates (5-7); recently, the coexistence of CTX-M-encoding genes with other *bla* genes on the same plasmids was emphasized (5,11). The strains harboring such plasmids might exhibt a high MIC

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Table 1. β-lactams susceptibility of *Enterobacter cloacae* and *Escherichia coli* strains expressing TEM-1, TEM-141, and CTX-M-22

β-lactams	MIC (μg/mL) for					
	E. cloacae EC002	*E. coli* JM109 (pEC002)	*E. coli* JM109 (pBK-TEM-1)	*E. coli* JM109 (pBK-TEM-141)	*E. coli* JM109 (pBK-CTX-M-22)	*E. coli* JM109 (pBK-cmv)
Ampicillin	> 256	> 256	> 256	> 256	> 256	1
Piperacillin	> 256	> 256	128	128	128	<0.25
Cefazolin	> 256	> 256	16	16	128	<0.25
Cefamandole	> 256	> 256	4	8	128	<0.25
Cefoperazone	> 256	128	0.25	0.5	64	<0.25
Cefoperazone-clavulanic acid[1)]	32	2	<0.25	<0.25	0.25	<0.25
Cefoperazone-tazobactam[1)]	32	2	<0.25	<0.25	0.25	<0.25
Cefoperazone-sulbactam (2:1)	32	4	<0.25	<0.25	0.5	<0.25
Cefotaxime	> 256	128	<0.25	<0.25	64	<0.25
Ceftazidime	32	2	<0.25	<0.25	2	<0.25
Cefepime	2	<0.25	<0.25	<0.25	<0.25	<0.25
Aztreonam	64	32	<0.25	<0.25	4	<0.25
Imipenem	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25

[1)]: Clavulanic acid and tazobactam were used at a fixed concentration of 2 and 4 μg/ml, respectively.

Table 2. Relative rates of hydrolysis of β-lactams by TEM-1, TEM-141, and CTX-M-22 β-lactamases[1)]

Substrate	*E. coli* JM109 (pBK-TEM-1)	*E. coli* JM109 (pBK-TEM-141)	*E. coli* JM109 (pBK-CTX-M-22)
Cephalothin	100	100	100
Cefazolin	37.96	49.56	70.29
Cefuroxime	2.86	8.62	61.23
Cefoperazone	11.57	16.74	24.99
Cefotaxime	ND	ND	24.84
Ceftazidime	ND	ND	2.10
Imipenem	ND	ND	ND

[1)]: Rates of hydrolysis of β-lactams were expressed relatively to that of cephalothin, which was set at 100. The hydrolysis rates of cephalothin by TEM-1, TEM-141, and CTX-M-22 were 938, 1,083, and 1,685 nmol/min/mg, respectively.
ND, not determined.

value against β-lactams, as *E. coli* JM109 (pBK-CTX-M-22) was found to have lower MIC values for piperacillin, cefazolin, cefamandole, cefoperazone/inhibitors, and aztreonam, as compared to those of *E. coli* JM109 (pEC002).

We demonstrated that TEM-141 is a novel TEM-1 variant with a conservative amino-acid substitution far from the catalytic site, and with little hydrolytic activity against oxyimino cephalosporins as well as TEM-1. The Lys34Glu in TEM-141 neither markedly altered the substrate profiles nor apparently rendered TEM-141 more active than TEM-1 against certain substrates. Even though the $bla_{CTX\text{-}M\text{-}22}$ gene was deposited in Genbank in 2002 (AY080894), and *E. coli* and *K. pneumoniae* strains producing the $bla_{CTX\text{-}M\text{-}22}$ gene were reported from China (12), this is the first report describing the enzymatic characterization of CTX-M-22 and the transferability of the plasmid encoding the $bla_{CTX\text{-}M\text{-}22}$ gene, which suggests the possibility of dissemination of this particular ESBL gene. CTX-M-22, a variant of CTX-M-1 (11), typically hydrolyzed cefotaxime more efficiently than ceftazidime. However, it was of interest that CTX-M-22 was able to hydrolyze ceftazidime, which suggests that the substitutions in CTX-M-22 (i.e., 80Ala near the conservative sequence 70STSK; 117Asn and 143Ala bilaterally and symmetrically laying aboard to the conservative sequence of 130SDN) might enhance the substrate profile against ceftazidime. Fortunately, imipenem was a poor substrate for CTX-M-22, as expected for the CTX-M-derived ESBLs. However, it should be noted that the biochemical characteristics of CTX-M-22 were not confirmed using a crude extract from *E. coli* JM109. As previous studies have confirmed, the hydrolytic activity against oxyimino cephalosporins of the CTX-M-type-ESBLs was related to the Ω-loop and certain mutations such as 167Ser or 240Asp (11); however, further study is still needed to determine how and why the substitutions in CTX-M-22 influence its hydrolytic activity against oxyimino cephalosporins.

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