Nucleotide Sequence and Characterization of a Novel Cefotaxime-Hydrolyzing β-Lactamase (CTX-M-10) Isolated in Spain

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A cefotaxime-resistant, ceftazidime-susceptible *Escherichia coli* isolate was obtained from a patient with sepsis in 1997, from which a β-lactamase with a pI of 8.1 was cloned. Cephaloridine and cefotaxime relative hydrolysis rates were 167 and 81, respectively (penicillin G rate = 100), whereas ceftazidime hydrolysis was not detected. The nucleotide sequence revealed a *bla* gene related to that coding for CTX-M-3. Despite 21 nucleotide substitutions, only 2 determined amino acid changes (Ala27Val and Arg38Gln). The amino acid sequence identity between this enzyme, designated CTX-M-10, and the chromosomal β-lactamase of *Kluyvera ascorbata* was 81%.

Plasmid-mediated expanded-spectrum β-lactamases (ESBLs) have been responsible for several outbreaks of enterobacteria resistant to expanded-spectrum cephalosporins since they were first reported in 1987 (7, 21). To date, the ESBLs found most frequently are those derived from the common TEM-1 and SHV-1 broad-spectrum β-lactamases (8, 24). These enzymes are characterized as conferring resistance, to a greater or lesser extent, to all expanded-spectrum cephalosporins but are most frequently associated with a higher level of resistance to ceftazidime than to cefotaxime. In 1992, a novel type of ESBL was reported, which in contrast to those found previously, determined high-level resistance to cefotaxime but did not significantly affect ceftazidime (3). ESBLs belonging to this new type, named cefotaximases (CTX-M), have caused outbreaks of cefotaxime-resistant enterobacteria mainly in South America, Eastern Europe, and Japan (6, 11, 14, 15; M. F. Galas, M. J. Rapoport, F. G. Pasteran, R. G. Melano, A. E. Petroni, P. G. Ceriana, W. Group, and M. A. Rosi, Abstr. 39th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 1474, 1999).

In 1997 a cefotaxime-resistant, ceftazidime-susceptible *Escherichia coli* isolate (97/38582) was obtained from blood and urine cultures from a patient with sepsis. DNA sequencing, isoelectric focusing (IEF), and determination of the resistance phenotype and antibiotic hydrolysis profile were performed. The strain harbored a novel CTX-M β-lactamase closely related to CTX-M-3, which was previously found in *Citrobacter freundii* (11).

Identification and initial antibiotic susceptibility testing were performed with the semiautomatic PASCO system (Difco, Detroit, Mich.). MICs of amoxicillin, amoxicillin-clavulanate, ticarcillin, piperacillin, piperacillin-tazobactam, cefazolin, cefuroxime, cefpodoxime, cefoxitin, cefotaxime, ceftazidime,

cefepime, aztreonam, and imipenem were determined by agar dilution according to the NCCLS guidelines (16). Additionally, MICs of cefuroxime, cefpodoxime, cefotaxime, and ceftazidime plus a 4-μg/ml fixed concentration of clavulanate were determined. The inoculum effect on antibiotic MICs was determined using a 10^3-CFU/ml inoculum (low inoculum) and a 10^7-CFU/ml inoculum (high inoculum) in addition to the 10^5-CFU/ml standard inoculum. The manufacturers provided all antibiotics as powders.

Conjugation experiments were performed by filter mating using *E. coli* JMG21 (MC4100 with kanamycin and nalidixic acid resistance markers) (12) as the recipient. Transconjugants were selected on Mueller-Hinton agar plates (MHAP) with 100 and 50 μg of ampicillin and kanamycin per ml, respectively. Plasmid DNA from *E. coli* clinical strain 97/38582 (High Pure plasmid isolation kit; Boehringer Manheim, Indianapolis, Ind.) was transformed into *E. coli* MC4100 competent cells. Selection of transformants was performed both on MHAP with 100 μg of ampicillin per ml and on MHAP with 2 μg of cefotaxime per ml. Plasmid DNAs obtained from strain 97/38582 and plasmid pBGS18⁻ (kanamycin resistance marker) (22) were digested with *Eco*RI and *Bam*HI. After enzyme inactivation, the digestion products were mixed, ligated overnight with T4 DNA ligase, and transformed into MC4100 competent cells. *E. coli* MC4100 transformants harboring the pBGS18⁻ plasmid containing the cloned β-lactamase gene were selected on MHAP with 2 and 50 μg of cefotaxime and kanamycin per ml, respectively. This plasmid was used as the DNA template, which was sequenced on both strands by the method of Sanger et al. (19). Sequencing was performed with a Big DyeDeoxyTerminator cycle sequencing kit (Perkin-Elmer, Foster City, Calif.) and specific primers for the coding sequence. The DNA sequence was analyzed in an automatic DNA sequencer (377 Abi-Prism: Perkin-Elmer).

