ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 2002, p. 1985–1988 Vol. 46, No. 6
0066-4804/02/$04.00+0 DOI: 10.1128/AAC.46.6.1985–1988.2002

CTX-M-14, a Plasmid-Mediated CTX-M Type Extended-Spectrum β-Lactamase Isolated from *Escherichia coli*

L. Ma,[1] Yoshikazu Ishii,[2] Feng-Yee Chang,[3] Keizo Yamaguchi,[2] Monto Ho,[1] and L. K. Siu[1]*

Division of Clinical Research, National Health Research Institutes,[1] and Division of Infectious Diseases and Tropical Medicine, Department of Internal Medicine, Tri-Service General Hospital, National Defense Medical Center,[3] Taipei, Taiwan, and Department of Microbiology, Toho University School of Medicine, Tokyo, Japan[2]

Received 25 July 2001/Returned for modification 23 November 2001/Accepted 16 March 2002

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 μM^{-1} s^{-1}) but low values for ceftazidime and aztreonam (<0.02 μM^{-1} s^{-1}) were observed in hydrolysis assays, indicating resistance to cefotaxime (MIC ≥ 64 μg/ml) but susceptibility to ceftazidime (MIC ≤ 2 μg/ml).

CTX-M type β-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 β-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 β-lactamases are endemic in Latin America and some northeastern European countries (24). Toho type β-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 β-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 *Eco*RI and *Hin*cII (Gibco BRL, Gaithersburg, Md.) were used.

* Corresponding author. Mailing address: Division of Clinical Research, National Health Research Institutes (99), 128 Yen-Chiu-Yuan Rd., Sec. 2, Taipei 11529, Taiwan. Phone: 886 2 26524094. Fax: 886 2 27890254. E-mail: lksiu@nhri.org.tw.

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 β-lactamase with known β-lactamase sequences revealed the consensus sequences STSK, SDN, and KTG, which are conserved motifs characteristic of class A β-lactamases. Amino acid alignments with other bla_{CTX} 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 β-lactamases differ by a single amino acid at position 231 (Ala→Val). For other CTX-M type extended-spectrum β-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 μg/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

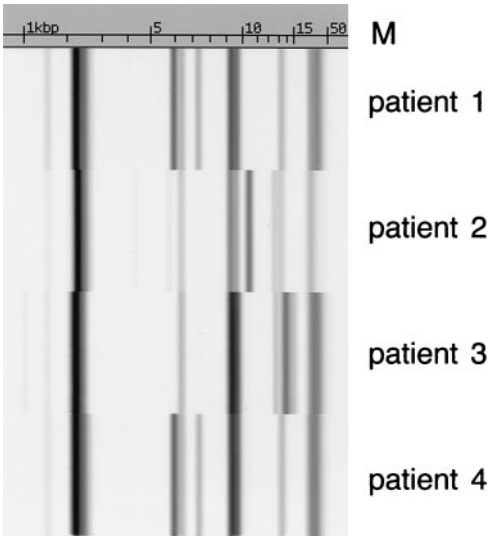

FIG. 1. Ribotyping of CTX-M-14 carriers isolated from four different patients. The scale at the top is measured in kilobase pairs.

μg/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 cefoxitin (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

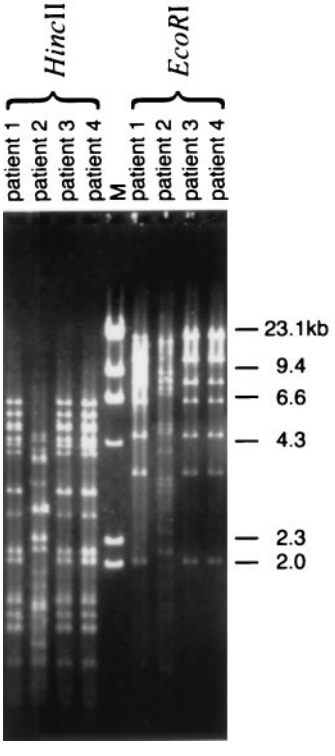

FIG. 2. *Hin*cII- or *Eco*RI-digested plasmid profiles of transconjugants of CTX-M-14 carriers isolated from four different patients.

