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Dissemination of CTX-M-Type β-Lactamases among Clinical Isolates of *Enterobacteriaceae* in Paris, France

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We analyzed 19 clinical isolates of the family *Enterobacteriaceae* (16 *Escherichia coli* isolates and 3 *Klebsiella pneumoniae* isolates) collected from four different hospitals in Paris, France, from 2000 to 2002. These strains had a particular extended-spectrum cephalosporin resistance profile characterized by a higher level of resistance to cefotaxime and aztreonam than to ceftazidime. The $bla_{\text{CTX-M}}$ genes encoding these β-lactamases were involved in this resistance, with a predominance of $bla_{\text{CTX-M-15}}$. Ten of the 19 isolates produced both TEM-1- and CTX-M-type enzymes. One strain (*E. coli* TN13) expressed CMY-2, TEM-1, and CTX-M-14. $bla_{\text{CTX-M}}$ genes were found on large plasmids. In 15 cases the same insertion sequence, IS*Ecp1*, was located upstream of the 5′ end of the $bla_{\text{CTX-M}}$ gene. In one case we identified an insertion sequence designated IS*26*. Examination of the other three $bla_{\text{CTX-M}}$ genes by cloning, sequencing, and PCR analysis revealed the presence of a complex *sul1*-type integron that includes open reading frame ORF513, which carries the *bla* gene and the surrounding DNA. Five isolates had the same plasmid DNA fingerprint, suggesting clonal dissemination of CTX-M-15-producing strains in the Paris area.

Plasmid-mediated extended-spectrum β-lactamases (ESBLs) are becoming increasingly frequent among clinical isolates of the family *Enterobacteriaceae* throughout the world. The emergence of new enzyme groups that have a typical ESBL resistance phenotype but that are non-TEM and non-SHV derivatives have recently been reported (11). CTX-M-type β-lactamases constitute a novel group of enzymes encoded by transferable plasmids. This novel family of plasmid-mediated ESBLs has been classified in Ambler class A (2) and in group 2be of the Bush, Jacoby, and Medeiros classification (12). They are capable of hydrolyzing broad-spectrum cephalosporins and are inhibited by clavulanic acid, sulbactam, and tazobactam. They confer a high level of resistance to cefotaxime but have a low level of activity against ceftazidime (37). The first two CTX-M-type enzymes were reported in Europe in 1989 (7, 8). So far, more than 30 CTX-M-type β-lactamases have been identified in various clinical isolates, but mostly in enterobacterial species such as *Escherichia coli*, *Klebsiella pneumoniae*, and *Salmonella enterica* serovar Typhimurium. These enzymes have been classified into four major phylogenetic branches on the basis of their amino acid sequence homologies (10). These clusters are CTX-M-1, CTX-M-2, CTX-M-8, and CTX-M-9. Two of them (the CTX-M-2 and CTX-M-8 types) were recently shown to be similar (95 to 100%) to the chromosomally encoded β-lactamase of *Kluyvera ascorbata* and *Kluyvera georgiana*, respectively (19, 29). Although CTX-M-producing strains were initially found in western Europe, they have now been observed over a wide geographic area, including Latin America (9, 10), Asia (14, 20, 23, 24, 27, 40), some parts of eastern Europe (6, 17, 18), and, recently, North America (34). In some countries, CTX-M-type enzymes are the ESBLs most frequently isolated from gram-negative strains (37). They have been involved in several outbreaks (6, 40), although the isolation of CTX-M-producing strains remains sporadic, including in France (16, 30, 32).

bla genes are often associated with transferable plasmids, and some of them are parts of transposons or constitute cassettes in integrons. The association of insertion sequences (e.g., IS*Ecp1*) that mobilize β-lactamase-encoding genes with these β-lactamase genes may be involved in their dissemination and expression (13). Insertion sequences such as IS*26* (32) and IS*903* (13, 27, 32) have also been associated with $bla_{\text{CTX-M}}$ genes. Unusual class 1 integrons, similar to In*6* and In*7*, have been reported to carry antibiotic resistance genes such as $bla_{\text{DHA-1}}$ (39). These *sul1*-type integrons with similar genetic organizations contain a partial duplication of the 3′ conserved segment (3′-CS) and have an open reading frame (ORF), ORF513, between the two *sul1* genes. Part of a novel complex *sul1*-type integron (In*60*), including the $bla_{\text{CTX-M-9}}$ gene and its downstream nucleotide sequence, was recently characterized (31). $bla_{\text{CTX-M-2}}$ and the surrounding DNA, which includes ORF513, have been reported in the complex *sul1*-type integron, designated In*35* (3) and InS*21* (15). Here we report on the diffusion of various CTX-M-type β-lactamases among clinical isolates of the family *Enterobacteriaceae* in Paris, France.

