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CTX-M-93, a CTX-M-variant lacking penicillin hydrolytic activity

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Extended-spectrum ß-lactamases (ESBLs) of the CTX-M-type are increasingly reported worldwide, with more than 90 known variants. Clinical isolate *Escherichia coli* Bre-1 was isolated in 2009 and displayed an unusual ESBL phenotype: made of a synergy image between expanded-cephalosporins and clavulanic acid disks, and susceptibility to penicillins. *E. coli* Bre-1 harbored a novel CTX-M-encoding gene, designated $bla_{\text{CTX-M-93}}$. CTX-M-93 differed from CTX-M-27 by only a single L169Q substitution. As compared to CTX-M-27, CTX-M-93 conferred higher MICs of ceftazidime to *E. coli* (MIC, 8 versus 1.5 µg/mL) and decreased MICs for other expanded-cephalosporins (MIC cefotaxime, 1 versus 32 µg/mL) and for penicillins (MIC ticarcillin, 0.5 versus >256 µg/mL). Comparison of enzymatic properties revealed that L169Q substitution lead to decreased K_m for ceftazidime (25.5 versus 330 µM), but decreased hydrolytic activity against good substrates, such as cefotaxime (k_{cat}, 0.6 versus 113 s^{-1}), probably owing to the alteration of omega loop positioning during the catalytic process. $Bla_{\text{CTX-M-93}}$ gene was surrounded by IS*Ecp1* and IS*903* elements and inserted onto a 150-kb non self-transferable IncF type plasmid. *E. coli* Bre-1 belongs to the phylogroup D, and is of MLST type 624, a ST-type found only in rare spanish CTX-M-14-producing *E. coli* isolates.

We have characterized a novel CTX-M-variant, CTX-M-93, lacking significant penicillin hydrolysis, but with increased ceftazidime hydrolysis.

A widely accepted definition of extended-spectrum β-lactamases (ESBLs) is: molecular Ambler class A or functional class 2be β-lactamases capable of conferring bacterial resistance to the penicillins, first-, second-, and third-generation cephalosporins, and aztreonam (but not to cephamycins or carbapenems) with hydrolysis rates of at least 10% of that for benzylpenicillin and activity inhibited by β-lactamase inhibitors such as clavulanic acid (1,7,23). ESBL-producing *Enterobacteriaceae* are mostly associated with urinary tract infections, but may also cause significant bloodstream-associated infections, that result in increased hospital costs, length of stay, and patient mortality (11,16). Plasmid-encoded ESBLs of the CTX-M-type are reported increasingly worldwide in Gram-negative rods and account now for most of the ESBLs found in *Enterobacteriaceae* (11,16). CTX-Ms form a rapidly growing family that comprises currently up to 90 variants that are divided into five groups according to amino-acid sequence identity (CTX-M-1, -2, -8, -9 and -25 groups) with different groups prevalent in different countries (16,24).

ß-lactamases of the CTX-M-types are structurally related to the naturally produced ß-lactamases of enterobacterial species such as *Kluyvera ascorbata* (CTX-M-2), *Kluyvera georgiana* (CTX-M-8), *Kluyvera cryocrescens* (CTX-M-1), *Kluyvera ascorbata* (CTX-M-3) and *Kluyvera* spp. isolated in Guyana (CTX-M-9) (31). The CTX-M enzymes usually have higher activity against cefotaxime than ceftazidime and aztreonam (31). Cefotaxime-hydrolyzing activity of CTX-M enzymes is related to the flexibility of the ß3 strand and omega loop, and residues Asparagine, Serine, Aspartate and Arginine at Ambler positions 104, 237, 240, and 276, respectively (1,13,14,31,33). Several CTX-Ms exhibiting an increased enzymatic activity against ceftazidime have been recently reported: the P167S mutant of CTX-M-18 (also called CTX-M-14),

designated CTX-M-19 (29), the P167Q in CTX-M-3 designated CTX-M-54 (2), the P167T in CTX-M-1 designated CTX-M-23 (34) and D240G mutants of CTX-M-3, CTX-M-9 and CTX-M-14, designated CTX-M-15, CTX-M-16 and CTX-M-27, respectively (3,4,27).

