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Three Cefotaximases, CTX-M-9, CTX-M-13, and CTX-M-14, among *Enterobacteriaceae* in the People's Republic of China

Aroonwadee Chanawong,[1] Fatima Hannachi M'Zali,[1] John Heritage,[1] Jian-Hui Xiong,[2,3] and Peter Michael Hawkey[1,2]*

Division of Microbiology, University of Leeds, Leeds LS2 9JT,[1] and Department of Immunity and Infection, The Medical School, University of Birmingham, Birmingham B15 2TT,[2] United Kingdom, and First Municipal People's Hospital of Guangzhou, Guangzhou 510180, People's Republic of China[3]

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Of 15 extended-spectrum β-lactamase (ESBL)-producing isolates of the family *Enterobacteriaceae* collected from the First Municipal People's Hospital of Guangzhou, in the southern part of the People's Republic of China, 9 were found to produce CTX-M ESBLs, 3 produced SHV-12, and 3 produced both CTX-M and SHV-12. Eleven isolates produced either TEM-1B or SHV-11, in addition to an ESBL. Nucleotide sequence analysis of the 12 isolates carrying $bla_{\text{CTX-M}}$ genes revealed that they harbored three different $bla_{\text{CTX-M}}$ genes, $bla_{\text{CTX-M-9}}$ (5 isolates), $bla_{\text{CTX-M-13}}$ (1 isolate), and $bla_{\text{CTX-M-14}}$ (6 isolates). These genes have 98% nucleotide homology with $bla_{\text{Toho-2}}$. The $bla_{\text{CTX-M}}$ genes were carried on plasmids that ranged in size from 35 to 150 kb. Plasmid fingerprints and pulsed-field gel electrophoresis showed the dissemination of the $bla_{\text{CTX-M}}$ genes through transfer of different antibiotic resistance plasmids to different bacteria, suggesting that these resistance determinants are highly mobile. Insertion sequence IS*Ecp1*, found on the upstream region of these genes, may be involved in the translocation of the $bla_{\text{CTX-M}}$ genes. This is the first report of the occurrence of SHV-12 and CTX-M ESBLs in China. The presence of strains with these ESBLs shows both the evolution of $bla_{\text{CTX-M}}$ genes and their dissemination among at least three species of the family *Enterobacteriaceae*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter cloacae*, isolated within a single hospital. The predominance of CTX-M type enzymes seen in this area of China appears to be similar to that seen in South America but is different from those seen in Europe and North America, suggesting different evolutionary routes and selective pressures. A more comprehensive survey of the ESBL types from China is urgently needed.

Until 1994, SHV-2 was the only extended-spectrum β-lactamase (ESBL) described in bacteria originating from the People's Republic of China (10). Recently, ESBL-producing bacteria have been reported from China, but molecular characterization of these ESBLs has not yet been undertaken (38, 45–47). During an antimicrobial resistance-monitoring project in the First Municipal People's Hospital of Guangzhou, which is in the southern part of China, in 1997, ESBL production rates in *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, and *Citrobacter freundii* were 18, 19, 16, and 18%, respectively (45). In 1998, the rates of resistance due to the production of ESBLs rose dramatically, to 33% for *E. coli*, 37% for *K. pneumoniae*, 18% for *E. cloacae*, and 25% for *C. freundii* (46). A limited number of these ESBL-producing isolates have been selected for investigation in the present study.

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MATERIALS AND METHODS

Bacterial strains. Fifteen nonduplicate ESBL-producing isolates of the family *Enterobacteriaceae* were selected from a collection of ESBL-producing strains isolated as part of an antimicrobial resistance monitoring project in the First Municipal People's Hospital of Guangzhou, a 900-bed hospital in the People's Republic of China, between 1997 and 1998. The isolates were chosen to represent the range of species and profiles of resistance to broad-spectrum cephalosporins seen in the hospital during the study period. These included eight *E. coli* isolates, three *K. pneumoniae* isolates, three *E. cloacae* isolates, and one *C. freundii* isolate. All isolates were positive by a double-disk diffusion test, confirming the production of an ESBL (23). The isolates were identified by conventional methods (25). Species identification of the *Enterobacter* isolates was achieved by the API 20E strip method (BioMerieux S.A., Marcy l'Etoile, France).

Susceptibility testing. A disk diffusion susceptibility test was performed on Iso-Sensitest agar (Oxoid, Basingstoke, England) by a comparative method (19). Cefepime, ceftazidime, cefotaxime, cefoxitin, imipenem, and piperacillin plus tazobactam disks were used (Oxoid). The MICs of aztreonam, cefotaxime, ceftazidime, and ceftazidime plus clavulanic acid (at a fixed concentration of 4 μg/ml) were determined by an agar dilution method (19). The antibiotics were supplied as follows: aztreonam, E. R. Squibb & Sons, Hounslow, England; cefotaxime, Roussel Laboratories, Uxbridge, England; ceftazidime, Glaxo Laboratories, Greenford, England; and clavulanic acid, SmithKline Beecham Pharmaceuticals, Brentford, England. *E. coli* strain NCTC 10418 was used as an antibiotic-sensitive control, and *E. coli* HB101(pAFF611) encoding SHV-5 was used as a positive control for the ESBL phenotype (22).

Transfer of cefotaxime and ceftazidime resistance markers. Plasmid transfer of cefotaxime or ceftazidime resistance markers was performed by a broth culture conjugation method (37). Laboratory *E. coli* strains UB1637 (*lys his trp lac recA* Str^r) and UB5201 (*pro met recA* Nal^r) were used as recipients. The mating time was 4 h. Transconjugants were selected on nutrient agar plates containing either cefotaxime or ceftazidime (2 μg/ml; Sigma, Poole, England) and either streptomycin (25 to 400 μg/ml; Sigma) to select for UB1637 transconjugants or nalidixic acid (25 μg/ml; Sigma) if UB5201 was used as the recipient strain.