MATERIALS AND METHODS

Bacterial strains and plasmids. The 19 strains of the family *Enterobacteriaceae* (16 *E. coli* strains and 3 *K. pneumoniae* strains) studied here are listed in Table 1; they were isolated in four different hospitals between 2000 and 2002 and were identified with API 20E systems (Bio-Mérieux SA, Marcy l'Etoile, France). *E. coli* J53-2 (*pro met* Rif^r) and *E. coli* DH10B (Invitrogen SARL, Cergy-Pontoise, France) were used for resistance transfer assays (conjugation and electroporation).

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TABLE 1. Cefotaxime-resistant clinical strains

Strain	Date of isolation (mo/day/yr or mo/yr)	Hospital	Specimen	Ward
E. coli TN03	03/26/2001	Tenon	Urine	Orthopedic surgery
E. coli TN05	07/04/2001	Tenon	Vaginal swab	Obstertics
E. coli TN06	09/30/2001	Tenon	Urine	Cardiology
E. coli TN07	10/03/2001	Tenon	Urine	Urology
E. coli TN08	07/31/2001	Tenon	Urine	Emergency
E. coli TN09	11/24/2001	Tenon	Urine	Emergency
E. coli TN12	12/19/2001	Tenon	Rectal swab	Nephrology ICU[a]
E. coli TN13	02/22/2002	Tenon	Blood	Digestive surgery
E. coli TN14	03/12/2002	Tenon	Urine	Nephrology
E. coli TN15	04/08/2002	Tenon	Vaginal swab	Maternity outpatient
E. coli TN16	05/29/2002	Tenon	Rectal swab	Nephrology ICU
E. coli TN17	06/04/2002	Tenon	Rectal swab	Nephrology ICU
E. coli TN18	03/05/2002	Tenon	Urine	Surgical ICU
E. coli TN19	08/18/2002	Tenon	Urine	Infectious diseases
E. coli RB-01	04/2002	R. Ballanger	Urine	Medical ward
E. coli LM-01	07/2002	L. Mourier	Rectal swab	ICU
K. pneumoniae KP-LT	06/2000	Saint-Michel	Urine	Surgery
K. pneumoniae KP-02	03/21/2002	Tenon	Wound	Respiratory ICU
K. pneumoniae KP-03	06/03/2002	Tenon	Urine	Nephrology ICU

[a] ICU, intensive care unit.

Antibiotic susceptibility. Susceptibilities to antimicrobial agents that are usually active against the *Enterobactericaeae* were determined by an antibiotic disk (Bio-Rad, Marnes-la-Coquette, France) diffusion method on Mueller-Hinton (MH) agar (Bio-Rad). The MICs of the antibiotics, including penicillins and cephalosporins with and without β-lactamase inhibitors (clavulanic acid at 2 μg/ml or tazobactam at 4 μg/ml), were determined by a dilution method on MH agar. An inoculum of 10^4 CFU per spot was delivered with a multipoint inoculator. ESBLs were detected by using the standard disk synergy test (21).

β-Lactam resistance transfer assays. Mating experiments were performed with *E. coli* J53-2 (*met pro* Rif^r). One-milliliter volumes of cultures of each donor and the rifampin-resistant *E. coli* recipient strain grown in Trypticase soy broth (Bio-Rad) were mixed and incubated for 18 h at 37°C. Transconjugants were then selected on Drigalski (Bio-Rad) agar plates containing rifampin (250 $\mu g \cdot ml^{-1}$) and cefotaxime (2.5 $\mu g \cdot ml^{-1}$).

E. coli strains liable to produce colicins were plated on MH agar that had been plated with *E. coli* J53-2 (32).

Plasmid DNA was isolated by the method of Takahashi and Nagano (35), and 2 μl was transformed into 20 μl of *E. coli* DH10B cells by electroporation according to the instructions of the manufacturer (Bio-Rad). Transformants were incubated for 1.5 h at 37°C and mated on Drigalski medium supplemented with cefotaxime (2.5 $\mu g \cdot ml^{-1}$) or cefoxitin (40 $\mu g \cdot ml^{-1}$).

Plasmid DNA analysis. Plasmid DNA from the *K. pneumoniae* and *E. coli* isolates and their corresponding *E. coli* transconjugants and transformants was obtained as described by Takahashi and Nagano (35) and Kado and Liu (22). Plasmid DNA was detected by electrophoresis in a 0.8% agarose gel. The molecular sizes of plasmid DNAs were estimated by comparison with the following plasmids of known sizes from the Institute Pasteur Collection: pIP112 (100.5 kb), pCFF04 (85 kb), and pIP173 (125.8 kb).

IEF of β-lactamases. Bacteria growing exponentially at 37°C in 50 ml of Trypticase soy broth were pelleted and sonicated with a Vibracell apparatus (twice for 30 s each time, 60 W). Isoelectric focusing (IEF) was performed on a pH 3.5 to 10 ampholine polyacrylamide gel. β-Lactamase activity was detected with the chromogen nitrocefin (Oxoid, Dardilly, France) (25). The pI values used as standards were those of TEM-1 (pI 5.4), TEM-2 (pI 5.6), SHV-4 (pI 7.8), and CMY-2b (pI 9.3).