TABLE 1. MICs of various antibiotics for strains producing CTX-M-14 β-lactamases

Antibiotic[a]	MIC (μg/ml) against:			
	Clinical isolates (KTC984167)	Trans-conjugant	Cloned strains	JP-995
AMP	≥32	≥32	≥32	2
LOT	≥32	≥32	≥32	4
CFX	8	2	8	4
CTX	≥64	≥64	≥64	≤0.25
CTX plus CAL	≤0.25	≤0.25	≤0.25	≤0.25
CTR	≥64	≥64	≥64	≤0.25
CTR plus CAL	≤0.5	≤0.5	≤0.5	≤0.5
CPD	≥64	≥64	≥64	≤0.5
CPD plus CAL	≤0.5	≤0.5	4	≤0.5
CAZ	2	1	16	≤0.25
CAZ plus CAL	≤0.25	≤0.25	0.5	≤0.25
ATM	8	4	≥64	≤0.25
IMP	≤0.25	0.5	0.5	0.5
GEN	>8	≤0.5	≤0.5	≤0.5
AMK	4	≤2	≤2	≤2
CIP	≤0.06	≤0.06	0.25	≤0.06

[a] Abbreviations: AMP, ampicillin; LOT, cephalothin; CFX, cefoxitin; 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 β-lactamases.

The β-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 β-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 μM. 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 β-lactam antibiotics (Table 2). The results showed that the β-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 β-lactam antibiotics

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

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

The CTX-M type β-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 β-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 oxiimino-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_{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 β-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 β-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.

This work was supported by a grant from the National Health Research Institutes.

We also thank Moreno Galleni, Centre d'Ingénierie des Protéines, Université de Liège, Liège, Belgium, for his critical review of the kinetics studies.

REFERENCES

1. **Ambler, R. P., A. F. Coulson, J. M. Frere, J. M. Ghuysen, B. Joris, M. Forsman, R. C. Levesque, G. Tiraby, and S. G. Waley.** 1991. A standard numbering scheme for the class A beta-lactamases. Biochem. J. **276:**269–270.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, and J. A. Smith (ed.).** 1995. Miniprep of bacterial genomic DNA, p. 2.4.2. *In* Current protocols in molecular biology. John Wiley & Sons, New York, N.Y.
3. **Barthelemy, M., J. Peduzzi, H. Bernard, C. Tancrede, and R. Labia.** 1992. Close amino acid sequence relationship between the new plasmid-mediated extended-spectrum beta-lactamase MEN-1 and chromosomally encoded enzymes of *Klebsiella oxytoca*. Biochim. Biophys. Acta **1122:**15–22.
4. **Bauernfeind, A., J. M. Casellas, M. Goldberg, M. Holley, R. Jungwirth, P. Mangold, T. Rohnisch, S. Schweighart, and R. Wilhelm.** 1992. A new plasmidic cefotaximase from patients infected with Salmonella typhimurium. Infection **20:**158–163.
5. **Bauernfeind, A., I. Stemplinger, R. Jungwirth, S. Ernst, and J. M. Casellas.** 1996. Sequences of beta-lactamase genes encoding CTX-M-1 (MEN-1) and CTX-M-2 and relationship of their amino acid sequences with those of other beta-lactamases. Antimicrob. Agents Chemother. **40:**509–513.
6. **Bonnet, R., J. L. Sampaio, R. Labia, C. De Champs, D. Sirot, C. Chanal, and J. Sirot.** 2000. A novel CTX-M beta-lactamase (CTX-M-8) in cefotaxime-resistant *Enterobacteriaceae* isolated in Brazil. Antimicrob. Agents Chemother. **44:**1936–1942.
7. **Bradford, P. A., Y. Yang, D. Sahm, I. Grope, D. Gardovska, and G. Storch.** 1998. CTX-M-5, a novel cefotaxime-hydrolyzing beta-lactamase from an outbreak of *Salmonella typhimurium* in Latvia. Antimicrob. Agents Chemother. **4:**1980–1984.
8. **Brisse, S., D. Milatovic, A. C. Fluit, K. Kusters, A. Toelstra, J. Verhoef, and F. J. Schmitz.** 2000. Molecular surveillance of European quinolone-resistant clinical isolates of *Pseudomonas aeruginosa* and *Acinetobacter* spp. using automated ribotyping. J. Clin. Microbiol. **38:**3636–3645.
9. **De Meester, F., B. Joris, G. Reckinger, C. Bellefroid-Bourguignon, J. M. Frere, and S. G. Waley.** 1987. Automated analysis of enzyme inactivation phenomena. Application to beta-lactamases and DD-peptidases. Biochem. Pharmacol. **36:**2393–2403.
10. **Galleni, M., G. Amicosante, and J. M. Frere.** 1988. A survey of the kinetic parameters of class C beta-lactamases. Cephalosporins and other beta-lactam compounds. Biochem. J. **255:**123–129.
11. **Gazouli, M., E. Tzelepi, A. Markogiannakis, N. J. Legakis, and L. S. Tzouvelekis.** 1998. Two novel plasmid-mediated cefotaxime-hydrolyzing beta-lactamases (CTX-M-5 and CTX-M-6) from Salmonella typhimurium. FEMS Microbiol. Lett. **165:**289–293.
12. **Gazouli, M., E. Tzelepi, S. V. Sidorenko, and L. S. Tzouvelekis.** 1998. Sequence of the gene encoding a plasmid-mediated cefotaxime-hydrolyzing class A beta-lactamase (CTX-M-4): involvement of serine 237 in cephalosporin hydrolysis. Antimicrob. Agents Chemother. **42:**1259–1262.
13. **Gniadkowski, M., I. Schneider, A. Palucha, R. Jungwirth, B. Mikiewicz, and A. Bauernfeind.** 1998. Cefotaxime-resistant *Enterobacteriaceae* isolates from a hospital in Warsaw, Poland: identification of a new CTX-M-3 cefotaxime-hydrolyzing beta-lactamase that is closely related to the CTX-M-1/MEN-1 enzyme. Antimicrob. Agents Chemother. **42:**827–832.
14. **Ho, M., L. C. McDonald, T. L. Lauderdale, L. L. Yeh, P. C. Chen, and Y. R. Shiau.** 1999. Surveillance of antibiotic resistance in Taiwan, 1998. J. Microbiol. Immunol. Infect. **32:**239–249.
15. **Ishii, Y., A. Ohno, H. Taguchi, S. Imajo, M. Ishiguro, and H. Matsuzawa.** 1995. Cloning and sequence of the gene encoding a cefotaxime-hydrolyzing class A beta-lactamase isolated from *Escherichia coli*. Antimicrob. Agents Chemother. **39:**2269–2275.
16. **Kado, C. I., and S. T. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145:**1365–1373.
17. **Kariuki, S., J. E. Corkill, G. Revathi, R. Musoke, and C. A. Hart.** 2001. Molecular characterization of a novel plasmid-encoded cefotaximase (CTX-M-12) found in clinical *Klebsiella pneumoniae* isolates from Kenya. Antimicrob. Agents Chemother. **45:**2141–2143.
18. **Knox, J. R.** 1995. Extended-spectrum and inhibitor-resistant TEM-type beta-lactamases: mutations, specificity, and three-dimensional structure. Antimicrob. Agents Chemother. **39:**2593–2601.
19. **Ma, L., Y. Ishii, M. Ishiguro, H. Matsuzawa, and K. Yamaguchi.** 1998.