The aim of the present work was to characterize the ß-lactamases produced by an *E. coli* clinical isolate displaying in vitro susceptibility to penicillins and resistance to third generation cephalosporins that is reversed by clavulanic acid addition. We have characterized a novel CTX-M-variant, CTX-M-93, which is an L169Q mutant of CTX-M-27. The biochemical characterization of the ß-lactamase CTX-M-93 gives insights into the role of the L169Q substitution in CTX-M enzymes.

Materials and methods

Bacterial strains, antimicrobial agents and susceptibility testing. Table 1 shows the strains, plasmids and primers used in this study. *E. coli* Bre-1 clinical strain was isolated from a urinary tract infection.

Bacterial identification was performed using the API 20E system (bioMérieux, Marcy-l'Etoile, France). Antibiograms were determined by the disc diffusion method on Mueller-Hinton agar (BioRad, Marnes-La-Coquette, France) and the susceptibility breakpoints were determined and interpreted as recommended by the Clinical and Laboratory Standards Institute (10). All plates were incubated at 37ºC for 18 h. MICs of ß-lactams were determined using the Etest technique (bioMérieux).

Nucleic acid extractions, PCR and DNA sequencing. Whole-cell DNAs were extracted using QIAamp DNA Mini Kit (Qiagen, Les Ulis, France). The $bla_{\text{CTX-M}}$, bla_{SHV}, bla_{TEM}, and minor ESBL genes (23) were searched for and characterized as

91 described previously (24). PCR experiments were performed on an ABI 2700
92 thermocycler (Applied Biosystems, Les Ulis, France) using laboratory-designed primers
93 (Table 1).

94 Both strands of the PCR products, were sequenced using laboratory-designed
95 primers with an automated sequencer (ABI PRISM 3100; Applied Biosystems). The
96 nucleotide and the deduced protein sequences were analyzed using software available at
97 the National Center of Biotechnology Information website (http: //www.
98 ncbi.nlm.nih.gov).

99 **ß-Lactamase gene cloning.** *bla*$_{\text{CTX-M-93}}$ was amplified using CTX-M-ATG and CTX-
100 M-stop primers (Table 1) and directly cloned into PCR-Script Cam cloning kit
101 (Stratagene, Agilent Technologies, Massy, France) as described by the manufacturer,
102 resulting into pM-93. The transformants harboring pM-93 were selected on Mueller
103 Hinton agar supplemented with ceftazidime 1 µg/ml. In order to over-express CTX-M-
104 93, a 893-bp PCR generated fragment using primers CTX-M-NdeI and CTX-M-BamHI,
105 containing an NdeI and a BamHI restriction site, respectively (Table 1), was cloned into
106 pPCRscript Cam+ plasmid, according to the manufacturer instruction resulting in
107 plasmid pM-93NB. Using standard techniques , the insert containing *bla*$_{\text{CTX-M-93}}$ of pM-
108 93NB was removed with NdeI and BamHI restriction enzymes and cloned into
109 NdeI/BamHI-restricted pET-9a expression vector (Stratagene, Amsterdam, The
110 Netherlands), resulting in pET-M-93, using standard techniques (32).

111 **Site-directed mutagenesis.** Recombinant plasmid pM-93 was used as a template for
112 site-directed mutagenesis as described by the manufacturer (QuickChange site-directed
113 mutagenesis kit, Stratagene). Primers CTX-M-93-Q169L-A and CTX-M-93-Q169L-B
114 (Table 1) were used to generate recombinant plamid pM-27 coding for CTX-M-27 β-

lactamase with a leucine residue at Ambler position 169. Similarly, primers CTX-M-93-Q169M-A and CTX-M-93-Q169M-B (Table 1) were used to generate plasmid pM-93-Q169M, where the glutamin residue at position 169 was replaced with a methionin residue, as found in PER-type ESBLs. Recombinant plasmid pM-27 was then used as a template with primers CTX-M-27-L169C-A and CTX-M-27-L169C-B to obtain recombinant plasmid pM-27-L169C (Table 1), where the leucine at position 169 was replaced with cysteine, as found in TLA-2 ESBL (15).