IEF was performed by applying the supernatant of a crude sonic extract to Phast gels (pH gradient of 3 to 9) in a Phast-System apparatus (Pharmacia AB, Uppsala, Sweden). β-Lactamases with known pIs were focused in parallel as controls.

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TABLE 1. MICs of different antibiotics for *E. coli* 97/38582, *E. coli* MC4100, the *E. coli* JMG21 (modified MC4100) transconjugant, and *E. coli* MC4100 with the cloned β-lactamase

Antimicrobial agent	MIC (μg/ml) for strain:					
	97/38582 (clinical strain)[a]			MC4100[b]	JMG21 (transconjugant)[b]	MC4100 (pBctx-M-10)[b]
	10^3	10^5	10^7			
Amoxicillin	256	256	>512	4	256	256
Amoxicillin-clavulanate[c]	8/4	8/4	8/4	2/1	4/2	8/4
Ticarcillin	>512	>512	>512	4	>512	512
Piperacillin	64	128	>512	1	32	32
Piperacillin-tazobactam[d]	1/4	2/4	2/4	1/4	2/4	2/4
Cefazolin	128	512	>512	4	128	512
Cefuroxime	512	512	>512	2	512	>512
Cefuroxime plus clavulanate[e]	2	2	4	2	4	4
Cefpodoxime	32	32	>512	≤1	16	256
Cefpodoxime plus clavulanate[e]	≤1	≤1	≤1	≤1	≤1	2
Cefoxitin	4	4	8	2	4	4
Cefotaxime	8	8	256	≤0.12	4	128
Cefotaxime plus clavulanate[e]	0.2	0.25	0.5	≤0.12	≤0.12	0.5
Ceftazidime	0.5	0.5	1	0.25	0.25	2
Ceftazidime plus clavulanate[e]	≤0.12	≤0.12	≤0.12	≤0.12	≤0.12	≤0.12
Aztreonam	1	2	64	≤0.12	2	16
Cefepime	2	4	512	≤0.12	2	32
Imipenem	≤0.1	≤0.12	≤0.12	≤0.12	≤0.12	0.25

[a] The inoculum effect was tested using 10^3, 10^5, and 10^7 CFU per spot.
[b] At 10^5 CFU per spot.
[c] 2:1 concentration ratio.
[d] Tazobactam was at a fixed concentration of 4 μg/ml.
[e] Clavulanate was at a fixed concentration of 4 μg/ml.

Gels were stained with 500 μg of nitrocefin (Oxoid, Basingstoke, Hampshire, England) per ml to identify the β-lactamase bands. An exponentially growing culture of the *E. coli* MC4100 strain harboring the cloned β-lactamase was sonicated, partially purified through a Sephadex G 100 column (Pharmacia AB), and concentrated with a Minicon concentrator (Amicon, Danvers, Mass.). Hydrolysis rates were determined spectrophotometrically (UVIKON 940; Kontron, Schlieren, Switzerland) in duplicate experiments at 25°C in 0.05 M phosphate buffer (pH 7.4) during 10 min using a 100 μM concentration of each antibiotic. Kinetic parameters were determined in duplicate experiments based on the initial steady-state rates using at least five different substrate concentrations (Lineweaver-Burk transformation). Fifty percent inhibitory concentrations (IC_{50}s) were determined for clavulanic acid, sulbactam, and tazobactam. The enzyme and inhibitor were preincubated for 10 min

at 25°C before addition of nitrocefin (100 μM, final concentration). Hydrolysis was measured spectrophotometrically at 482 nm, and IC_{50}s were determined graphically.

IEF of the *E. coli* clinical strain 97/38582 crude sonic extract revealed two β-lactamases with pIs of 5.4 and 8.1. Both β-lactamases were cotransferred by conjugation into the JMG21 *E. coli* strain. A few transformants obtained on MHAP with ampicillin carried only the pI 5.4 β-lactamase (suggestive of TEM-1), and those selected on MHAP with cefotaxime carried only the pI 8.1 β-lactamase, suggesting that the two β-lactamases were encoded by different plasmids. Enzymatic restriction of the pBGS18− plasmid harboring the pI 8.1 β-lactamase revealed an insert of approximately 3 kb.

