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CTX-M-1, CTX-M-3, and CTX-M-14 β-Lactamases from *Enterobacteriaceae* Isolated in France

C. Dutour,[1] R. Bonnet,[1]* H. Marchandin,[2] M. Boyer,[3] C. Chanal,[1] D. Sirot,[1] and J. Sirot[1]

Laboratoire de Bactériologie, Faculté de Médecine, 63001 Clermont-Ferrand Cedex,[1] Laboratoire de Bactériologie, Hôpital Arnaud de Villeneuve, 34295 Montpellier Cedex 5,[2] and Laboratoire de Bactériologie, CHR de Roanne, 42 328 Roanne,[3] France

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Six clinical CTX-M-producing isolates of the family *Enterobacteriaceae* were detected between 1999 and 2000 in different French hospitals. Two strains produced CTX-M-1 and CTX-M-3 and four strains produced CTX-M-14, a mutant Ala-231→Val of CTX-M-9. A putative transposable element, ISEcp-1, was located 43 bp upstream of all the bla_{CTX-M} genes. Two CTX-M-14-encoding plasmids exhibited similar restriction patterns. The CTX-M-1- and CTX-M-3-encoding plasmids were related to the CTX-M-1- and CTX-M-3-encoding plasmids previously reported in 1990 in France and in 1998 in Poland, respectively.

The early extended-spectrum β-lactamases (ESBLs) arose as the result of a few amino acid substitutions from the common plasmid-mediated TEM and SHV-1 β-lactamases. At the beginning of the 1990s, a new class A type of ESBL was characterized in the first reports of the CTX-M-1 (MEN-1) enzyme (2, 3). These CTX-M-type enzymes are typical ESBLs, characterized by much greater hydrolytic activity against cefotaxime than against ceftazidime. Thus, they confer a high level of resistance to cefotaxime and have only marginal effects on the MIC of ceftazidime.

The family of CTX-M-type ESBLs comprises at least 12 members belonging to four major phylogenetic branches on the basis of their amino acid sequence similarities (8): the CTX-M-1 (MEN-1) branch (CTX-M-1, CTX-M-3, and CTX-M-10), the CTX-M-2 branch (CTX-M-2, Toho-1, and CTX-M-4 to CTX-M-6), the CTX-M-9 branch (CTX-M-9 and Toho-2), and the CTX-M-8 branch. These enzymes have been reported in several species of the family *Enterobacteriaceae* and in *Vibrio cholerae* serovar El Tor and have been isolated in three main geographical areas: South America (7, 8; M. Galas, F. Pasteran, R. Melano, A. Petroni, G. Lopez, A. Corso, A. Rossi, and Whonet Collaborative Group, Abstr. 38th Intersci. Conf. Antimicrob. Agents Chemother., abstr. E-109, p. 201, 1998), Eastern Europe (11, 12, 13, 20), and Japan (14, 19).

We describe six clinical strains of the family *Enterobacteriaceae* (Table 1) which were collected in different French hospitals on the basis of their positive synergy test results (16) and their higher levels of resistance to cefotaxime than ceftazidime (MICs, 16 to 128 and 2 to 8 μg/ml, respectively). *Escherichia coli* CF-1 and *Enterobacter cloacae* CF-2 were isolated at the teaching hospital of Clermont-Ferrand, France, from the urine of a patient hospitalized in 1999 and from a pulmonary sample of a patient admitted in 2000, respectively. *E. coli* Mnt-1 and *Klebsiella pneumoniae* Mnt-2 were isolated from blood and stool samples, respectively, of a Vietnamese child admitted to Montpellier hospital, Montpellier, France, in 1999. *E. coli* Roa-1 was isolated in Roannes hospital, Roannes, France, in 1999 from blood, and *E. cloacae* Ver-1 was isolated in Versailles hospital, Versailles, France, in 1999 from a urine sample (10). CTX-M-1-producing strain MEN (2) and CTX-M-3-encoding plasmid A1 (20) were used as references.

Analytical isoelectric focusing was performed as described previously (7). The following β-lactamases with known pIs were used as standards: TEM-1 (pI 5.4), SHV-1 (pI 7.6), and CTX-M-1 (pI 8.4). All strains tested produced an enzyme of pI 5.4, associated with a second β-lactamase of alkaline pI: pI 8.4 for strains Ver-1 and CF-2 and pI 7.9 for strains CF-1, Mnt-1, Mnt-2, and Roa-1.