Characterization of β-lactamase-encoding (*bla*) genes. Detection of gene sequences coding for the TEM, CMY, and CTX-M-type enzymes was performed by PCR with genomic DNA. The oligonucleotide primer sets specific for the β-lactamase genes used in the PCR assays are listed in Table 2. To characterize the 3′ end of $bla_{CTX\text{-}M\text{-}15}$, we used primers ORF1 pol M3 and M3 int upp (Table 2); primer ORF1 pol M3 was designed by using the sequence of ORF1 described downstream of the CTX-M-3 gene of *Citrobacter freundii* (GenBank accession no. AF550415). The bla_{TEM} genes were characterized by PCR-restriction fragment length polymorphism (RFLP) analysis (4).

The PCR products of bla_{CMY} and $bla_{CTX\text{-}M}$ were subjected to direct sequencing by the dideoxy chain termination method of Sanger et al. (33). Both strands of the PCR products were sequenced twice with an Applied Biosystems sequencer (model ABI 377).

The nucleotide sequences and deduced protein sequences were analyzed with the BLAST and Clustal W programs (for preparation of multiple-sequence alignments, pairwise comparisons of sequences, and preparation of dendrograms) (1, 36).

Genetic environment of $bla_{CTX\text{-}M}$ genes. The genetic organization of the $bla_{CTX\text{-}M}$ genes was investigated by PCR, cloning, and sequencing of the regions surrounding these genes. The internal IS*26* and IS*Ecp1* forward primers and the CTX-M reverse consensus primer (MA1 reverse) were used to investigate the promoter regions of the $bla_{CTX\text{-}M}$ genes. PCR primers corresponding to sequences upstream of the $bla_{CTX\text{-}M}$ genes (ORF513) and downstream of the $bla_{CTX\text{-}M}$ genes (IS*903*, ORF1005, ORF1, and *sul1*) were also used.

β-Lactamase gene cloning was performed with plasmid DNA digested and ligated in the EcoRI or HindIII (Ozyme; New England Biolabs Inc., Saint Quentin en Yvelines, France) site of phagemid pBK-CMV (Stratagene, La Jolla, Calif.). *E. coli* DH10B was transformed by electroporation. The transformants harboring the recombinant CTX-M-encoding plasmids were selected on MH agar supplemented with 2.5 μg of cefotaxime per ml and 25 μg of kanamycin per ml. The molecular sizes of the inserts were estimated from the results of restriction enzyme digestion and electrophoresis in 1% agarose gels. Finally, inserts were investigated by sequencing the ends and then by PCR.

Rep-PCR and ERIC-PCR. DNA was extracted by using the Qiagen Mini kit (Qiagen, Courtaboeuf, France). Repetitive extragenic palindromic sequence PCR (Rep-PCR) was performed with primers rep-1R and rep-2T; enterobacterial repetitive intergenic repetitive consensus sequence PCR (ERIC-PCR) was performed with primer ERIC-2, as described previously (26). The resulting products were run in 1.5% agarose gels.

Plasmid DNA fingerprinting. Plasmid DNA was purified from transformant cells with the Qiagen Plasmid Midi kit (Qiagen), according to the recommendations of the manufacturer. For fingerprinting analysis, plasmid DNA was digested with the BamHI restriction enzyme (New England Biolabs Inc.) and subjected to electrophoresis in a 1% agarose gel at 80 V for 4 h.

RESULTS

Description of clinical isolates. Sixteen *E. coli* strains and 3 *K. pneumoniae* strains were recovered between June 2000 and August 2002. Most isolates were isolated at Tenon Hospital. The strains were associated with urinary tract infections (11 strains), blood infection (1 strain), a wound infection (1 strain), and vaginal or gastrointestinal colonization (6 strains) (Table 1).

TABLE 2. Sequences of the primers used to detect *bla* genes and their promoter regions