Cloning and sequencing of the gene encoding Toho-2, a class A beta-lactamase preferentially inhibited by tazobactam. Antimicrob. Agents Chemother. **42:**1181–1186.
20. **Matthew, M., and A. M. Harris.** 1976. Identification of beta-lactamases by analytical isoelectric focusing: correlation with bacterial taxonomy. J. Gen. Microbiol. **94:**55–67.
21. **National Committee for Clinical Laboratory Standards.** 1999. Performance standards for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A4. National Committee for Clinical Laboratory Standards, Wayne, Pa.
22. **Sabate, M., R. Tarrago, F. Navarro, E. Miro, C. Verges, J. Barbe, and G. Prats.** 2000. Cloning and sequence of the gene encoding a novel cefotaxime-hydrolyzing beta-lactamase (CTX-M-9) from *Escherichia coli* in Spain. Antimicrob. Agents Chemother. **44:**1970–1973.
23. **Siu, L. K., P. L. Lu, P. R. Hsueh, F. M. Lin, S. C. Chang, K. T. Luh, M. Ho, and C. Y. Lee.** 1999. Bacteremia due to extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* in a pediatric oncology ward: clinical features and identification of different plasmids carrying both SHV-5 and TEM-1 genes. J. Clin. Microbiol. **37:**4020–4027.
24. **Tzouvelekis, L. S., E. Tzelepi, P. T. Tassios, and N. J. Legakis.** 2000. CTX-M-type beta-lactamases: an emerging group of extended-spectrum enzymes. Int. J. Antimicrob. Agents **14:**137–142.
25. **Yagi, T., H. Kurokawa, K. Senda, S. Ichiyama, H. Ito, S. Ohsuka, K. Shibayama, K. Shimokata, N. Kato, M. Ohta, and Y. Arakawa.** 1997. Nosocomial spread of cephem-resistant *Escherichia coli* strains carrying multiple Toho-1-like beta-lactamase genes. Antimicrob. Agents Chemother. **41:**2606–2611.
26. **Yan, J. J., W. C. Ko, S. H. Tsai, H. M. Wu, Y. T. Jin, and J. J. Wu.** 2000. Dissemination of CTX-M-3 and CMY-2 beta-lactamases among clinical isolates of *Escherichia coli* in southern Taiwan. J. Clin. Microbiol. **38:**4320–4325.