Plasmid content, mating out and electroporation experiments. Direct transfer of resistance into azide-resistant *E. coli* J53 was attempted as previously reported (22). Plasmids were introduced by electroporation into *E. coli* TOP10 (22) using a Gene Pulser II (BioRad). Plasmid DNAs were extracted with Qiagen plasmid DNA maxi kit (Qiagen, Courtaboeuf, France) and analyzed by agarose gel electrophoresis (Invitrogen, Paris, France). Natural plasmids were extracted using Kieser extraction method (18) and subsequently analyzed by electrophoresis on a 0.7% agarose gel.

MLST typing. MLST with seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, *recA*) was performed according to Wirth *et al.* (37). Allele sequences and sequence types (STs) were verified at the http:// mlst.ucc.ie/mlst/dbs/Ecoli web site. A different allele number was given to each distinct sequence within a locus, and a distinct sequence type (ST) number was attributed to each distinct combination of alleles.

Phylogenetic grouping. *E. coli* phylogenetic group was determined by a triplex PCR technique using a combination of two genes *chuA* and *yjaA* and a DNA fragment TspE4.C2, as described by Clermont *et al.* (9).

Replicon typing and genetic environment of CTX-M-93. PCR-based replicon typing of the main plasmid incompatibility groups reported in *Enterobacteriaceae* was performed as described (8). Genetic structures surrounding the $bla_{\text{CTX-M-93}}$ gene were determined as previously described (19,24,30). Primers specific of the known genetic environment of group 9 CTX-M variants were used (Table 1).

Isoelectrofocusing (IEF). Crude β-lactamase extracts, obtained from 10-mL cultures of clinical isolate *E. coli* Bre-1, electroporants and recombinant clones were subjected to analytical IEF as previously described (20,25)

Overexpression and ß-Lactamase purification. A two liter culture of *E. coli* BL21 (pET-M93) was IPTG induced (1 mg/ml) as previously described (20). The ß-lactamase extract was obtained after sonification, as described previously (20), was dialyzed overnight against 50 mM DEA buffer (pH 9.7) and was then loaded onto a preequilibrated Q-Sepharose column (Amersham Pharmacia Biotech) in 50 mM DEA buffer (pH 9.7). The ß-lactamase activities, as determined qualitatively for each fraction using nitrocefin hydrolysis (Oxoid), were detected in the flow through. The fractions containing the highest ß-lactamase activity were pooled and dialyzed overnight against 50 mM sodium phosphate buffer (pH 7.0). It was filtrated onto a polyethersulfone membrane (Vivaspin 50,000 MW, Sartorius Stedim, Aubagne, France) that removes molecules with a molecular weight higher than 50 kDa, prior to a 10-fold concentration (Vivaspin 10,000 MW, Sartorius Stedim) as described by the manufacturer. The protein content was measured using the Bio-Rad DC protein assay, and the specific activities of the crude extract and of the purified ß-lactamase were determined as previously reported (20) with 100 µM of cephalothin as the substrate. One unit of enzyme activity

was defined as the amount of enzyme that hydrolyses 1 µmol of substrate per min. The purity of the enzymes was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (20).

Biochemical properties. The purified β-lactamase CTX-M-93 was used for kinetic measurements performed at 30°C in 100 mM sodium phosphate (pH 7.0). The rates of hydrolysis were determined with a spectrophotometer ULTROSPEC 2000 (Amersham Pharmacia Biotech). The wavelengths and absorption coefficients of β-lactams have been previously referenced (25).

Kinetic parameters (maximal velocity, Vmax and Michaelis-Menten constant, *Km*) were determined by using the Eadie-Hoffstee linearization (V = Vmax – V *Km* / [S]) of the Michaelis-Menten equation (V = Vmax [S] / (*Km* + [S])) as previously described (12,25). *Kcat* values were determined by dividing the Vmax (µM s-1) for each substrate by the concentration of purified enzyme.

Various concentrations of clavulanic acid and tazobactam were preincubated with the enzyme for 3 min at 30°C before testing the rate of cephalothin (100 µM) hydrolysis. The 50% inhibitory concentrations (IC_{50}) of these inhibitors were determined as the concentration of these inhibitors that inhibited hydrolytic activity by 50%.

Nucleotide sequence accession number. The nucleotide sequences reported in this paper have been assigned to the GenBank nucleotide database under the accession number HQ166709.