PCR target	Primer name	Primer sequence[a]
bla_{CTX-M} (CTX-M-1 group)	M13 upper	5′-GGT TAA AAA ATC ACT GCG TC-3′
	M13 lower	5′-TTG GTG ACG ATT TTA GCC GC-3′
bla_{CTX-M} (CTX-M-2 group)	M25 upper	5′-ATG ATG ACT CAG AGC ATT CG-3′
	M25 lower	5′-TGG GTT ACG ATT TTC GCC GC-3′
bla_{CTX-M} (CTX-M-9 group)	M9 upper	5′-ATG GTG ACA AAG AGA GTG CA-3′
	M9 lower	5′-CCC TTC GGC GAT GAT TCT C-3′
bla_{TEM}	OT3	5′-ATG AGT ATT CAA CAT TTC CG-3′
	OT4	5′-CCA ATG CTT AAT CAG TGA GG-3′
bla_{CMY}	CF1	5′-ATG ATG AAA AAA TCG TTA TGC-3′
	CF2	5′-TTG TAG CTT TTC AAG AAT GCG C-3′
*tnp*IS*Ecp1*[b]	IS*Ecp1*	5′-AAA AAT GAT TGA AAG GTG GT-3′
	MA1 reverse	5′-ACY TTA CTG GTR CTG CAC AT-3′
*tnp*IS*26*	IS*26*	5′-AGC GGT AAA TCG TGG AGT GA-3′
	MA1 reverse	5′-ACY TTA CTG GTR CTG CAC AT-3′
IS*903*	IS*903* reverse	5′-CGG TTG TAA TCT GTT GTC CA-3′
	M1 IS*903*	5′-CGT CGG CGG CTA AAA TCG TC-3′
	M9 IS*903*	5′-CTA CGG CAC CAC CAA TGA TA-3′
ORF513	ORF513	5′-TGG AAG AGG GCG AAG ACG AT-3′
	M2 reverse upper	5′-CGA ATG CTC TGA GTC ATC AT-3′
	M9 reverse upper	5′-TGC ACT CTC TTT GTC ACC AT-3′
ORF1005	ORF1005 reverse	5′-ATC CAT AAT AGC ATC CAT CAT-3′
	M9 reverse lower	5′-GAG AAT CAT CGC CGA AGG G-3′
sul1	*sul1* reverse	5′-GCT CAA GAA AAA TCC CAT CCC C-3′
	M2 reverse lower	5′-GCG GCG AAA ATC GTA ACC CA-3′
	M9 reverse lower	5′-GAG AAT CAT CGC CGA AGG G-3′
ORF1	M3 int upp	5′-TCA CCC AGC CTC AAC CTA AG-3′
	ORF1 pol M3	5′-GCA CCG ACA CCC TCA CAC CT-3′

[a] Y = C or T; R = A or G.
[b] *tnp*, transposase.

β-Lactam susceptibility profile and associated resistance (Table 3). All the strains were resistant to penicillins at high concentrations (MICs, ≥128 μg/ml), but clavulanic acid and tazobactam partially restored the activities of amoxicillin (MICs, 4 to 16 μg/ml) and piperacillin (MICs, 2 to 8 μg/ml) against all except three strains (strains TN13, TN17, and TN18). One of these strains (strain TN13) was also resistant to cefoxitin (MIC, 128 g/ml), but its transconjugant was not. All but one of the strains (strain TN13) had a higher level of resistance to cefotaxime, cefepime, and aztreonam than to ceftazidime. Similar results were observed with the transconjugants or electroporants. The disk diffusion method showed synergy between ceftazidime, cefotaxime, aztreonam, cefepime, and clavulanic acid against all the strains and their transconjugants or electroporants: these results agreed with the MICs of the extended-spectrum cephalosporins and aztreonam combined with clavulanic acid. The non-β-lactam antibiotic resistance markers are also listed in Table 3. Fourteen isolates were resistant to aminoglycosides, and this resistance was transferred for 12 of them.

Transfer of resistance. Cefotaxime resistance transfer by conjugation was obtained for only seven *E. coli* isolates and two *K. pneumoniae* isolates. The probable production of colicins by three *E. coli* isolates (isolates TN07, TN16, and TN19), suggested by observation of a growth inhibition zone, possibly explained why the mating-out assays failed to yield transconjugants of these three strains. Electroporation of plasmid DNA from the other 10 strains into *E. coli* DH10B successfully transferred cefotaxime resistance. Cefoxitin resistance was not cotransferred with the ESBL in *E. coli* TN13 but was independently transferred by electroporation by selection on cefoxitin.

Plasmids encoding ESBLs. Plasmid DNA was isolated from all the strains and their transconjugants or electroporants. All 19 wild-type isolates had one or more plasmids. Analysis of *E. coli* transconjugants or electroporants expressing ESBLs revealed the presence of large plasmids, with estimated molecular sizes of 80 to >130 kb (data not shown).

IEF. All clinical strains and their transconjugants or electroporants exhibited a band of β-lactamase activity with an alkaline pI (7.6 to 8.4). In addition to enzymes with these pIs, 11 isolates had another band of β-lactamase activity with a pI of 5.4. One strain (strain TN13) also had an additional band of β-lactamase activity with a pI of >9. IEF of sonic extracts of

TABLE 3. Characteristics of ESBL-producing clinical isolates and their transformants, including β-lactam MICs for the clinical strains, their *E. coli* J53-2 transconjugants, and *E. coli* DH10B transformants and non β-lactam antibiotic resistance markers