PCR TEM and direct sequencing of the PCR product (23) identified the β-lactamase of pI 5.4 as being the TEM-1 penicillinase. No PCR products were obtained with primers specific for bla_{SHV} genes. Positive amplifications were obtained with primers CTX-MA (5′-CGCTTTGCGATGTGCAG-3′) and CTX-MB (5′-ACCGCGATATCGTTGGT-3′), which amplified 550-bp internal fragments of the bla_{CTX-M} genes (8). From the results obtained for the sequence of this 550-bp fragment, the complete bla_{CTX-M} open reading frames (ORFs) of strains Ver-1 and CF-2 were amplified and sequenced with primers specific for the $bla_{CTX-M-1}$ and $bla_{CTX-M-3}$ genes (primers CTX-M1A [5′-CTTCCAGAATAAGGAATC-3′] and CTX-M-1B [5′-CCGTTTCCGCTATTACAA-3′]; temperature of annealing, 52°C). The sequence obtained had 100% identity with those of $bla_{CTX-M-3}$ (13) and $bla_{CTX-M-1}$ (4) for strains Ver-1 and CF-2, respectively. The bla_{CTX-M} gene of strain CF-1 was cloned in plasmid vector pBK-CMV (Stratagene, La Jolla, Calif.) by partial digestion of its plasmid content with *Sau*3A, as reported previously (7, 22). A 13-kb recombinant plasmid, designated pClCF-1, was sequenced with primers CTX-M-A and CTX-M-B, which are specific for the internal bla_{CTX-M} sequence. The sequence of the bla_{CTX-M} gene was identical to that of $bla_{CTX-M-14}$ sequenced by another group (GenBank accession number AF252622). Strains Mnt-1, Mnt-2, and Roa-1 harbored the same $bla_{CTX-M-14}$ gene.

The deduced amino acid sequence of the CTX-M-14 β-lactamase differed from that of CTX-M-9 (21) by the amino acid

* Corresponding author. Mailing address: Faculté de Médecine, Service de Bactériologie-Virologie, 28, Place Henri Dunant, 63 001 Clermont-Ferrand Cedex, France. Phone: 33 (0) 4 73 60 80 18. Fax: 33 (0) 4 73 27 74 94. E-mail: Richard.Bonnet@u-clermont1.fr.

TABLE 1. Clinical strains and plasmids used in the study

Strain or plasmid	Relevant genotype	Source or reference
Clinical strains		
E. coli CF-1	Clinical strain harboring natural plasmid pCF-1 (Clermont-Ferrand, France, 1999)	This study
E. cloacae CF-2	Clinical strain harboring natural plasmid pCF-2 (Clermont-Ferrand, France, 2000)	This study
E. coli Roa-1	Clinical strain harboring natural plasmid pRoa-1 (Roanne, France, 1999)	This study
E. coli Mnt-1	Clinical strain harboring natural plasmid pMnt-1 (Montpellier, France, 1999)	This study
K. pneumoniae Mnt-2	Clinical strain producing CTX-M-14 (Montpellier, France, 1999)	This study
E. cloacae Ver-1	Clinical strain harboring natural plasmid pVer-1 (Versailles, France, 1999)	10
E. coli MEN	Clinical strain harboring natural plasmid pMEN (Paris, France, 1999)	2
Plasmids		
pCF-1	150-kb natural plasmid from *E. coli* strain CF-1 containing the $bla_{CTX\text{-}M\text{-}14}$ gene	This study
pCF-2	55-kb natural plasmid from *E. cloacae* strain CF-2 containing the $bla_{CTX\text{-}M\text{-}1}$ gene	This study
pMnt-1	110-kb natural plasmid from *E. coli* strain Mnt-1 containing the $bla_{CTX\text{-}M\text{-}14}$ gene	This study
pRoa-1	120-kb natural plasmid from *E. coli* strain Roa-1 containing the $bla_{CTX\text{-}M\text{-}14}$ gene	This study
pVer-1	180-kb natural plasmid from *E. cloacae* strain Ver-1 containing the $bla_{CTX\text{-}M\text{-}3}$ gene	10
pMEN	40-kb natural plasmid from *E. coli* strain MEN containing the $bla_{CTX\text{-}M\text{-}1}$ gene	2
A1	110-kb natural A1 plasmid containing the $bla_{CTX\text{-}M\text{-}3}$ gene	20
pClCF-1	13-kb recombinant plasmid of pBK-CMV containing the $bla_{CTX\text{-}M\text{-}14}$ gene	This study
pBK-CMV	Phagemid vector; kanamycin resistance phenotype	Stratagene, La Jolla, Calif.

substitution Ala-231→Val (numbering of Ambler et al. [1]). Position 231 is located at the beginning of CTX-M beta sheet β3 (15), which contains at position 234 the KTG conserved element of class A β-lactamases. Val is usually encountered at positions 231 of CTX-M enzymes.