Analytical isoelectric focusing. Crude β-lactamase preparations were extracted by a sonication method (11). Analytical isoelectric focusing was performed as described previously (17), except that agarose gels containing Pharmalyte (pH 3 to 10; Pharmacia Biotech, Uppsala, Sweden) were used. The

* Corresponding author. Mailing address: Department of Immunity and Infection, The Medical School, University of Birmingham, Birmingham B15 2TT, United Kingdom. Phone: 44 121 424 1240. Fax: 44 121 772 6229. E-mail: HawkeyP@heartsol.wmids.nhs.uk.

TABLE 1. Oligonucleotide primers used in the study

Gene or gene region	Primer	Oligonucleotide sequence (5′→3′)	GenBank accession no. (reference)	Position of primer[a]
bla_{CTX-M}	C-1	AACACGGATTGACCGTCTTG	D89862 (29)	82–101
	C-2	TTACAGCCCTTCGGCGAT	D89862	981–964
	C-3	GCTGGTTCTGGTGACCTATTTTACCC	D89862	881–907
	C-5	CCAGCAGCAGCGGAATGCAC	D89862	175–156
Downstream	C-4	GTTGTCGGGAAGATACGTGA	AF252622 (this study)	2683–2664
	C-6	CATCACGACTGTGCTGGTCATTAAAC	AF252622	2860–2885
	C-7	GAAATCGCACACCTGGTAATTGATTC	AF252622	3035–3050
Upstream	C-8	CACTCACCTCACAAGCAACGAA	AJ242809 (unpublished)	902–923
	C-9	AAATTGACATCCATTACGATTGA	AJ242809	553–575
	C-10	TGAGAATGCAAAAAGCAATGGG	AJ242809	252–273
	C-11	CTTCAAAAAGCATAATCAAAGCCT	AJ242809	19–43
	C-12	ACCATATATCGAGTCATAAAAGCTGA	AJ242809	146–121
	C-13	AAAAATCCCATTGCTTTTTGCATTCT	AJ242809	279–254

[a] Position counted from the first nucleotide of the entire sequence, as numbered for the GenBank submission.

β-lactamase activity was detected by overlaying the gel with filter paper containing nitrocefin (0.5 mg/ml).

PCR amplification. All isolates were first screened for the presence of bla_{SHV} and bla_{TEM} by previously described PCR protocols (18, 30, 32). Isolates carrying neither bla_{SHV} nor bla_{TEM} were further screened for the presence of bla_{Toho-1} and $bla_{CTX-M-1}$ as described previously (15, 48). In addition, primers C-1 and C-2 (Table 1), designed from the nucleotide sequence of bla_{Toho-2} (29), were used to amplify a 906-bp gene fragment. Bacterial DNA was prepared by suspending one or two fresh colonies in 50 μl of sterile distilled water and heating at 95°C for 5 min. PCR amplification was carried out under the following conditions: 95°C for 3 min, followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 5 min. Primer C-11, designed from the nucleotide sequence of the *tnpA* gene of IS *Ecp1* (P. D. Stapleton, GenBank accession no. AJ242809), and primer C-5, an internal bla_{CTX-M} primer, were used to amplify the flanking regions upstream of the bla_{CTX-M} genes. All primers designed for this study are shown in Table 1.

Differentiation of bla_{TEM} genes. The bla_{TEM} genes were identified by PCR-restriction fragment length polymorphism (RFLP) analysis (2). The restriction endonucleases used included *Alu*I, *Bcl*I, *Bpm*I, *Bsma*I, *Hha*I, *Hpa*II, *Hph*I, *Mse*I, *Nla*III, and *Sau*3AI. Restriction endonuclease *Alu*I was supplied by Gibco BRL (Life Technologies, Paisley, United Kingdom), *Bcl*I was obtained from Boehringer Mannheim (Mannheim, Germany), and the remaining restriction endonucleases were purchased from New England Biolabs. Four *E. coli* reference strains, C600(pCFF04) encoding TEM-3, C600(pUD16) encoding TEM-4, C600(pCFF14) encoding TEM-5, and C600(pIF100) encoding TEM-7 (22), were used as controls.

Nucleotide sequence determination. The nucleotide sequences of the bla_{SHV} and bla_{TEM} genes were determined as described previously (30, 32). The 906-bp fragments of the bla_{CTX-M} genes were also sequenced. To determine the remaining bla_{CTX-M} sequences, plasmid DNAs digested with either *Bam*HI or *Eco*RI, depending on which enzyme gave the smaller fragment containing bla_{CTX-M}, were used as templates; and the sequence of the entire open reading frame (ORF) was determined. Primers C-1, C-2, and C-3 (Table 1) were used as primers for determination of the bla_{CTX-M} gene sequences. The digested plasmids or amplification products were purified with the QIAquick PCR purification kit (QIAGEN, Crawley, England), and their nucleotide sequences were determined with an ABI PRISM automated sequencing machine (model 373, version 3.3), according to the manufacturer's instructions. The nucleotide sequences of both DNA strands and two independently generated amplimers were determined.

The nucleotide sequences of the flanking regions of the $bla_{CTX-M-14}$ gene in *E. coli* strain 8 were determined by using the upstream primers shown in Table 1. The primers used to sequence the upstream region, primers C-8 to C-13, were derived from the nucleotide sequence of the *tnpA* gene of IS *Ecp1* (P. D. Stapleton, GenBank accession no. AJ242809). Walking primers, primers C-4, C-6, and C-7, were designed from data generated in the present study for determination of the sequence of the downstream region. In addition, the nucleotide sequences of the downstream regions of the $bla_{CTX-M-9}$ and $bla_{CTX-M-13}$ genes in *E. coli* strain 2 and *K. pneumoniae* strain 1, respectively, were determined with primer C-3 (Table 1).