Strain[a]	pI by IEF	Result by:				CTX-M identified by sequencing	MIC (μg/ml)[b]									Associated antibiotic resistance markers[c]
		PCR for bla_{CTX-M}	PCR for bla_{CMY}	PCR for bla_{TEM}	PCR-RFLP analysis		CTX	CTX-CA	CAZ	CAZ-CA	FEP	FEP-CA	ATM	ATM-CA	IPM	
TN03	5.4, 7.6	M-1/M-3		+	TEM-1	CTX-M-15	128	0.06	64	0.5	128	0.12	128	0.06	0.12	G, T, Nt, Te
Ep TN03	5.4, 7.6			+			64		32		32		64			G, T, Nt, Te
TN05	8.4	M-9		−		CTX-M-9	32	0.12	0.5	0.03	32	0.12	4	0.06	0.12	G, T, Nt, Te, Tp, Su
Ep TN05	8.4						32		0.5		4		4			G, T, Nt, Tp, Su
TN06	5.4, 8.2	M-2/M-5	−	+	TEM-1	CTX-M-2	128	0.5	64	0.5	64	1	128	1	0.12	G, T, Nt, Te, C, Tp, Su
Ep TN06	5.4, 8.2			+			64		0.5		32		64			G, T, Nt, Su
TN07	8.4	M-9		−		CTX-M-14	32	0.12	0.5	0.12	4	0.12	8	0.06	0.12	G, T, Te, C, Tp, Su
Ep TN07	8.4						4		0.5		4		4			None
TN08	5.4, 7.6	M-1/M-3		+	TEM-1	CTX-M-15	128	0.25	64	2	128	0.12	64	0.25	0.12	T, Nt, A, Te, Tp, Su
Tc TN08	5.4, 7.6			+			32		32		16		16			T, Nt, A, Te, Tp, Su
TN09	5.4, 7.6	M-1/M-3		+	TEM-1	CTX-M-15	128	0.25	64	2	64	0.12	128	0.25	0.12	G, T, Nt, Te
Ep TN09	5.4, 7.6			+			128		64		64		128			G, T, Nt, Te
TN12	5.4, 7.6	M-1/M-3		+	TEM-1	CTX-M-15	128	0.25	64	1	32	0.5	64	0.5	0.12	G, T, Nt, Te, C, Tp, Su
Tc TN12	5.4, 7.6			+			64		32		32		64			C
TN13	5.4, 8.4, >9	M-9	+	+	TEM-1	CTX-M-14	128	32	128	64	16	1	32	32	0.25	Te, C
Tc TN13	8.4		−	−			4		0.12		0.5		0.5			C
TN14	5.4, 7.6	M-1/M-3		+	TEM-1	CTX-M-15	128	0.5	64	2	128	0.25	128	0.5	0.12	G, T, Nt, Te
Ep TN14	5.4, 7.6			+			128		64		64		128			G, T, Nt, Te
TN15	5.4, 7.6	M-1/M-3		+	TEM-1	CTX-M-15	128	0.25	64	1	32	0.5	64	0.5	0.12	G, T, Nt, Te, C, Tp, Su
Tc TN15	5.4, 7.6			+			32		32		16		16			G, T, Nt, Te, Tp, Su
TN16	7.8	M-1/M-3		−		CTX-M-1	32	0.25	2	0.25	8	0.12	16	0.12	0.12	Tp, Su
Ep TN16	7.8						32		2		8		16			Tp, Su
TN17	5.4, 7.6	M-1/M-3		+	TEM-1	CTX-M-15	64	16	32	8	32	16	64	32	0.12	G, T, Nt, Te, C, Tp, Su
Tc TN17	7.6			−			8		2		4		8			G, T, Nt, Te
TN18	5.4, 7.6	M-1/M-3		+	TEM-1	CTX-M-15	128	32	64	32	64	16	128	32	0.12	G, T, Nt, Te
Ep TN18	5.4, 7.6			+			64		32		64		64			G, T, Nt, Te
TN19	8.2	M-2/M-5		−		CTX-M-2	128	4	16	2	64	4	128	4	0.25	Te, Tp, Su
Ep TN19	8.2						64		16		64		64			Te, Tp, Su
RB-01	7.8	M-1/M-3		−		CTX-M-1	64	0.06	4	0.12	64	0.12	32	0.06	0.25	Te, C, Tp, Su
Tc RB-01	7.8						4		0.12		4		2			Tp, Su
LM-01	7.6	M-1/M-3		−		CTX-M-15	>128	4	64	2	64	2	128	2	0.12	T, Nt, A, Te
Tc LM-01	7.6						64		16		32		32			T, Nt, A, Te
KP-LT	8	M-1/M-3		−		CTX-M-3	32	2	1	4	16	0.25	32	0.5	0.12	G, T, Nt, Te, C, Tp, Su
Tc KP-LT	8						4		1		1		1			G, T, Nt, Tp
KP-02	7.8	M-1/M-3		−		CTX-M-1	4	0.06	0.25	0.25	2	0.12	1	0.03	0.12	Tp, Su
Tc KP-02	7.8						4		1		1		1			Tp
KP-03	5.4, 7.6	M-1/M-3		+	TEM-1	CTX-M-15	>128	4	>128	32	128	4	>128	8	0.25	G, T, Nt, A, C, Tp, Su
Ep KP-03	5.4, 7.6			+			128		64		16		128			T, Nt, A, Tp, Su

[a] Ep, electroporant; Tc, transconjugant.
[b] CTX, cefotaxime; CA, clavulanic acid (2 μg/ml); CAZ, ceftazidime; FEP, cefepime; ATM, aztreonam; IPM, imipenem.
[c] G, gentamicin; T, tobramycin; Nt, netilmicin,; A, amikacin; Te, tetracycline; C, chloramphenicol; Tp, trimethoprim; Su, sulfamides.

