

## CTX-M-14, a Plasmid-Mediated CTX-M Type Extended-Spectrum $\beta$ -Lactamase Isolated from *Escherichia coli*

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**Four *Escherichia coli* isolates harboring CTX-M-14, with a single Ala231→Val substitution compared to CTX-M-9, had three different ribotypes. Cefotaxime resistance was plasmid encoded and conjugatively transferable. Three isolates had the same plasmid restriction enzyme digestion profile, suggesting clonal spread of a resistant plasmid. A high  $k_{cat}/K_m$  value for cefotaxime ( $20.3 \mu\text{M}^{-1} \text{s}^{-1}$ ) but low values for ceftazidime and aztreonam ( $<0.02 \mu\text{M}^{-1} \text{s}^{-1}$ ) were observed in hydrolysis assays, indicating resistance to cefotaxime (MIC  $\geq 64 \mu\text{g/ml}$ ) but susceptibility to ceftazidime (MIC  $\leq 2 \mu\text{g/ml}$ ).**

CTX-M type  $\beta$ -lactamases constitute a novel group of class A plasmid-encoded enzymes, and their carriers are highly resistant to cefotaxime but sometimes susceptible to ceftazidime. This family of enzymes is well inhibited by clavulanate and tazobactam (24). Recently, CTX-M type  $\beta$ -lactamases have been described in various members of the *Enterobacteriaceae* family, mostly in *Salmonella enterica* serovar Typhimurium and *Escherichia coli*; these include MEN-1, CTX-M-1 to CTX-M-12, Toho-1, and Toho-2. CTX-M type  $\beta$ -lactamases are endemic in Latin America and some northeastern European countries (24). Toho type  $\beta$ -lactamases have been found only in Japan (15, 19, 25). In Taiwan, CTX-M-3 has been previously described in *E. coli* (26).

According to GenBank, the CTX-M-14 sequence was submitted from China in 2000 (A. Chanawong, F. H. M'Zali, J. Heritage, J.-H. Xiong, and P. M. Hawkey, accession no. AF252622). However, the characteristics of this enzyme have not been described. In this study, we characterize this new CTX-M type  $\beta$ -lactamase obtained from *E. coli* in Taiwan. Four isolates were isolated from patients in three different hospitals in 1998 during an island-wide survey of antibiotic resistance (14).

To study the epidemiology, ribotyping, plasmid isolation, resistance transferal, and restriction enzyme digestion profiles of plasmids were performed. Ribotyping was performed with an automated Riboprinter microbial characterization system (Qualicon, Wilmington, Del.) according to the manufacturer's instructions. The results of ribotyping were analyzed as previously described (8). Plasmid isolation and plasmid profile analysis were performed by the alkaline extraction method (16). Resistance transferal was carried out by conjugation (23). For restriction enzyme digestion profiles of plasmids, plasmid DNA from the transconjugant was prepared as described previously (2). The restriction enzymes *EcoRI* and *HincII* (Gibco BRL, Gaithersburg, Md.) were used.

Among the four isolates, three different ribotypes were identified (Fig. 1). Two isolates with the same ribotype were obtained from two different hospitals and were geographically unrelated. The cefotaxime resistance of all isolates was found to be conjugatively transferable. Only one plasmid was transferred for each strain, and the resistant gene in each transconjugant was found to be located in a plasmid of  $>90$  kb (data not shown). Plasmid typing of the transconjugants revealed that three isolates, including the two isolates with the same ribotype, had the same restriction enzyme digestion profile (Fig. 2).

The CTX-M-14 resistance gene was amplified by PCR with the primers CTX-F (5'-AAAAATGATTGAAAGGTGGTTG T-3') and CTX-R (5'-TTACAGCCCTTCGGCGATGA-3') and cloned into a vector (PCR-ScriptCamSK) according to the instructions for a PCR-ScriptCam cloning kit (Stratagene, La Jolla, Calif.). Our sequence data indicated an open reading frame of 876 bp, corresponding to 291 amino acids. A comparison of the nucleotide and deduced amino acid sequences of the CTX-M-14  $\beta$ -lactamase with known  $\beta$ -lactamase sequences revealed the consensus sequences STSK, SDN, and KTG, which are conserved motifs characteristic of class A  $\beta$ -lactamases. Amino acid alignments with other *bla*<sub>CTX</sub> types revealed 99, 87, and 87% similarity with CTX-M-9, Toho-2, and CTX-M-1, respectively. CTX-M-9 and CTX-M-14  $\beta$ -lactamases differ by a single amino acid at position 231 (Ala→Val). For other CTX-M type extended-spectrum  $\beta$ -lactamases (ESBLs), homology of not more than 85% was observed. The amino acid relationships of CTX-M type proteins can be found at the following website: <http://www.lahey.org/studies/webt.htm>.