Plasmid DNA analysis, Southern blotting, and hybridization. Plasmid DNA was extracted by a rapid method (4) and was separated by agarose gel electrophoresis. Plasmids isolated from *E. coli* strains NCTC 50192 and NCTC 50193 were used as standard size markers. In addition, plasmid DNA from transconjugants was digested with either *Bam*HI or *Eco*RI. The digested fragments were analyzed by electrophoresis with a 1-kb ladder (Promega) as a DNA size marker. Either plasmid DNAs or restriction fragments were transferred from the agarose gel to a nylon membrane (Boehringer Mannheim) by the method of Southern (39) and were hybridized with digoxigenin-labeled bla_{SHV}, bla_{TEM}, or bla_{CTX-M} gene fragments with the PCR DIG detection system (Boehringer Mannheim).

PFGE. Pulsed-field gel electrophoresis (PFGE) of chromosomal DNA for strain typing was carried out as described by Gautom (12). Chromosomal DNA was digested with 10 U of *Xba*I (Gibco BRL) at 37°C for 4 h, according to the supplier's instructions. The DNA fragments were separated by electrophoresis in a 1.2% (wt/vol) agarose gel (Agarose NA; Amersham Pharmacia Biotech AB, Sweden) with an LKB Pharmacia (Uppsala, Sweden) system at 175 V and 12°C for 20 h. The pulse time was increased from 5 to 35 s. A 48.5-kb bacteriophage λ ladder (Bio-Rad, Hemel Hempstead, England) was used as a DNA size marker. The DNA patterns obtained by PFGE were classified visually, and only fragments larger than 48.5 kb, which is the smallest rung of the bacteriophage λ DNA ladder, were considered when the PFGE patterns were compared. The patterns were considered to belong to the same type if there was a difference of no more than three bands (43).

Nucleotide sequence accession numbers. The nucleotide sequence data for $bla_{CTX-M-9}$, $bla_{CTX-M-13}$, and $bla_{CTX-M-14}$ genes were submitted to the GenBank nucleotide sequence database and assigned accession numbers AF252621, AF252623, and AF252622, respectively.

RESULTS

Bacterial strains. Each isolate was collected from a different patient, and clinical details are given in Table 2.

Susceptibility testing. All isolates were sensitive to imipenem, and all isolates except *E. coli* strains 5 and 6 and *E. cloacae* strains 1 and 3 were intermediately susceptible to cefepime and piperacillin plus tazobactam; *E. coli* strains 5 and 6 and *E. cloacae* strains 1 and 3 were resistant to cefepime. *E. coli* strain 1 and *K. pneumoniae* strain 2 were resistant to cefoxitin, as were the *E. cloacae* isolates and the *C. freundii* strain. On the basis of the MICs of ceftazidime and cefotaxime, three resistance phenotypes were observed among the 15 ESBL producers (Table 3). Nine isolates were more resistant to cefotaxime than to ceftazidime, while three isolates were more resistant to ceftazidime than to cefotaxime. For the remaining three isolates, high cefotaxime and ceftazidime MICs were observed. The presence of clavulanic acid at a fixed con-

TABLE 2. Sources of the 15 clinical isolates used in the study

Organism	Date collected (day/mo/yr)	Ward	Specimen[a]	Clinical diagnosis	Antibiotic therapy[b]
E. coli 1	13/06/97	Respiratory	Sputum	Bronchopneumonia secondary to chronic bronchitis	Unknown
E. coli 2	15/08/97	Cardiology	Sputum	Bronchopneumonia secondary to chronic bronchitis and emphysema	None
E. coli 3	19/03/97	Pediatric	NS	Unknown	Unknown
E. coli 4	24/10/97	Pediatric	US	Neonatal intracerebral bleeding	Penicillin
E. coli 5	21/10/98	Respiratory	Sputum	Low differentiation squamous cell cancer of right lung and chronic pneumonia	None
E. coli 6	23/10/98	Respiratory	Sputum	Multiple thrombi, cerebral embolus, and aspiration pneumonia	None
E. coli 7	30/10/98	Gastroenterology	Blood	Rectal carcinoma and pneumonia secondary to obstruction	Ceftriaxone
E. coli 8	06/03/98	Hematology	Urine	Myelodysplastic syndrome and urinary tract infection	None
K. pneumoniae I	05/10/98	Neurology	Sputum	Thrombic cerebral embolus and pneumonia	None
K. pneumoniae 2	21/10/98	Urology	Sputum	Diabetes mellitus and peripheral neuritis	Unknown
K. pneumoniae 3	28/10/98	Respiratory	Sputum	Chronic bronchitis and chronic pulmonary emphysema	Ceftriaxone
E. cloacae 1	07/03/97	Neurology	Sputum	Thrombic cerebral embolus and pneumonia	Unknown
E. cloacae 2	24/04/97	Hematology	Sputum	Acute myeloblastic leukemia with pneumonia	Unknown
E. cloacae 3	07/10/98	Chinese medicine	Sputum	Pneumonia secondary to Parkinson's disease	None
C. freundii	18/03/97	Surgery	Sputum	Left temporal bone defect	Unknown

[a] NS, nasopharyngeal swab; US, umbilical swab.
[b] Clinical outcome data were not available.

centration of 4 μg/ml greatly reduced the MICs of both cefotaxime and ceftazidime except for those for *K. pneumoniae* strains 1 and 3.

Transfer of cefotaxime and ceftazidime resistance markers. Plasmid transfer of the ESBL phenotype to a laboratory strain of *E. coli* was successful for 9 of 15 isolates (60%). The susceptibility testing results for the transconjugants are shown in Table 3.