TABLE 4. Genotypic characterization of bla_{CTX-M} surrounding DNA

Strain	Distance (kb) by PCR with the indicated primer:						
	Upstream				Downstream		
	IS*Ecpl*	IS*26*	ORF513	IS*903*	ORF1	*sul1* reverse	ORF1005
E. coli TN03	+0.8				+0.45		
E. coli TN05			+1.1				+1.6
E. coli TN06			+1.3			+2	
E. coli TN07	+0.8						
E. coli TN08	+0.8				+0.45		
E. coli TN09	+0.8				+0.45		
E. coli TN12	+0.8				+0.45		
E. coli TN13	+2.4						
E. coli TN14	+0.8				+0.45		
E. coli TN15	+0.8				+0.45		
E. coli TN16	+0.8						
E. coli TN17		+0.6 kb			+0.45		
E. coli TN18	+0.8 kb				+0.45		
E. coli TN19						+2	
E. coli RB-01	+0.8 kb				+0.45		
E. coli LM-01	+0.8 kb				+0.45		
K. pneumoniae KP-LT	+0.8 kb				+0.45		
K. pneumoniae KP-02	+0.8 kb						
K. pneumoniae KP-03	+0.8 kb				+0.45		

E. coli transconjugants or electroporants revealed that all but two strains (strains TN13 and TN17) transferred the β-lactamase activity with a pI of 5.4 (Table 3).

Characterization of β-lactamase-encoding (*bla*) genes. The results of PCR and sequence analysis are summarized in Table 3. PCR experiments were positive for bla_{CTX-M} for all isolates and their transconjugants or electroporants. These results corresponded to pI values of 7.6 to 8.4 for CTX-M-type enzymes.

Sequence analysis of the deduced amino acid sequences showed the presence of various CTX-M-type enzymes: CTX-M-1, -2, -3, -9, and -14 and particularly CTX-M-15 (with a glycine at position 240) (23), produced by TN03, TN08, TN09, TN12, TN14, TN15, TN17, TN18, LM-01, and KP-03.

The 11 isolates harboring the enzyme with a pI of 5.4 were found to carry TEM-1 by PCR-RFLP analysis. Amplification was obtained with the CMY-specific primers for *E. coli* TN13 producing a β-lactamase of pI >9, and sequencing showed that this β-lactamase was CMY-2.

Exploration of the regions surrounding bla_{CTX-M} genes. PCR identified the insertion sequence IS*Ecp1* upstream of the bla_{CTX-M} gene in 15 strains (Table 4). The sizes of the PCR products were about 0.8 kb in all except one strain (strain TN13). For this strain, the PCR fragment was about 2.4 kb, suggesting the insertion of an additional DNA fragment. The upstream region of $bla_{CTX-M-15}$ in *E. coli* strain TN17, analyzed by PCR, contained the transposase gene of the insertion sequence IS*26*. PCR with the IS*903*-specific primer produced no amplicons.

The genetic organization of the bla_{CTX-M} genes of *E. coli* TN05, TN06, and TN19 was investigated by cloning, partial sequencing of the regions surrounding these genes, and PCR analysis. Upstream of the bla_{CTX-M} gene, these strains harbored a region common to the complex *sul1*-type integron (38, 39). This region includes ORF513, which is present in In*6* and In*7*. Analysis of the nucleotide sequence of the region downstream of the $bla_{CTX-M-9}$ gene from *E. coli* TN05 revealed the presence of ORF1005. In strains TN06 and TN19, we found part of the 3′-CS complex *sul1*-type integron downstream of the $bla_{CTX-M-2}$ stop codon.

Epidemiological analysis. ERIC-PCR and Rep-PCR analyses were used to analyze the molecular epidemiology of the 19 clinical isolates. Rep-PCR showed that five *E. coli* clinical isolates were clonally related (Fig. 1A). Plasmids isolated from transconjugants or electroporants of these five *E. coli* strains yielded similar restriction patterns after digestion with BamHI (Fig. 2) These strains were isolated from five different patients with no apparent relationships in time or space. ERIC-PCR of the three *K. pneumoniae* strains gave different restriction patterns (Fig. 1B).

DISCUSSION

We studied 19 enterobacterial strains collected in four Paris hospitals between June 2000 and August 2002. The antimicrobial susceptibility patterns showed that the strains harbored ESBLs responsible for resistance to penicillin, broad-spectrum cephalosporins, and aztreonam. The ESBLs were inhibited by clavulanate and tazobactam. Importantly, the isolates were more resistant to cefotaxime and aztreonam than to ceftazidime, suggesting that they were CTX-M producers. Some CTX-M ESBLs confer high-level resistance to ceftazidime, and this was the case for 10 of our strains. Cefotaxime resistance could be transferred by conjugation for 9 of the 19 isolates and by electroporation for 10 isolates. The transconjugants or electroporants showed similar resistance profiles.