Results

Characterization of clinical isolate *E. coli* Bre-1 and β-lactam susceptibility. *E. coli*

Bre-1 was isolated in the urines of a 61-year-old patient hospitalized at the University Hospital of Brest, France in June 2009. *E. coli* Bre-1 exhibited resistance to narrow and broad spectrum cephalosporins (MICs of cephalothin 16 µg/ml, cefotaxime 6 µg/ml, ceftazidime 8 µg/ml) with synergy images between ceftazidime and co-amoxiclav, suggesting the presence of an ESBL. However, *E. coli* Bre-1 showed low MIC for amoxicillin (6 µg/ml), ticarcillin (2 µg/ml), and piperacillin (8 µg/ml), which is very unusual for an ESBL phenotype (Table 2). *E. coli* Bre-1 belongs to phylogenetic group D and was identified by MLST-typing as ST624.

Resistance gene determination. Preliminary PCR amplification experiments with primers designated to amplify internal fragments of ESBL genes (Table 1) gave positive results for $bla_{\text{CTX-M}}$. External primers were used to amplify the $bla_{\text{CTX-M}}$ gene by using whole cell DNAs of *E. coli* Bre-1. DNA sequence analysis of the corresponding PCR amplimers revealed an open reading-frame (ORF) of 876-bp encoding a 291-amino-acid protein. On the basis of the protein alignments, a new CTX-M-type enzyme was identified, CTX-M-93, which differed from CTX-M-27 by a leucine-to-glutamine substitution at position 169 (1).

Genetic environment and support of the $bla_{\text{CTX-M-93}}$ gene. Analysis of the sequences surrounding $bla_{\text{CTX-M-93}}$ gene identified two insertion sequences in the same orientation named IS*Ecp1* and IS*903* and located respectively upstream and downstream of the gene.

Analysis of the plasmid content of *E. coli* Bre-1 identified a single 150-kb plasmid named pNBRE-1. Electrotransformation of this plasmid into *E. coli* TOP10 reference strain indicated this natural plasmid encoded CTX-M-93 as well as resistance determinants for tetracycline, cotrimoxazole, and aminoglycosides. Plasmid pNBRE-1

was categorized into the IncF group by PCR-based replicon typing.

Conjugation experiments failed to transfer pNBRE-1 from *E. coli* Bre-1 or from *E.coli* TOP10 (pNBRE-1) to azide-resistant *E. coli* J53 recipient strain.

Biochemical properties of CTX-M-93. IEF analysis of β-lactamase extracts of *E. coli* Bre-1, *E. coli* TOP10 (pNBRE-1) and clone *E. coli* TOP10 (pM-93) clone revealed a pI value of 8.9 (data not shown) for CTX-M-93. The specific activity of the purified β-lactamase CTX-M-93 was 3.8 µmol min^{-1} mg^{-1} of protein with 100 µM cephalothin as a substrate and its purification factor was 19 fold. The purity of the enzyme was estimated to be 99% according to sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis and its apparent molecular mass was about 30 kDa (Fig. 1).

Kinetic parameters of purified CTX-M-93 β-lactamase revealed activity against restricted and expanded-spectrum cephalosporins (Table 3). The enzyme showed the highest affinity for penicillin G (*Km* value of 22.5 µM) and ceftazidime (Km value of 25.5 µM), and the highest level of activity against ceftazidime (*kcat* value of 2.5 s^{-1}) and cephalothin (*kcat* value of 1.8 s^{-1}). Interestingly, catalytic efficiency for ceftazidime (*kcat*/*Km* of 0.1 μM^{-1} s^{-1}) is better than for cefotaxime (*kcat*/*Km* of 0.009 μM^{-1} s^{-1}) and also higher than for CTX-M-27. However, the other catalytic efficiencies of CTX-M-93 are lower than those of CTX-M-27. As expected, CTX-M-93 had no detectable hydrolysis for amoxicillin, ticarcillin and piperacillin. Determinations of IC50 performed with cephalothin (100 µM) as substrate showed that CTX-M-93 is inhibited by amoxicillin (IC50 of 16 µM) and ticarcillin (IC50 of 0.11 µM).