PCR amplification of β-lactamase genes. Of the 15 ESBL-producing isolates, 4 carried bla_{TEM}, 2 carried bla_{SHV}, and 6 carried both bla_{SHV} and bla_{TEM} (Table 3). The remaining three isolates did not harbor either bla_{SHV} or bla_{TEM}. With Toho-1-specific primers (48), no amplification products were detected from any isolate, but predicted PCR products were obtained with CTX-M-1-specific primers (15). A bla_{CTX-M} amplimer from *K. pneumoniae* strain 1 was selected for nucleotide sequence analysis. The gene was more closely related to bla_{Toho-2} than to $bla_{CTX-M-1}$. Primers C-1 and C-2, whose sequences were derived from the nucleotide sequence of the bla_{Toho-2} gene, were used instead of primers specific for CTX-M-1. Twelve isolates were found to carry bla_{CTX-M} genes, of which three harbored both bla_{CTX-M} and bla_{SHV-12}.

Characterization of SHV β-lactamase genes. Nucleotide sequence analysis of the eight isolates carrying bla_{SHV} genes revealed that six isolates harbored bla_{SHV-12} and that the remaining isolates carried bla_{SHV-11}. The bla_{SHV-12} gene has a silent mutation (CT**A**→CT**G** [the silent mutation is indicated in boldface]) in the codon encoding the amino acid at position 138, as described previously (33). The bla_{SHV-11} sequence of *K. pneumoniae* strain 1 contains five silent mutations (indicated in boldface), at amino acid positions 112 (CA**C**→CA**T**), 123 (TG**C**→TG**T**), 138 (CT**A**→CT**G**), 268 (AC**G**→AC**C**), and 287 (GA**G**→GA**A**), using the numbering convention of Ambler et al. (1), whereas that of strain 2 carries four silent mutations at amino acid positions 112, 138, 268, and 287, as described above. The silent mutations at amino acid positions 123, 268, and 287 have not previously been described in bla_{SHV-11}, as far as the authors can ascertain.

Characterization of TEM β-lactamase genes. By using 10 restriction endonucleases, all amplimers of the bla_{TEM} genes from 10 isolates yielded identical RFLP profiles, in which only one silent mutation at nucleotide position 436, using the numbering system of Sutcliffe (41), was detected (data not shown). These profiles were consistent with those of bla_{TEM-1} variants. Nucleotide sequence analysis of the bla_{TEM} genes from two representative isolates, *E. coli* strains 1 and 8, showed that both genes were identical to the bla_{TEM-1B} gene described by Goussard and Courvalin (16).

Characterization of CTX-M β-lactamase genes. Among the 12 bla_{CTX-M}-carrying isolates, $bla_{CTX-M-9}$ (5 isolates), $bla_{CTX-M-13}$ (1 isolate), and $bla_{CTX-M-14}$ (6 isolates) were identified by nucleotide sequence analysis (Fig. 1). The gene encoding CTX-M-14 differs from that encoding CTX-M-9 by only one amino acid change, at position 231 (Ala→Val), whereas CTX-M-13 differs from CTX-M-9 by four amino acid substitutions, at positions 3 (Val→Met), 53 (Val→Lys), 154 (Ala→Glu), and 231 (Ala→Val). Between CTX-M-13 and CTX-M-14, there are three amino acid substitutions at positions 3, 53, and 154, as described above.

Nucleotide sequence analysis of the upstream region of $bla_{CTX-M-14}$ in *E. coli* strain 8 revealed the presence of at least part of a putative insertion sequence, which showed 100% identity to the *tnpA* gene of IS *Ecp1* (P. D. Stapleton, GenBank accession no. AJ242809) and 99.9% identity to the insertion sequence found upstream of bla_{CMY-5} (44). PCR amplification with primers C-5 and C-11 (Table 1) was performed to screen for the presence of the insertion sequence in the bla_{CTX-M}-carrying isolates. The predicted amplimers were obtained from

TABLE 3. Phenotypic and genotypic characterization of the 15 clinical isolates

Genus species	MIC[a] (μg/ml)					pI	β-Lactamase(s) detected by PCR[b]	PFGE pattern	Plasmid profile (kb)
	ATM	CTX	CTX+	CAZ	CAZ+				
E. coli 1	128	8	0.5	64	4	5.4, 8.2	SHV-12, TEM-1B*	Eco-1	130, 10, 8.5
Transconjugant[c]	32	2	0.5	16	2	NT[d]	SHV-12		130
E. coli 2	8	32	≤0.25	2	≤0.25	5.4, 8.1	CTX-M-9, TEM-1B	Eco-2	150, 7.0, 5.0, 4.0
Transconjugant	4	16	≤0.25	1	≤0.25	NT	CTX-M-9		150, 5.0, 4.0
E. coli 3	4	16	≤0.25	1	≤0.25	5.4, 8.1	CTX-M-9, TEM-1B	Eco-2	150, 7.0, 5.0, 4.0
Transconjugant	2	8	≤0.25	0.5	≤0.25	NT	CTX-M-9		150, 5.0, 4.0
E. coli 4	2	32	NT	0.5	≤0.25	8.1	CTX-M-9	Eco-3	150
E. coli 5	>128	128	NT	>128	0.25	8.1	CTX-M-14, SHV-12, TEM-1B	Eco-4	130, 3.5
E. coli 6	128	64	NT	64	1	8.1	CTX-M-14, SHV-12, TEM-1B	Eco-4	130, 3.5
E. coli 7	4	32	≤0.25	1	≤0.25	5.4, 8.1	CTX-M-14, TEM-1B	Failed[e]	130
Transconjugant	4	16	≤0.25	0.5	≤0.25	NT	CTX-M-14		130
E. coli 8	16	64	≤0.25	4	0.5	5.4, 8.1	CTX-M-14, TEM-1B*	Eco-5	130, 5.5, 4.8, 3.5, 2.5, 1.9
Transconjugant	4	8	≤0.25	1	≤0.25	NT	CTX-M-14		130
K. pneumoniae 1	16	128	16	32	64	5.4, 7.6, 8.2	CTX-M-13, TEM-1B, SHV-11	Kp-1	55, 35, 7.0
Transconjugant	8	32	≤0.25	2	≤0.25	NT	CTX-M-13		35
K. pneumoniae 2	128	>128	≤0.25	32	1	7.6, 8.1	CTX-M-14, SHV-11	Kp-2	150, 60, 7.0
Transconjugant	4	32	≤0.25	1	≤0.25	NT	CTX-M-14		60
K. pneumoniae 3	16	32	NT	8	16	8.1	CTX-M-9	Kp-3	150, 7.0, 2.7, 1.9
E. cloacae 1	32	>128	0.5	8	0.5	8.1, 9.25	CTX-M-9	Ecl-1	85, 2.4
Transconjugant	8	32	≤0.25	2	≤0.25	NT	CTX-M-9		Plasmid not found
E. cloacae 2	64	16	0.5	64	≤0.25	5.4, 9.25	SHV-12, TEM-1B	Ecl-2	Plasmid not found
Transconjugant	8	0.5	≤0.25	8	≤0.25	NT	SHV-12, TEM-1B		Plasmid not found
E. cloacae 3	>128	128	NT	128	4	8.1	CTX-M-14, SHV-12	Ecl-3	150, 2.4
C. freundii	128	8	NT	128	≤0.25	5.4, 8.2	SHV-12, TEM-1B	NT	7.0