We used primers specific to each cluster of CTX-M-type enzymes. We obtained amplification of all the strains, confirming the presence of such enzymes. Analysis of the deduced amino acid sequences showed the diversity of the CTX-M-type enzymes. Indeed, we found six different enzymes, including CTX-M-15 (23), produced by 10 isolates. This member of the CTX-M family showed increased activity against ceftazidime. Sequence analysis revealed an Asp-240 Gly substitution. This substitution has already been reported in CTX-M-16 and is known to confer high-level resistance to ceftazidime (9, 23). Another substitution known to increase the hydrolyzing activity of ceftazidime is due to the Pro-167 Ser substitution in CTX-M-19 (30).

Of the 19 strains producing cefotaximases and screened for the presence of TEM β-lactamases genes, 11 were found to express TEM-1. This association is frequent and has already been described in the literature (9, 30, 32). Lastly, *E. coli* strain TN13, which had a high level of resistance to cefoxitin (MIC, 128 $mg \cdot liter^{-1}$), harbored both CTX-M-14 and CMY-2; to our knowledge this is only the third report of such a combination (5, 40). All the strains yielded an enzyme with an alkaline pI, as observed for the CTX-M-type enzymes. Some had an additional band of β-lactamase activity with a pI of 5.4 (11 strains) and, in one case (strain TN13), a third band of pI >9; these bands correspond to TEM-1 and CMY-2, respectively.

Previously reported analyses of the surrounding regions have shown the frequent association of β-lactamase genes with the insertion sequence IS*Ecp1*. This element was first de-

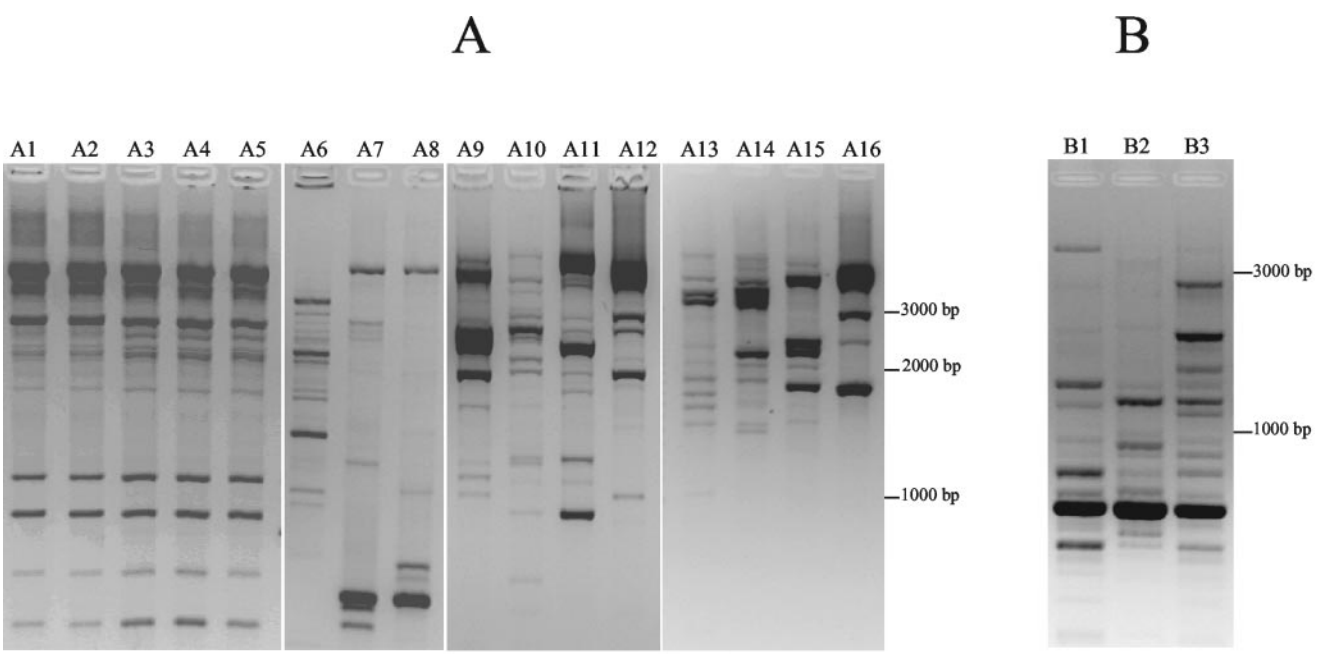

FIG. 1. Fingerprinting analysis of ESBL-producing strains. (A) Rep-PCR of *E. coli* clinical isolates carrying bla_{CTX-M} genes. Lane A1, *E. coli* TN18; lane A2, *E. coli* TN03; lane A3, *E. coli* TN08; lane A4, *E. coli* TN09; lane A5, *E. coli* TN14; lane A6, *E. coli* TN15; lane A7, *E. coli* RB-01; lane A8, *E. coli* TN12; lane A9, *E. coli* TN13; lane A10, *E. coli* TN05; lane A11, *E. coli* TN06; lane A12, *E. coli* TN07; lane A13, *E. coli* TN16; lane A14, *E. coli* TN17; lane A15, *E. coli* LM-01; lane A16, *E. coli* TN19. (B) ERIC-PCR of *K. pneumoniae* isolates. Lane B1, *K. pneumoniae* KP-LT; lane B2, *K. pneumoniae* KP-02; lane B3, *K. pneumoniae* KP-03.