MICs of several antibiotics against the *E. coli* clinical strain 97/38582, the transconjugant, and the MC4100 strain with the pI 8.1 cloned β-lactamase are shown in Table 1. β-Lactam

TABLE 2. Kinetic parameters of CTX-M-10

Antibiotic	Relative hydrolysis rate[a] (%)	Relative V_{max} (%)	K_m (μM)	Relative V_{max}/K_m ratio	IC_{50} (μM)
Penicillin G	100	100	34	100	
Cephaloridine	167	200	96	71	
Cefuroxime	95	100	38	88	
Cefotaxime	81	50	28	61	
Ceftazidime	NH[b]	ND[c]	ND	ND	
Cefepime	5	ND	ND	ND	
Clavulanic acid					0.006
Tazobactam					0.0009
Sulbactam					0.32

[a] Hydrolysis rates (micromoles per minute per microliter) were determined using a 100 μM concentration of each substrate; relative values were determined by considering the penicillin G rate to be 100.
[b] NH, no hydrolysis detected.
[c] ND, not done.

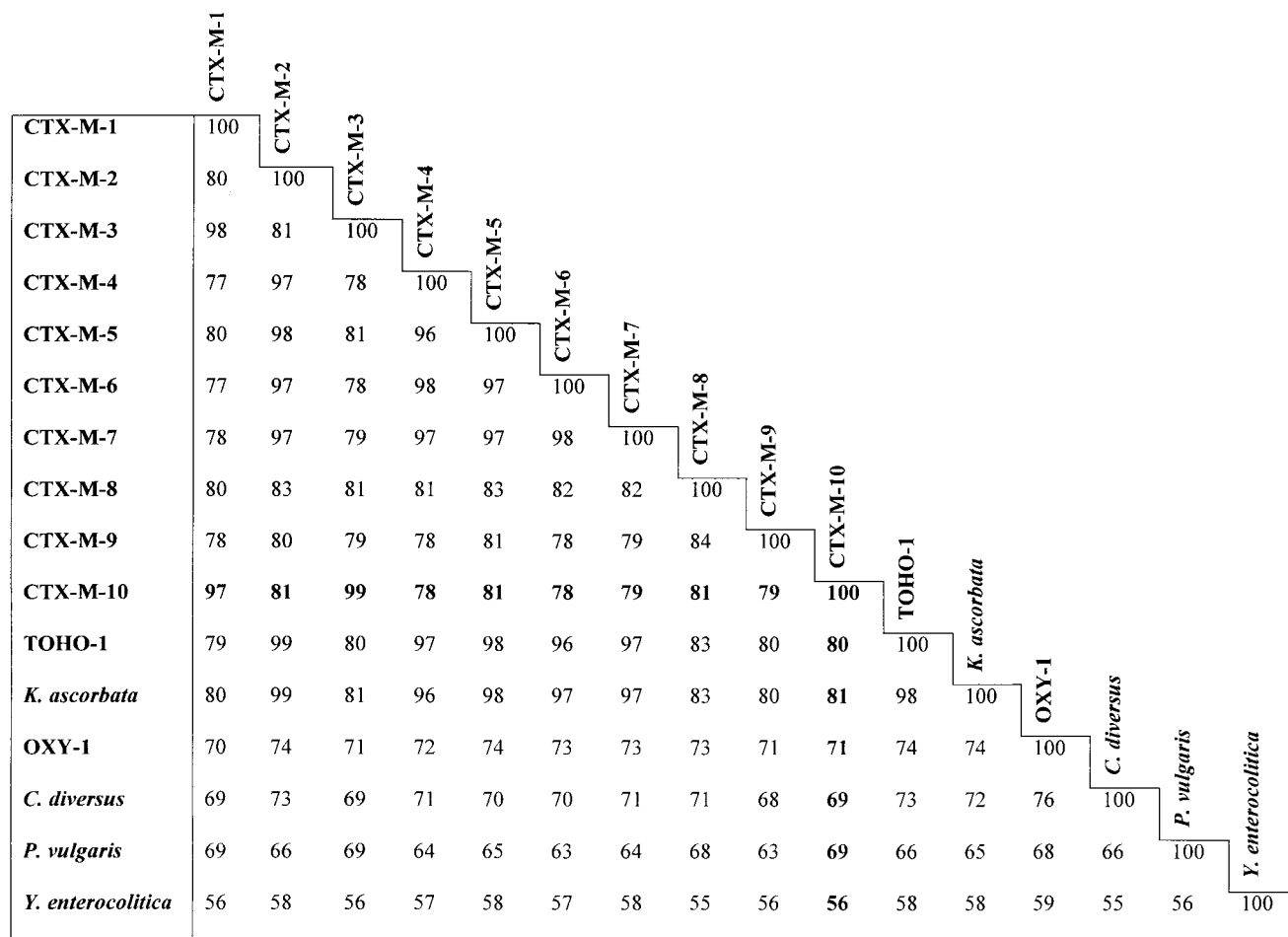|	CTX-M-1	CTX-M-2	CTX-M-3	CTX-M-4	CTX-M-5	CTX-M-6	CTX-M-7	CTX-M-8	CTX-M-9	CTX-M-10	TOHO-1	K. ascorbata	OXY-1	C. diversus	P. vulgaris	Y. enterocolitica
CTX-M-1	100															
CTX-M-2	80	100														
CTX-M-3	98	81	100													
CTX-M-4	77	97	78	100												
CTX-M-5	80	98	81	96	100											
CTX-M-6	77	97	78	98	97	100										
CTX-M-7	78	97	79	97	97	98	100									
CTX-M-8	80	83	81	81	83	82	82	100								
CTX-M-9	78	80	79	78	81	78	79	84	100							
CTX-M-10	97	81	99	78	81	78	79	81	79	100						
TOHO-1	79	99	80	97	98	96	97	83	80	80	100					
K. ascorbata	80	99	81	96	98	97	97	83	80	81	98	100				
OXY-1	70	74	71	72	74	73	73	73	71	71	74	74	100			
C. diversus	69	73	69	71	70	70	71	71	68	69	73	72	76	100		
P. vulgaris	69	66	69	64	65	63	64	68	63	69	66	65	68	66	100	
Y. enterocolitica	56	58	56	57	58	57	58	55	56	56	58	58	59	55	56	100