[a] ATM, aztreonam; CAZ, ceftazidime; CAZ+, ceftazidime plus 4 μg of clavulanic acid per ml; CTX, cefotaxime; CTX+, cefotaxime plus 4 μg of clavulanic acid per ml.

[b] The bla_{SHV} and bla_{CTX-M} genes were characterized by nucleotide sequence analysis, and the bla_{TEM} gene was characterized by PCR-RFLP (2). An asterisk indicates the bla_{TEM} gene that was confirmed by nucleotide sequence analysis.

[c] All transconjugants except those of *E. cloacae* 2 were *E. coli* UB1637 transconjugants; the *E. cloacae* 2 transconjugant was an *E. coli* UB5201 transconjugant.

[d] NT, not tested.

[e] Despite retesting, no DNA fragments were seen.

the isolates carrying $bla_{CTX-M-13}$ and $bla_{CTX-M-14}$, but no PCR products were detected from the isolates carrying $bla_{CTX-M-9}$. Analysis of the nucleotide sequence of the region downstream of the $bla_{CTX-M-9}$, $bla_{CTX-M-13}$, and $bla_{CTX-M-14}$ genes from *E. coli* strain 2, *K. pneumoniae* strain 1, and *E. coli* strain 8, respectively, revealed that the sequence of the $bla_{CTX-M-14}$ gene is different from those of the $bla_{CTX-M-9}$ and $bla_{CTX-M-13}$ genes. Three hundred forty-five nucleotide bases of the downstream regions of the latter two genes were determined and showed 99% identity. These showed 99 to 100% similarity with the nucleotide sequence downstream of the CTX-M-9 gene (M. Sabate, F. Navarro, J. Barbe, E. Miro, B. Mirelis, and G. Prats, GenBank accession no. AF373104). In addition, analysis of the downstream region of the $bla_{CTX-M-14}$ gene showed that the first 486 nucleotides had 99% similarity to a part of IS*903.B* (31). Furthermore, a 5-bp directed repeat sequence was found immediately upstream from the left inverted repeat of IS*Ecp1* and immediately downstream from the part of the IS*903.B* gene.

Isoelectric focusing. Detection of β-lactamases with pIs of 5.4 and 7.6 corresponded with the presence of TEM-1B and SHV-11, respectively. The three CTX-M β-lactamases found