The CTX-M-14 enzyme was extracted from recombinant *E. coli* DH5α(pClCF-1) by sonification and was purified to homogeneity as described previously (7). The purities of the CTX-M extracts (≥97%) were estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, as described previously (7).

The kinetic constants of CTX-M-14 were obtained by a computerized microacidimetric method described elsewhere (18) and were compared with those of CTX-M-9 (Table 2). The concentrations of the inhibitors (clavulanate and tazobactam) required to inhibit enzyme activity by 50% (IC_{50}s) were determined with penicillin G as described previously (7). IC_{50}s and K_i values were monitored with penicillin G (200 mM) as the reporter substrate. CTX-M-14 and CTX-M-9 had similar kinetic constants (Table 2). High catalytic efficiencies (k_{cat}/k_m values) were observed against penicillin G (11.8 to 14.5 $\mu M^{-1} \cdot s^{-1}$), amoxicillin (4.5 to 5 $\mu M^{-1} \cdot s^{-1}$), piperacillin (4.2 to 5.5 $\mu M^{-1} \cdot s^{-1}$), cephalothin (15.4 to 20 $\mu M^{-1} \cdot s^{-1}$), and cefuroxime (7 to 8 $\mu M^{-1} \cdot s^{-1}$). As reported previously for the other CTX-M enzymes, CTX-M-14 and CTX-M9 had better catalytic activities against methoximino cephems such as cefuroxime, cefotaxime, and cefpirome (k_{cat} values, 320 to 950 s^{-1}) than against carboxylic propyloximino β-lactams such as ceftazidime and aztreonam (k_{cat} values, 2 to 10 s^{-1}). CTX-M-14 and CTX-M-9 were susceptible to the β-lactam inhibitors clavulanate (IC_{50}s, 0.033 and 0.036 μM, respectively), tazobactam (IC_{50}s, 0.008 and 0.007 μM, respectively), and, to a lesser extent, sulbactam (IC_{50}s, 3.4 and 3.0 μM, respectively).

Five *E. coli* transconjugants of the six clinical strains were selected on agar containing cefotaxime (2 μg/ml) and rifampin (30 μg/ml). All these transconjugants produced the CTX-M enzyme associated with the TEM-1 penicillinase.

Table 3 lists the MICs of the β-lactams alone and in combination with β-lactamase inhibitors for the CTX-M-producing transconjugants. They were determined by a dilution method on Mueller-Hinton agar (Sanofi Diagnostics Pasteur, Marnes la Coquette, France) with an inoculum of 10^4 CFU per spot. The antibiotics were provided as powders by SmithKline Beecham Pharmaceuticals (amoxicillin, ticarcillin, and clavulanate); Lederle Laboratories (piperacillin and tazobactam); Eli Lilly, Paris, France (cephalothin); Roussel-Uclaf (cefo-

TABLE 2. Substrate profile of CTX-M-14 and CTX-M-9 β-lactamases

Substrate	CTX-M-14			CTX-M-9		
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)
Penicillin G	290	20	14.5	295	25	11.8
Amoxicillin	100	20	5.0	90	20	4.5
Ticarcillin	45	24	1.9	60	35	1.7
Piperacillin	200	48	4.2	110	20	5.5
Cephalothin	2,700	175	15.4	3,000	150	20.0
Cefuroxime	320	40	8.0	350	50	7.0
Cefotaxime	415	130	3.2	450	120	3.7
Cefpirome	940	1,000	0.9	950	800	1.2
Aztreonam	10	200	0.05	10	220	0.04
Ceftazidime	3	610[a]	0.005	2	600[a]	0.003

[a] K_m values were determined as the K_i values by substrate competition with penicillin G.