FIG. 1. Percent identity between CTX-Ms and related chromosomal class A β-lactamases CTX-M-2 (4), CTX-M-3 (11), CTX-M-4 (9), CTX-M-5 (6), CTX-M-6 (10), CTX-M-7 (previously designated CTX-M-5) (10), CTX-M-8 (5), and CTX-M-9 (18), and chromosomal β-lactamases of *Kluyvera ascorbata* (C. Humeniuk et al., unpublished data [GenBank accession number CAB59824]), *Klebsiella oxytoca* (OXY-1) (2), *Proteus vulgaris* (23), and *Yersinia enterocolitica* (20).

MICs against the transformant carrying only the pI 5.4 β-lactamase resembled those expected against a TEM-1 β-lactamase-producing *E. coli* strain (data not shown). Interestingly, a high resistance level was found for cefotaxime, while MICs of ceftazidime were only slightly increased. Clavulanate restored the MIC to the basal value in all combinations tested. A high inoculum effect was observed with all cephalosporins except ceftazidime. For example, when the inoculum was increased from 10^3 to 10^7 CFU/ml, the cefepime MICs increased from 2 to 512 μg/ml. Hydrolysis rates and kinetic parameters for penicillin G, cephaloridine, cefuroxime, cefotaxime, ceftazidime, and cefepime are shown in Table 2. The hydrolysis rates for cephalosporins were high compared to that for penicillin G, with the exception of ceftazidime, for which hydrolysis could not be detected.

DNA sequencing of the 3-kb fragment carrying the β-lactamase gene revealed an 873-bp open reading frame. The deduced amino acid sequence revealed a 291-amino-acid protein with the typical motifs of serine β-lactamases. Nucleotide sequence analysis showed that this *bla* gene was related to those coding for the CTX-M-3 (11) and CTX-M-1 (4) β-lactamases,

with 21 and 23 nucleotide substitutions, respectively. The deduced amino acid sequence of this new β-lactamase, designated CTX-M-10, revealed that despite the high number of nucleotide substitutions, only two and six of them determined amino acid changes compared with the CTX-M-3 (Ala27Val and Arg38Gln) and the CTX-M-1 (Ala27Val, Arg38Gln, Asp114Asn, Ser140Ala, Val177Ala, and Asn288Asp) enzymes, respectively. Amino acid numbers were assigned as described by Ambler et al. (1). Sequence identity with other CTX-M β-lactamases is far lower than that with CTX-M-1 or CTX-M-3; CTX-M-2, CTX-M-5, and CTX-M-8 were the next three most related CTX-M β-lactamases, and each had only 81% amino acid identity to CTX-M-10 (4, 5, 6). This result suggests the existence of different subgroups with different phylogenetic origins within the CTX-M β-lactamase group.