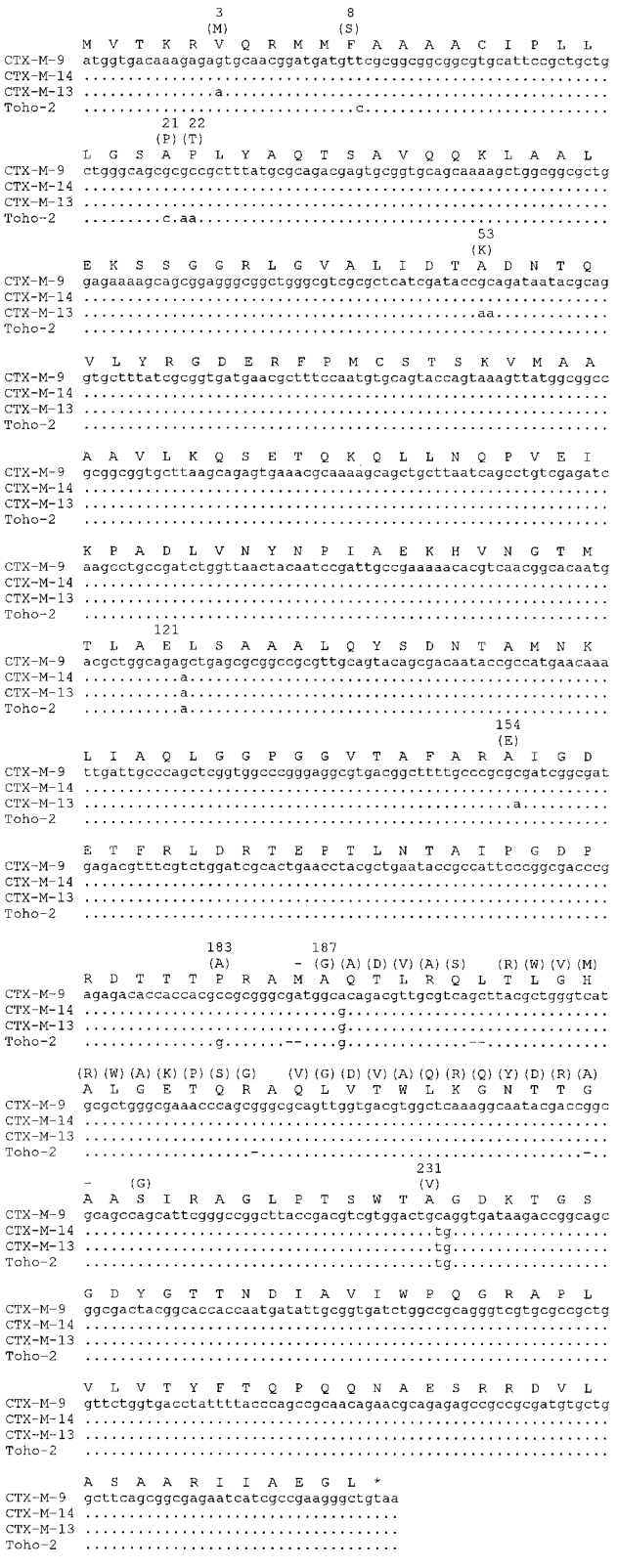

FIG. 1. Nucleotide sequence alignment of the open reading frames of CTX-M-9 36; this study), CTX-M-13 (this study), CTX-M-14 (this study), and Toho-2 (29). Dots indicate identical nucleotides. Hyphens show nucleotide deletions. Amino acids in parentheses indicate substitutions compared with the sequence of CTX-M-9. The numbering is according to Ambler et al. (1). The asterisk at the end indicates the stop codon.

in the present study have the same pI, 8.1. The enzyme with a pI of 8.2 was the SHV-12 β-lactamase. The presence of enzymes with pIs of >9.0 was consistent with the production of AmpC enzymes. Only one band with a pI of 8.1 was seen for β-lactamases extracted from the three isolates carrying both the bla_{SHV-12} and the $bla_{CTX-M-14}$ genes. The single band detected may indicate the presence of more than one enzyme with identical or, rather, similar pIs. In addition, the absence of bands representing TEM-1 in *E. coli* strains 5 and 6, SHV-12 in *E. cloacae* strain 2, and the AmpC β-lactamase in the *C. freundii* strain and *E. cloacae* strain 3 may be because enzyme expression was minimal.

Strain typing. PFGE analysis of the 15 isolates revealed that each of the *K. pneumoniae* isolates and the *E. cloacae* isolates gave unique PFGE DNA patterns, whereas five PFGE patterns were seen among the eight *E. coli* isolates (Table 3). *E. coli* strains 2 and 3 were indistinguishable, as were strains 5 and 6. Attempts to type *E. coli* strain 7 failed.

Plasmid DNA analysis. Among the six isolates carrying bla_{SHV-12}, a large plasmid, of approximately 130 kb, from *E. coli* strain 1 hybridized with the bla_{SHV} probe, but no hybridization with any plasmid from the remaining five isolates was detected (data not shown). Only chromosomal DNAs from these isolates, including the transconjugant of *E. cloacae* strain 2, hybridized with the bla_{SHV}-specific probe. For the five $bla_{CTX-M-9}$-carrying isolates, plasmids of approximately 150 kb from *E. coli* strains 2 and 3 and *K. pneumoniae* strain 3 hybridized with the bla_{CTX-M}-specific probe, as did chromosomal DNAs from *E. coli* strain 4 and *E. cloacae* strain 1. No hybridization with plasmids of either *E. coli* strain 4 or the *E. cloacae* strain was observed. Restriction digestion of the plasmids carrying this gene with either *Bam*HI or *Eco*RI revealed that plasmids from *E. coli* strains 2 and 3 yielded the same restriction profiles, whereas that from the *K. pneumoniae* strain showed a unique profile (data not shown). Subsequent Southern blotting and hybridization with the bla_{CTX-M}-specific probe showed that the $bla_{CTX-M-9}$ genes of the two *E. coli* isolates were encoded on the same *Bam*HI or *Eco*RI fragment, whereas that of the *K. pneumoniae* strain was found on a different fragment. In the present study, only *K. pneumoniae* strain 1 carried a $bla_{CTX-M-13}$ gene. This gene was encoded on a single plasmid, of approximately 35 kb (Fig. 2). All CTX-M-14-producing isolates had a plasmid of approximately 60 to 150 kb that hybridized with the bla_{CTX-M}-specific probe (Fig. 2). Restriction digestion of the plasmids with either *Bam*HI or *Eco*RI showed that each plasmid except those from *E. coli* strains 5 and 6 carried the bla_{CTX-M} gene on a unique restriction fragment; plasmids from *E. coli* strains 5 and 6 yielded the same restriction profiles (data not shown). Consistent with this finding was the fact that the bla_{CTX-M} gene was found on fragments of different sizes from all isolates except those from *E. coli* strains 5 and 6 when the fragments which hybridized with the bla_{CTX-M}-specific probe were indistinguishable.

DISCUSSION

Recently, several reports have described ESBLs that do not belong to either the TEM or the SHV family and that are more active against cefotaxime than ceftazidime but that are inhibited by clavulanic acid (3, 5–7, 13–15, 20, 29, 34, 36, 42, 48, 51).