Antimicrobial susceptibility was determined by a broth microdilution test (TREK Diagnostic Systems Ltd., West Sussex, United Kingdom) in serial twofold concentrations from 0.025 to 64  $\mu\text{g/ml}$ , and the results were interpreted according to the method of the NCCLS (21). The CTX-M-14 carriers, transconjugants, and cloned strains were found to be resistant to ampicillin, cephalothin, and cefotaxime. They were susceptible to ceftazidime, aztreonam, ciprofloxacin, amikacin, and imipenem. When clavulanic acid at a fixed concentration of 4

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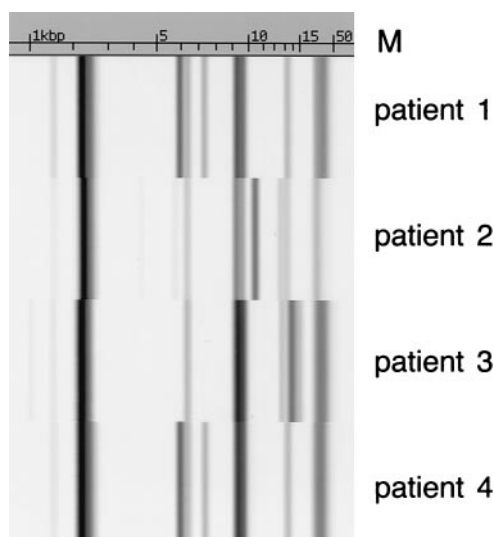


FIG. 1. Ribotyping of CTX-M-14 carriers isolated from four different patients. The scale at the top is measured in kilobase pairs.

$\mu\text{g/ml}$  was combined with cefotaxime, ceftriaxone, ceftazidime, or cefpodoxime, >4-fold reductions in the MIC were observed, a characteristic of ESBL. The CTX-M-14 cloned strain was also susceptible to ceftazidime (Table 1).

Isoelectric focusing (IEF) was performed in ampholine gel (pH 3.0 to 10.0; Pharmacia). Preparations from standard strains known to harbor CTX-M-3, SHV-1, and SHV-5 were used as standards (20). IEF revealed that CTX-M-14 had a pI

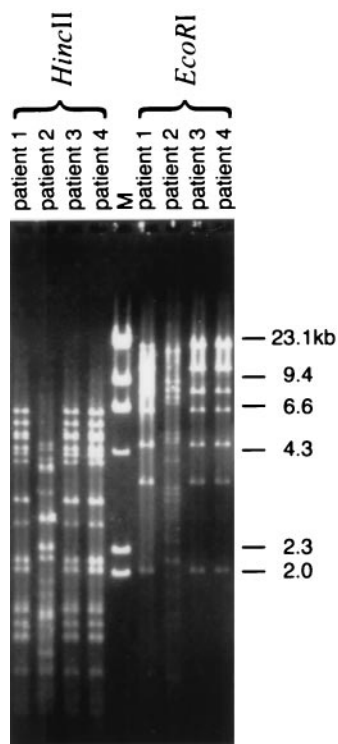


FIG. 2. *HincII*- or *EcoRI*-digested plasmid profiles of transconjugants of CTX-M-14 carriers isolated from four different patients.