scribed upstream of bla_{CMY-4} (P. D. Stapleton, Abstr. 39th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 1457, 1999), and then IS*Ecp1* was detected upstream of several bla_{CTX-M} genes (13, 14, 16, 23, 32). Of the 19 clinical isolates studied here, 15 had this same insertion sequence upstream of the bla_{CTX-M} gene. Another insertion sequence, IS*26*, was described by Saladin et al. (32) to be upstream of a $bla_{CTX-M-1}$ gene. This structure was found upstream of the bla_{CTX-M} gene in one of our strains (strain TN17). It is worth mentioning that none of the 19 strains harbored the IS*903* insertion sequence. Interestingly, PCR amplification with primers specific for the *tnpA* genes of IS*Ecp1* or IS*26* was negative for three strains. Analysis of the surrounding regions after cloning and end sequencing allowed us to draw some primers. PCR analysis showed the presence of a structure-type integron. The sizes of the fragments were compatible with the genetic organization described in the literature for two $bla_{CTX-M-2}$ sequences and one $bla_{CTX-M-9}$ sequence inserted in novel complex *sul1*-type integrons In*35*, InS*21*, and In*60*, respectively (3, 15, 31). Our three bla_{CTX-M} genes (two $bla_{CTX-M-2}$ genes and one $bla_{CTX-M-9}$ gene) were located in unusual class 1 integrons. ORF1005 was found in the 3′ end of $bla_{CTX-M-9}$ (strain TN05) when PCR amplification with the *sul*1-specific primer was positive with strains TN06 and TN19. ORF513 was found at the 5′ ends of these *bla* genes in these three strains and has already been reported; ORF513 may be a transposase (38). This is the first report of a bla_{CTX-M} gene in a complex *sul1*-type integron in France. These findings confirm the diversity of transposable elements and integrons associated with bla_{CTX-M} genes.

Ten of the 19 strains harbored a CTX-M-15 enzyme, enabling us to determine whether these clinical isolates were genetically related. The results of Rep-PCR strongly suggested that five *E. coli* isolates with no epidemiological relationship were identical. In addition, plasmids extracted from transconjugants or electroporants yielded similar restriction patterns, supporting evidence of the dissemination of these enterobacterial strains. The corresponding patients were from three long-term-care facilities near Tenon Hospital, suggesting the presence of this enzyme in these institutions (28).

CTX-M β-lactamases constitute a novel and rapidly growing family of plasmid-mediated ESBLs. Outbreaks have been described in several countries (6, 40). These enzymes have been

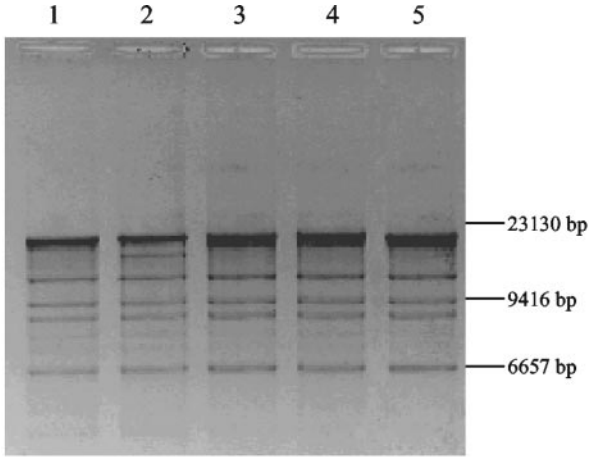

FIG. 2. BamHI-digested plasmid profiles of transconjugants or electroporants producing CTX-M-type β-lactamases. Lane 1, *E. coli* TN03; lane 2, *E. coli* TN08; lane 3, *E. coli* TN09; lane 4, *E. coli* TN14; lane 5, *E. coli* TN18.

reported at a much lower frequency in France (16, 30, 32). However, CTX-M β-lactamase producers represented 50% of the ESBL-harboring *E. coli* clinical isolates in Tenon Hospital in 2001 and 2002 (unpublished data). A previous study described 9 CTX-M-producing strains collected over 11 years (32), while we found 19 CTX-M-producing isolates of the *Enterobacteriaceae* during a 2-year survey of four Paris hospitals. This work confirms the emergence of CTX-M-type enzymes and their spread in the Paris area.

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