To date, CTX-M enzymes have been thought to be derived from the chromosomal β-lactamase of *Klebsiella oxytoca*, although a direct relationship is not likely, given a sequence identity lower than 80%. The amino acid sequence identities between this new β-lactamase and chromosomal enzymes from *Kluyvera ascorbata* (C. Humeniuk et al., unpublished data

[GenBank accession number CAB59824]), *K. oxytoca* (2), and *Citrobacter diversus* (17) were 81, 71, and 69%, respectively (Fig. 1). Therefore, if this β-lactamase is derived from one of the known sequenced chromosomal enzymes, *K. ascorbata* is the most likely candidate. In fact, the CTX-M-2 β-lactamase differs from that of *K. ascorbata* in only two amino acids, making the direct relationship clearly established.

The β-lactam resistance phenotypes conferred by CTX-M-10 and the closely related CTX-M-3 β-lactamase (11) in their respective conjugation recipient strains are very similar. As found for CTX-M-3, for CTX-M-10, MICs of cefotaxime are at least 8 times greater than those of ceftazidime, which in both cases remain in the susceptible category. Both amino acid substitutions (Ala27Val and Arg38Gln) are located in the mature protein that starts at residue 25 (4). Residue 27 has been found to be nonvisible in the recently crystallized Toho-1 β-lactamase, but residue 38 was found to be solvent oriented (13). Both amino acids are divergent in CTX-M β-lactamases. Alanine 27 in CTX-M-1 and CTX-M-3 is replaced by an asparagine in CTX-M-2 and closely related CTX-M β-lactamases such as TOHO-1, CTX-M-4, CTX-M-5, and CTX-M-6, whereas it is replaced by a serine in CTX-M-9 and by a valine in CTX-M-10 (4, 6, 9, 10, 14, 18). On the other hand, arginine 38 of CTX-M-1 and CTX-M-3 is replaced by a lysine in CTX-M-2 and closely related CTX-Ms and by a glutamine in CTX-M-10 (4, 11).

Several *E. coli*, *Klebsiella pneumoniae*, and *Enterobacter cloacae* isolates from different patients at our hospital, as well as five isolates (four *E. coli* and one *Salmonella*) with similar susceptibility profiles from four other Spanish hospitals, were found to harbor a β-lactamase with the same 8.1 pI value. Moreover, in a 10-year survey approximately 50% of *E. coli* isolates harboring ESBLs at our institution produced a CTX-M-like β-lactamase with the same 8.1 pI value (data not shown), suggesting that CTX-M-10 may be widely distributed. Different outbreaks involving CTX-M β-lactamase-producing *Enterobacteriaceae* have been reported. CTX-M β-lactamases are now recognized as an important cause of cefotaxime resistance in *Enterobacteriaceae*. In Poland, several *E. coli* and *C. freundii* isolates from the same institution were found to harbor the CTX-M-3 β-lactamase (11). In Argentina, the CTX-M-2 β-lactamase is widely distributed and is currently the ESBL most frequently found in *K. pneumoniae* (69% of all ESBLs found) (Galas et al., 39th ICAAC) as well as in *Enterobacter*, *Citrobacter*, *Serratia*, *Morganella*, and *Providencia* (E. G. Pasteran, R. G. Melano, M. F. Galas, M. M. Rodriguez, W. Group, and M. A. Rosi, Abstr., 39th Intersci. Conf. Antimicrob. Agents Chemother., abstr 1475, 1999).

It is presently not clear whether isolates harboring CTX-M β-lactamases should be considered resistant to all cephalosporins as recommended for ESBLs (16). In vitro susceptibility testing shows that MICs of ceftazidime are not much increased in isolates harboring these β-lactamases, and a significant inoculum effect is not found (Table 1). Moreover, the enzymatic hydrolysis profile demonstrates that ceftazidime is highly stable to hydrolysis by CTX-M β-lactamases. Finally, the chromosomal β-lactamases from which CTX-M is thought to be derived (from *K. ascorbata*, *K. oxytoca*, and *C. diversus*), even when hyperproduced, hardly affect ceftazidime and are routinely reported with the NCCLS criteria as being susceptible to this antibiotic. Determination of whether to report isolates

harboring CTX-M β-lactamases as susceptible or resistant to ceftazidime can only be done with clinical experience.

In summary, we describe the characterization of a novel cefotaxime-hydrolyzing β-lactamase, CTX-M-10, that is closely related to CTX-M-3 and is widely distributed in our institution.

Nucleotide sequence accession number. The GenBank accession number for the CTX-M-10 β-lactamase is AF255298.

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