TABLE 1. MICs of various antibiotics for strains producing CTX-M-14  $\beta$ -lactamases

Antibiotic <sup>a</sup>	MIC ( $\mu\text{g/ml}$ ) against:			
	Clinical isolates (KTC984167)	Trans-conjugant	Cloned strains	JP-995
AMP	$\geq 32$	$\geq 32$	$\geq 32$	2
LOT	$\geq 32$	$\geq 32$	$\geq 32$	4
CFX	8	2	8	4
CTX	$\geq 64$	$\geq 64$	$\geq 64$	$\leq 0.25$
CTX plus CAL	$\leq 0.25$	$\leq 0.25$	$\leq 0.25$	$\leq 0.25$
CTR	$\geq 64$	$\geq 64$	$\geq 64$	$\leq 0.25$
CTR plus CAL	$\leq 0.5$	$\leq 0.5$	$\leq 0.5$	$\leq 0.5$
CPD	$\geq 64$	$\geq 64$	$\geq 64$	$\leq 0.5$
CPD plus CAL	$\leq 0.5$	$\leq 0.5$	4	$\leq 0.5$
CAZ	2	1	16	$\leq 0.25$
CAZ plus CAL	$\leq 0.25$	$\leq 0.25$	0.5	$\leq 0.25$
ATM	8	4	$\geq 64$	$\leq 0.25$
IMP	$\leq 0.25$	0.5	0.5	0.5
GEN	>8	$\leq 0.5$	$\leq 0.5$	$\leq 0.5$
AMK	4	$\leq 2$	$\leq 2$	$\leq 2$
CIP	$\leq 0.06$	$\leq 0.06$	0.25	$\leq 0.06$

<sup>a</sup> Abbreviations: AMP, ampicillin; LOT, cephalothin; CFX, ceftazidime; CTX, cefotaxime; CAL, clavulanic acid; CTR, ceftriaxone; CPD, cefpodoxime; CAZ, ceftazidime; ATM, aztreonam; IMP, imipenem; GEN, gentamicin; AMK, amikacin; CIP, ciprofloxacin.

of 8.0, which was similar to those of CTX-M-9 and CTX-M-2 but different from those of other CTX-M type  $\beta$ -lactamases.

The  $\beta$ -lactamase from each cloned bacterial strain was purified as described previously (19). The purity of enzyme was >95%. The rate of hydrolysis of antibiotics by CTX-M-14 was determined by monitoring the variation in the absorbance of the  $\beta$ -lactam in 50 mM phosphate buffer (pH 7.0). The peak wavelength for each antibiotic used for the measurement was set according to those described in previous reports (15). The steady-state kinetic parameters ( $K_m$  or  $K_i$  and  $k_{cat}$ ) were determined by analyzing the complete hydrolysis time courses as described by De Meester et al. (9) and Galleni et al. (10). Lower values of  $K_m$  were determined as  $K_i$  with the help of a substrate reporter.  $K_m$  and  $k_{cat}$  values were obtained at different substrate concentrations ranging from 10 to 100  $\mu\text{M}$ . Kinetic parameters for poor substrates ( $k_2$ ,  $k_3$ ,  $K$ , or  $k_2/K$ ) were determined by using nitrocefin as a reporter substrate (10).

The  $k_{cat}$  and  $K_m$  values were determined for a representative set of  $\beta$ -lactam antibiotics (Table 2). The results showed that the  $\beta$ -lactamase exhibited a broad-spectrum activity profile, although with notable differences for different substrates. Penicillin G, cephalothin, cephaloridine, cefotaxime, and nitroce-

TABLE 2. Kinetic parameters ( $k_{cat}$  and  $K_m$ ) and physiologic efficiency ( $k_{cat}/K_m$ ) of CTX-M-14 enzyme against various  $\beta$ -lactam antibiotics

Antibiotic	$k_{cat}$ ( $\text{s}^{-1}$ )	$K_m$ ( $\mu\text{M}$ )	$k_{cat}/K_m$	Relative $k_{cat}/K_m$
Penicillin G	$1,200 \pm 60$	$52 \pm 4.3$	22.9	100
Cephalothin	$6,900 \pm 320$	$98 \pm 8.0$	70.5	308
Cephaloridine	$6,500 \pm 430$	$168 \pm 4.0$	38.7	169
Cefotaxime	$1,100 \pm 40$	$54 \pm 3.2$	20.3	87
Ceftazidime	<0.01	$440 \pm 26$	<0.01	<0.04
Aztreonam	<0.01	$0.48 \pm 0.06$	<0.02	<0.04
Nitrocefin	$150 \pm 1.9$	$1.12 \pm 0.02$	133	581