Surprisingly, CTX-M-93 was weakly inhibited in vitro by β-lactam inhibitors (IC50 of 140 µM for tazobactam and 870 µM for clavulanic acid) although MICs of cephalosporins were significantly lowered by the addition of β-lactam inhibitors (Table

4). The synergy image observed on disc diffusion antibiograms between ceftazidime and co-amoxiclav, is in fact the result of an inhibition of CTX-M-93 by amoxicillin, and not clavulanic acid. Indeed, amoxicillin discs alone give the same synergy images with ceftazidime as co-amoxiclav (data not shown).

Site-directed mutagenesis and effects for MICs. The leucine residue at position 169 located in the omega-loop is fully conserved among CTX-M enzymes (data not shown). To investigate the role of the glutamine residue of CTX-M-93, pM-27 plasmid was generated coding for CTX-M-27 β-lactamase, which differed from CTX-M-93 by the Gln169Leu substitution. *E. coli* TOP10 (pM-27), compared to *E. coli* TOP10 (pM-93), showed higher MICs of amoxicillin (> 256 *vs* 4 µg/ml), ticarcillin (> 256 *vs* 0.5 µg/ml) and piperacillin (48 *vs* 12 µg/ml) but lower MICs of ceftazidime (1.5 *vs* 8 µg/ml) (Table 2).

Analysis of amino acid residues found at Ambler position 169 among class A ESBLs (Fig. 2) revealed a leucine or a methionine residue for most ESBLs. Only CTX-M-93 and TLA-2 differed at position 169, with a glutamine and a cysteine, respectively. Recombinant plasmid pM-93-Q169M and pM-27-L169C were generated to evaluate the role of these methionine and cysteine residues at position 169. MICs for recombinant strains *E. coli* TOP10(pM-93-Q169M) and *E. coli* TOP10(pM-27-L169C) were compared to *E. coli* TOP10(pM-93) and *E. coli* TOP10(pM-27) respectively (Table 2). MICs of *E. coli* TOP10 (pM-93-Q169M) were higher for amoxicillin (96 vs 4 mg/l), ticarcillin (256 *vs* 2 µg/ml) and piperacillin (24 *vs* 12 µg/ml) than MICs of *E. coli* TOP10(pM93), whereas MICs of ceftazidime were identical. MICs of *E. coli* TOP10(pM-27-L169C) were lower for amoxicillin (8 *vs* > 256 µg/ml), ticarcillin (16 *vs* >256 µg/ml) and piperacillin (3 *vs* 48 µg/ml) than MICs of *E. coli* TOP10(pM-27), so as

for ceftazidime (0.38 *vs* 1.5 µg/ml).

Discussion

A novel CTX-M variant, CTX-M-93, lacking significant penicillin hydrolysis has been identified in a clinical strain of *E. coli* responsible for urinary tract infection. CTX-M-93 conferred to *E. coli* Bre-1 an ESBL profile, which remained susceptible to amoxicillin, ticarcillin, and piperacillin. CTX-M-93 differed from the CTX-M-9-group-variant CTX-M-27 by a Leu169Gln substitution located in the omega loop and from CTX-M-14, only by two substitutions (Asp240Gly and Leu169Gln) (4). CTX-M-27 is yet rarely isolated as compared to CTX-M15 or CTX-M-14 but epidemic situations have also been described (6,16,36).

The genetic environment of $bla_{\text{CTX-M-93}}$ gene, made of the insertion sequences IS*Ecp1* and IS*903* upstream and downstream, respectively, was similar to the genetic environment previously described for $bla_{\text{CTX-M-14}}$ (19,24). IS*Ecp1* insertion sequences have been involved in the mobilization of $bla_{\text{CTX-M}}$ genes and in providing the promoter for expression of these genes (30,31). $Bla_{\text{CTX-M-93}}$ was inserted on a non self-transferable 150-kb plasmid of broad spectrum IncF type. It was isolated from a clinical strain of *E. coli* ST624, an ST found only in rare Spanish CTX-M-14 producing *E. coli* isolates (36).