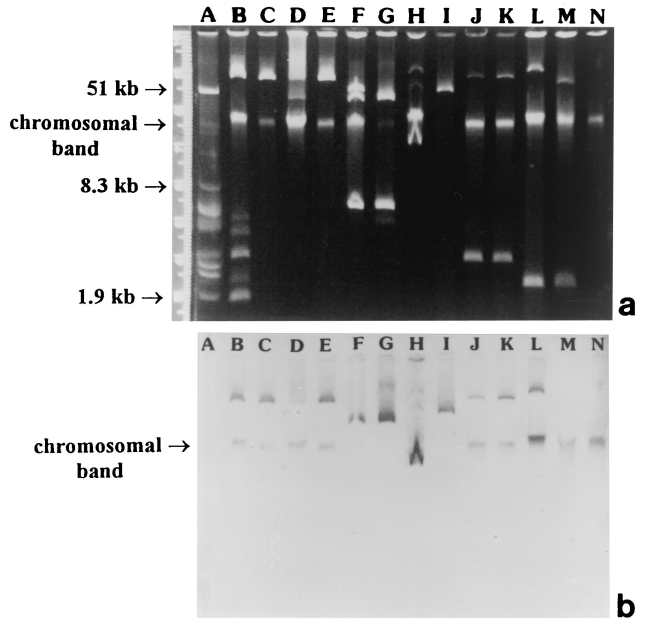

FIG. 2. (a) Plasmid DNA profiles of the clinical isolates carrying bla_{CTX-M} genes and their transconjugants; (b) Southern blots hybridized with a bla_{CTX-M}-specific probe. Lanes A, V517 plasmid size marker; lanes B and C, *E. coli* strain 8 and its transconjugant ($bla_{CTX-M-14}$), respectively; lanes D and E, *E. coli* strain 7 and its transconjugant ($bla_{CTX-M-14}$), respectively; lanes F and G, *K. pneumoniae* strain 1 and its transconjugant ($bla_{CTX-M-13}$), respectively; lanes H and I, *K. pneumoniae* strain 2 and its transconjugant ($bla_{CTX-M-14}$), respectively; lanes J and K, *E. coli* strains 5 and 6, respectively ($bla_{CTX-M-14}$); lanes L, *E. cloacae* strain 3 ($bla_{CTX-M-14}$); lanes M and N, *E. cloacae* strain 1 and its transconjugant ($bla_{CTX-M-9}$), respectively.

They are more closely related to the chromosomal β-lactamase of *Klebsiella oxytoca* than to the TEM or the SHV β-lactamases (5). These cefotaxime-hydrolyzing β-lactamases include the CTX-M and Toho families, of which four subgroups have been recognized on the basis of amino acid sequence homology (6). The first subgroup, the CTX-M-1 subgroup, includes CTX-M-1 (MEN-1) from *E. coli* in France (5) and Germany (3), CTX-M-3 from *C. freundii* and *E. coli* in Poland (15) and from *E. coli* in Taiwan (51), and CTX-M-10 from *E. coli* in Spain (34). The second subgroup is represented by CTX-M-2 and includes CTX-M-2 from *Salmonella enterica* serovar Typhimurium, *E. coli*, and *Proteus mirabilis* in Argentina and from *K. pneumoniae* in Israel and Paraguay (3); Toho-1 from *E. coli* in Japan (20); CTX-M-4 from *S. enterica* serovar Typhimurium in Russia (13); CTX-M-5 from *S. enterica* serovar Typhimurium in Latvia (7); and CTX-M-6 and CTX-M-7 (previously designated CTX-M-5) from *S. enterica* serovar Typhimurium in Greece (14). The third subgroup, the Toho-2 subgroup, includes Toho-2 and CTX-M-9 from *E. coli* in Japan (29) and Spain (36), respectively. The fourth subgroup, the CTX-M-8 subgroup, has only one member, CTX-M-8 from *Citrobacter amalonaticus*, *E. cloacae*, and *Enterobacter aerogenes*, and was first seen in Brazil (6). In addition, kluA-1 and kluA-2, class A chromosomal β-lactamases, have been reported recently from *Kluyvera ascorbata* in France (C. Humeniuk, G. Arlet, R. Labia, and A. Philippon, GenBank accession nos. AJ272538 and AJ251722, respectively). The kluA-1 β-lactamase has 97 to 99% amino acid identity to members of the CTX-M-2 subgroup, and the nucleotide sequence of kluA-2 is identical to that of CTX-M-5. This finding indicates that the kluA-1 and kluA-2 enzymes from *K. ascorbata* are likely the progenitors of some plasmid-mediated CTX-M-type enzymes (34).

The clinical isolates used in the present study were screened for the presence of ESBLs by the double-disk diffusion test, and all were positive. Therefore, this report concentrates on the ESBLs that are inhibited by clavulanic acid. All isolates were first screened for the presence of SHV and TEM β-lactamase genes, which are commonly found in members of the family *Enterobacteriaceae*. As many isolates lacked genes for the TEM and SHV ESBLs but definitely had a phenotype for ESBL production and were found to be more resistant to cefotaxime than to ceftazidime, they were further screened for the presence of bla_{Toho-1} and $bla_{CTX-M-1}$. Amplification products were detected with CTX-M-1-specific primers only. This may be due to the sequences at the positions that the primers were designed to target, since the amino acid identities among cefotaxime-hydrolyzing enzymes vary from 77 to 99%. It was found that the primers designed to amplify $bla_{CTX-M-1}$ have nucleotide sequences that are shared by both bla_{Toho-1} and bla_{Toho-2}, although some nucleotides are different. Thus, these primers are not specific for the detection of bla_{CTX-M} genes in the CTX-M-1 subgroup only.

This paper describes the occurrence of TEM and SHV β-lactamases in strains from the southern area of mainland China. The TEM β-lactamases that have been reported in the Far East include TEM-52 in Korea (35), TEM-26 in Japan (49), TEM-1 and TEM-10 in southern Taiwan (50, 51), and TEM-1B in Thailand (8). Only TEM-1B was found in the present study. Until 1994, SHV-2 was the only ESBL to have been reported from China (10). Recently, SHV-28, a novel SHV variant, has been reported from China (Y. Yu, W. Zhou, and Y. Chen, GenBank accession no. AF299299). SHV ESBLs that have been described among other Asian countries include SHV-2a and SHV-12 in Korea (26); SHV-2, SHV-12, and SHV-24 in Japan (27, 49); SHV2, SHV-5, and SHV-12 in Taiwan (28, 40, 50); SHV-5 in Singapore (21); and SHV-2a, SHV-5, and SHV-12 in Thailand (8). Recently, two novel SHV variants, SHV-25 and SHV-26, have been reported in Taiwan (9). This paper represents the first report of SHV-12 production by bacteria isolated from mainland China. In addition, species from three different genera of the *Enterobacteriaceae*, *E. coli*, *E. cloacae*, and *C. freundii*, were found to produce SHV-12, indicating horizontal transfer of this resistance gene.