fin were good substrates for CTX-M-14, with  $k_{\text{cat}}$  values ranging from 150 to 6,900  $\text{s}^{-1}$ . The catalytic efficiency of CTX-M-14 against these drugs was greater than 20  $\mu\text{M}^{-1} \text{s}^{-1}$ . On the other hand, ceftazidime and aztreonam were poorly hydrolyzed ( $k_{\text{cat}}/K_m \leq 0.02 \mu\text{M}^{-1} \text{s}^{-1}$ ). For the reporter substrate of nitrocefin,  $K_m$  and  $k_{\text{cat}}$  were 1.12  $\mu\text{M}$  and 150  $\text{s}^{-1}$ , respectively (Table 2). Cefoxitin and imipenem were also poor substrates for CTX-M-14. The  $k_2/K$  values of cefoxitin and imipenem were 0.059 and 0.072  $\mu\text{M}^{-1} \text{s}^{-1}$ , respectively. However, the  $k_3$  values of the deacylation constant for these two substrates were 0.01 and 0.0007  $\text{s}^{-1}$ , respectively.

The CTX-M type  $\beta$ -lactamase is considered an enzyme with a potent hydrolytic activity against cefotaxime. Substitution of Ser-237 (1), which is known to enhance hydrolysis of cefotaxime, is observed in CTX-M-14 (3). Comparison with other CTX-M type enzymes revealed 99 and 87% similarity with CTX-M-9 and Toho-2, respectively, and less similarity with other CTX-M type  $\beta$ -lactamases. The amino acid substitutions at positions 104, 164, 179, 238, and 240 (1), which are associated with expansion of the spectrum of activity towards oximino-cephalosporins and aztreonam in TEM and SHV type ESBLs (18), were not observed in CTX-M-14, for which the MICs of aztreonam and ceftazidime were low. IEF revealed a pI value similar to those of CTX-M-9 and CTX-M-2. Substrate profiles showed that cefotaxime is a good substrate with a high  $k_{\text{cat}}/K_m$  value, such that hydrolysis by CTX-M-14 resulted in resistance of the organism to cefotaxime. The reverse was the case with ceftazidime.

Cefoxitin and imipenem were poorly hydrolyzed by CTX-M-14. In the case of cefoxitin, we determined the  $K$ ,  $k_2$ ,  $k_3$ , and  $k_2/K$  values. These values explained why these were poor substrates for CTX-M-14. The  $k_2$  value, a constant for the acylation speed from the Michaelis-Menten complex to the acyl intermediate, was four times greater than  $k_3$ , a constant for the deacylation speed. Accordingly, the acyl enzyme accumulated in the reaction mixture. The  $k_2/K$  and  $k_3$  values of imipenem were close to those of cefoxitin. These values also indicated that the deacylation speed of imipenem was slower than the acylation speed.

Bacterial strains harboring CTX-M type  $\beta$ -lactamases have been identified for over 10 years as isolated incidents or outbreaks in various geographic regions. Since the first reports of CTX-M-1/MEN-1 in 1989 in Germany and France (3, 4), 13 CTX-M type  $\beta$ -lactamases have been reported: CTX-M-2 in Argentina (5), CTX-M-3 in Poland (13) and Taiwan (26), CTX-M-4 in Russia (12), CTX-M-5 in Latvia (7), CTX-M-6 in Greece (11), CTX-M-8 in Brazil (6), CTX-M-9 in Spain (22), CTX-M-12 in Kenya (17), and Toho-1 and Toho-2 in Japan (15, 19). In 2000, one report of CTX-M-3 was documented in southern Taiwan (26). In our laboratory, apart from CTX-M-3, CTX-M-14 is the most frequent CTX-M type ESBL detected in *E. coli* (unpublished data), and it has also been isolated in central and northern Taiwan. In the present study, the CTX-M-14 in all isolates was encoded on a transferable plasmid of >90 kb. Molecular typing revealed that two isolates had the same ribotype and that three had the same plasmid type, suggesting clonal and plasmid spread. To our knowledge, this is the first report of CTX-M-14 in Taiwan, and our results raise the important question of whether this enzyme has disseminated to other geographic areas. Unfortunately, the isolates in

the present study were retrieved from a nonselective antibiotic surveillance study (14) and we cannot estimate the magnitude of this clonal or plasmid spread. Thus, collection of isolates with specific selection criteria should be undertaken to delineate the epidemiology of CTX-M-14 in Taiwan.

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