TABLE 3. Comparison of β-lactam MICs for CTX-M-producing *E. coli* transconjugants

	MIC (μg/ml) for *E. coli* C600 with plasmid (pI):				
	pCF-2[a] (5.4, 8.4)	pVer-1[b] (5.4, 8.4)	pCF-1[c] (5.4, 7.9)	pMnt-1[d] (5.4, 7.9)	pRoa-1[e] (5.4, 7.9)
Amoxicillin	2,048	>2,048	>2,048	>2,048	>2,048
Amoxicillin + CLA[f]	8	16	16	32	32
Ticarcillin	2,048	>2,048	>2,048	>2,048	>2,048
Ticarcillin + CLA	8	64	32	32	64
Piperacillin	128	512	256	1,024	1,024
Piperacillin + TZB[g]	0.5	1	1	1	1
Cephalothin	1,024	1,024	1,024	1,024	1,024
Cephalothin + CLA	8	8	8	8	8
Cefuroxime	1,024	1,024	1,024	1,024	1,024
Cefuroxime + CLA	4	8	4	4	8
Cefotaxime	16	16	16	32	32
Cefotaxime + CLA	0.06	0.06	0.06	0.06	0.06
Cefpirome	2	8	8	8	16
Cefpirome + CLA	0.03	0.06	0.06	0.03	0.03
Ceftazidime	1	2	1	1	2
Ceftazidime + CLA	0.12	0.25	0.12	0.12	0.12
Aztreonam	4	8	4	4	8
Aztreonam + CLA	0.06	0.12	0.06	0.06	0.06

[a] Natural CTX-M-1-encoding plasmid of strain CF-2.
[b] Natural CTX-M-3-encoding plasmid of strain Ver-1.
[c] Natural CTX-M-14-encoding plasmid of strain CF-1.
[d] Natural CTX-M-14-encoding plasmid of strain Mnt-1.
[e] Natural CTX-M-14-encoding plasmid of strain Roa-1.
[f] CLA, clavulanate at a fixed concentration of 2 μg/ml.
[g] TZB, tazobactam at a fixed concentration of 4 μg/ml.

taxime and cefpirome); Glaxo Wellcome Research and Development (ceftazidime); and Bristol-Myers Squibb (cefepime). The *E. coli* transconjugants exhibited a high level of resistance to amoxicillin, ticarcillin, cephalothin, and cefuroxime (MICs, >1,024 μg/ml). The MICs of cefotaxime (16 to 32 μg/ml) were 8- to 32-fold higher than those of ceftazidime (1 to 2 μg/ml) and 2- to 8-fold higher than those of aztreonam (4 to 8 μg/ml) and cefpirome (2 to 16 μg/ml). Clavulanate restored partially or totally the activities of the β-lactams. All strains were susceptible to associations of clavulanate and broad-spectrum cephalosporins (MICs, 0.06 to 0.12 μg/ml).

The plasmid contents of the transconjugants after extraction by the method of Kado and Liu (17) are shown in Fig. 1A. Plasmid sizes were determined by comparison with 39.5-, 65-, 85-, and 180-kb reference plasmids (6). The CTX-M-encoding plasmids were 55-kb plasmid pCF-2 for strain CF-2 (CTX-M-1), 180-kb plasmid pVer-1 for strain Ver-1 (CTX-M-3), 150-kb plasmid pCF-1 for strain CF-1 (CTX-M-14), 110-kb plasmid pMnt-1 for strain Mnt-1 (CTX-M-14), and 120-kb plasmid pRoa-1 for strain Roa-1 (CTX-M-14) (Table 1).

These CTX-M-encoding plasmids were extracted by the method of Birnboim and Doly (5) and were digested with restriction endonucleases *Hpa*I, *Eco*RI, and *Sal*I (Boehringer Mannheim), according to the manufacturer's recommendations. The restriction patterns are shown in Fig. 1B. The CTX-M-1-, CTX-M-3-, and CTX-M-9-type enzymes were encoded by plasmids with distinct restriction patterns. Of the three CTX-M-14-encoding plasmids, pCF-1 presented a distinct restriction pattern, whereas plasmids pRoa-1 and pMnt-1 exhibited related restriction patterns. CTX-M-1-encoding plasmid pCF-2 had a restriction pattern related to that of $bla_{\text{CTX-M-1}}$-harboring plasmid pMEN of strain MEN, which was isolated in Paris, France, from an Italian patient in 1990 (2). Likewise, CTX-M-3-encoding plasmid pVer-1 was related to $bla_{\text{CTX-M-3}}$-harboring plasmid A1, which was characterized in 1997 in Poland (20). Closely related plasmids encoding the same CTX-M ESBL were thus observed in different geographical