In the present study, three different CTX-M ESBLs were found, and they were found to be associated with three genera of the family *Enterobacteriaceae*. One to four amino acid substitutions were found among these enzymes (Fig. 1), but the substitutions did not significantly alter the MICs of cefotaxime (Table 3). Thus, these substitutions do not appear to play an important role in cefotaxime hydrolysis. The kinetics of these enzymes, however, were not studied. It is of interest that CTX-M-13 has a difference of three amino acids from CTX-M-9 and CTX-M-14 at positions 3, 53, and 154. Mutants with intermediate changes must exist, but either these mutations have not yet been discovered or the substitutions alter the efficacy of the β-lactamase, making them difficult to detect.

Both CTX-M-9 and CTX-M-14 enzymes were produced by

bacteria of three different genera, suggesting horizontal transfer. PFGE analysis of the clinical isolates indicates the dissemination of these resistance genes in different strains. *E. coli* strains 2 and 3 were, however, clonal, as were *E. coli* strains 5 and 6. Seven of the 12 isolates carrying $bla_{\text{CTX-M}}$ genes were able to transfer the ESBL phenotype in conjugation experiments, suggesting the spread of $bla_{\text{CTX-M}}$ genes by conjugative plasmids. Plasmid fingerprinting of the transconjugants carrying these genes followed by hybridization with the $bla_{\text{CTX-M}}$-specific probe further demonstrated the dissemination of the $bla_{\text{CTX-M}}$ genes by different plasmids carrying resistance determinants. This suggests that the genes encoding these β-lactamases are highly mobile. The IS*Ecp1* insertion sequence observed upstream of the $bla_{\text{CTX-M}}$ genes examined in the present study may be involved in the translocation and dissemination of these β-lactamase genes, as was found in the $bla_{\text{CMY-4}}$ gene (P. D. Stapleton, Abstr. 39th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 1457, 1999). In the present study, the $bla_{\text{CTX-M}}$ genes were encoded on the chromosome in two isolates, as were the $bla_{\text{SHV-12}}$ genes in five isolates and their transconjugants. This may be explained by the same reasoning that has been applied to the chromosome-encoded Toho-1-like genes, in that mobile elements such as transposons or integrons may be involved in the translocation of these genes (48). This suggestion is strengthened by the occurrence of the *bla* genes on the chromosomes of transconjugants.

The three enzymes found in the present study have 98% nucleotide homology with Toho-2 (29). However, they share only 86 to 87% amino acid homology since there are nucleotide deletions at four positions in the Toho-2 sequence which are not found in the sequences of the enzymes described here (Fig. 1). In addition, the three enzymes have 78 to 85% amino acid homology with the amino acid sequences of other CTX-M types. Closely related enzymes such as CTX-M-2 in Argentina (3) and Toho-1 in Japan (20), as well as CTX-M-9 in Spain (36) and CTX-M-9, CTX-M-13, and CTX-M-14 in China (this study), have been reported from geographically distant areas. In contrast, Toho-1 (20) and Toho-2 (29), as well as CTX-M-9 (36) and CTX-M-10 (34), which are classified in different subgroups, originated from the same locations. These observations suggest that these genes are the result of divergent evolution from a common ancestor. It also demonstrates the rapid dissemination of these resistance determinants on diverse replicons and through wide-ranging geographical locations.

The strains used for the present study illustrate the widespread dissemination of the $bla_{\text{CTX-M}}$ genes on different replicons and among at least three species of the family *Enterobacteriaceae*. It also highlights the evolution of these genes within a single hospital; other variants will almost certainly exist elsewhere in China. This is supported by a recent submission to GenBank of the sequence of CTX-M-11 from China (C. Minjun, X. Yingchun, and W. Hui, GenBank accession no. AY005110). It was found that CTX-M-11 is closely related to the CTX-M-1 subgroup, whereas the enzymes found in the present study are closely related to the Toho-2 subgroup. Similar divergences of genes within a single locality have been reported elsewhere (20, 29, 34, 36). Recently, CTX-M-3 has been reported from southern Taiwan (51) and two novel cefotaxime-hydrolyzing β-lactamases have been described, CTX-M-12 from *K. pneumoniae* in Kenya (24) and UOE-1 from *E. coli* in Japan (T. Muratani, K. Takahashi, and T. Matsumoto, GenBank accession no. AY013478). Both novel enzymes are closely related to the CTX-M-1 subgroup. These findings suggest that variants of cefotaxime-hydrolyzing β-lactamases are becoming more common.

The ESBL-producing isolates evaluated in the present study were classified into three groups on the basis of their resistance phenotypes. The nine isolates that were predominately resistant to cefotaxime produced CTX-M ESBLs, whereas the three isolates that were characterized by high ceftazidime MICs produced SHV-12. In addition, the three isolates resistant to both antibiotics were found to produce both CTX-M and SHV ESBLs. It is worrying that bacteria that harbor multiple β-lactamase genes are being reported with increasing frequency, and we describe the occurrence of SHV and CTX-M ESBLs produced by single strains. The high MICs of both cefotaxime and ceftazidime for strains with more than one ESBL that are reported here, particularly compared with the MICs for bacteria that produce a single ESBL, illustrate the increasing levels of resistance to β-lactams of bacteria of clinical significance. The ease with which such bacteria can be isolated should be a cause for grave concern and indicate the need for more detailed surveillance and epidemiological surveys in this region, which has increasing contact with the rest of the world.

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