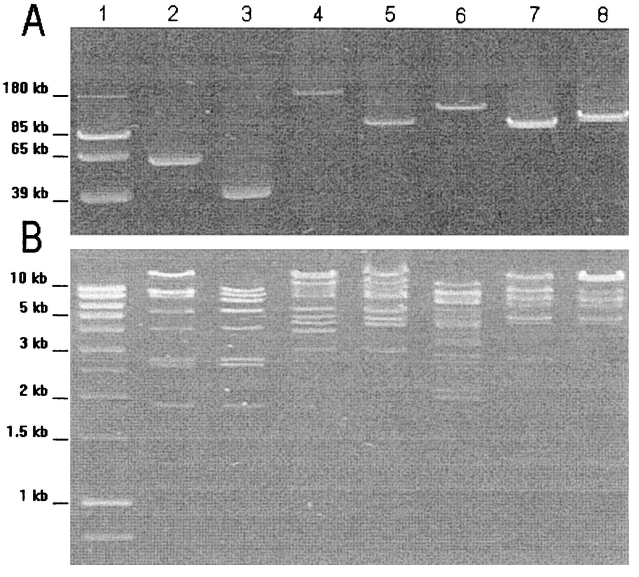

FIG. 1. (A) Agarose (0.7%) electrophoresis of plasmid DNA from CTX-M-producing *E. coli* transconjugants. (B) Agarose (0.7%) electrophoresis of *Hpa*I-restricted plasmid DNA from CTX-M-producing *E. coli* transconjugants. Lanes: 1, molecular size marker; 2, CTX-M-1-encoding plasmid pCF-2; 3, CTX-M-1-encoding plasmid pMEN (2); 4, CTX-M-3-encoding plasmid pVer-1; 5, CTX-M-3-encoding plasmid A1 (20); 6, CTX-M-14-encoding plasmid pCF-1; 7, CTX-M-14-encoding plasmid pMnt-1; 8, CTX-M-14-encoding plasmid pRoa-1.

areas (central France and the south of France for CTX-M-14; France and Poland for CTX-M-3) and at different times (in 1999 and 1987 for CTX-M-1). These findings suggest the establishment and diffusion of the CTX-M-encoding plasmids in Europe.

Genes encoding an identical CTX-M enzyme could be harbored by different plasmids, as observed for $bla_{CTX\text{-}M\text{-}3}$ (20), $bla_{CTX\text{-}M\text{-}8}$ (8), and $bla_{CTX\text{-}M\text{-}14}$ (this work). The CTX-M-5 enzyme is encoded by genes located on plasmids in different isolates of the family *Enterobacteriaceae* (9) and in the chromosome of *Kluyvera ascorbata*. In addition, the putative insert sequence ISEcp-1 initially reported upstream of the plasmid-encoded CMY-type cephalosporinases (24) was observed upstream of the gene of the CTX-M-5 enzyme in the chromosome (C. Humeniuk, G. Arlet, R. Labia, P. Grimont, and A. Philippon, 20th Réunion Interdisciplinaire Chimiothér. Anti-Infectieuse, abstr. 20/C4, 2000). We used PCR and hybridization of the restricted plasmids to detect ISEcp-1 in our CTX-M-encoding plasmids: the ISEcp-1 probe and the amplifications were performed at an annealing temperature of 50°C with primers ISEcp1A (5′-AATCTAACATCAAATGCAGG-3′) and ISEcp-1B (5′-TTTTGCTGCAAGAAATACATA-3′), whose sequences are located in the transposase gene of ISEcp-1 (24). The specificities of the primers were confirmed by sequencing of the PCR products. ISEcp-1 was observed upstream of the CTX-M-encoding ORF in plasmids pCF1, pCF-2, pRoa-1, pVer-1, pMnt-1, and A1; but only one copy was detected. These results confirm the close association of ISEcp-1 with the $bla_{CTX\text{-}M}$ genes and suggest that the mobility of the $bla_{CTX\text{-}M}$ genes involves the ISEcp-1 element. If ISEcp-1 is really involved in the transfer of $bla_{CTX\text{-}M}$ genes, this element should be able to mobilize adjacent DNA sequences alone. Another possibility is that ISEcp-1 is part of an altered composite transposon from which the second ISEcp-1, initially located downstream of the $bla_{CTX\text{-}M}$ gene, was deleted, perhaps by a transposition event. Besides, the location of ISEcp1 only 43 bp upstream of $bla_{CTX\text{-}M}$ genes may contribute to $bla_{CTX\text{-}M}$ gene expression.

In this work, we have reported on six clinical strains of the family *Enterobacteriaceae* which produced CTX-M-1, CTX-M-3, and CTX-M-14 ESBLs. The study of their genetic support suggests that the CTX-M-encoding plasmids have become established in Europe and are spreading. In addition, the $bla_{CTX\text{-}M}$ genes were associated with ISEcp-1, which could be implicated in their spread and/or expression.

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