Amino acids of the omega loop play a major role in the enzyme substrate profiling particularly for cephalosporins (2,13,17,27,29,34). Substitutions in the omega loop might affect the expression, the stability and the activity of the enzymes. Position 169 is well-conserved among ESBLs, most of them showing a leucine or a methionine residue (Fig. 2). No natural class A β-lactamase have a glutamine residue at position 169 as CTX-M-93 does. Site-directed mutagenesis experiments showed that the

glutamine at position 169 was responsible for weak activity against penicillins of CTX-M-93. Interestingly, Leu169Cys substitution conferred also a weak activity against these substrates. Cys residue at position 169 was found in TLA-2 (15), an Ambler class A ESBL identified in a wastewater treatment plant, having also a weak activity against amino-, carboxy-, and ureido-penicillins. As CTX-M-93, TLA-2 was weakly inhibited in vitro by clavulanic acid and tazobactam although MICs of cephalosporins were lowered by their addition. Random mutagenesis technique applied to CTX-M-9 (13) to investigate substitutions, which increased activity against ceftazidime, selected novel amino acid substitutions. A mutant with a Leu169Gln substitution was selected and resulted in an increased activity against ceftazidime but a concomitant compromised activity against penicillins. In vitro mutagenesis experiment with TEM-1 enzyme (35) showed similar behavior against ceftazidime and penicillins in mutants with Leu169Pro substitution. According to these different studies, position 169 in the omega loop of class A β-lactamase may be implicated in the enzyme's activity against amino-, carboxy-, and ureidopenicillins and also against ceftazidime. It was shown that conserved water molecules associated with the omega loop could reduce its flexibility and have a role in its stabilization (5). Some of these water molecules have been shown to interact especially with backbone atoms such as Leu169 residue.

Finally, this work identified the first clinical isolate expressing an ESBL without activity against penicillins. This substrate profile was selected *in vivo* through the single amino acid substitution Leu169Gln located in the omega loop. This report raised also the question whether penicillins could be options for treating urinary tract infections associated with such CTX-M variant.

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431 **Table 1:** Strains, plasmids, and primers used in this study

Strains, plasmids, primers	Relevant genotype and produced ß-lactamase (pI)	Source
Strains		
E. coli Bre-1	Clinical *E. coli* strain producing CTX-M-93 (pI 8.9)	This study
E. coli Top10	SupE44 hsdS20 (r_B^-, m_B^-) recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1	Stratagene
E. coli J53	Azide resistant *E. coli* strain	22
E. coli 50192	*E. coli* NTCC 50192 harbouring 154-, 66-, 48-, and 7-kb plasmids	22
Plasmids		
pNBre-1	natural plasmid encoding CTX-M-93	This study
pPCRscript	recombinant pPCRScript plasmid	Stratagene
pET-9a	recombinant pET-9a plasmid	Stratagene
pM-93	recombinant pPCRScript plasmid which contains a 0.9-kb fragment encoding CTX-M-93 (pI 8.9)	This study
pM-93NB	recombinant pPCRScript plasmid which contains a 0.9-kb fragment encoding CTX-M-93 with NdeI and BamHI restriction sites	This study
pM-27	recombinant pPCRScript plasmid which contains a 0.9-kb fragment encoding CTX-M-27 (Gln169Leu)	This study
pM-93-Q169M	recombinant pPCRScript plasmid which contains a 0.9-	This study

	kb fragment encoding a modified CTX-M-93 protein with a Gln169Met substitution	
pM-27-L169C	recombinant pPCRScript plasmid which contains a 0.9-kb fragment encoding a modified CTX-M-27 protein with a Leu169Cys substitution	This study
pET-M-93	recombinant pET-9a plasmid which contains a 0.9-kb fragment encoding CTX-M-93	This study
Primers		
TEM-a	5-GAGTATTCAACATTTCCGTGT-3	24
TEM-β	5-TAATCAGTGAGGCACCTATCT-3	24
SHV-A	5-ATGCGTTATWTTCGCCTGTGT-3	24
SHV-B	5-TTAGCGTTGCCAGTGCTCG-3	24
CTX-M-A	5-CGCTTTGCGATGTGCAG-3	24
CTX-M-B	5-ACCGCGATATCGTTGGT-3	24
TLA-2A	5 -TCCCTGGAGCACTTATGAAT-3	15
TLA-2B	5 -ATTAAGGATAAACTCATCCGC-3	15
TLA-1A	5 -GGCTAAAGGTACGGATTCGC -3	This study
TLA-1B	5 - ACGCTTTTGCAAATTTCGGC-3	This study
SFO-A	5 - TTACGTCAAACCACCCTGATGG-3	This study
SFO-B	5 - TTCTGCATTCTGCTGTGGCTG-3	This study
BES-A	5 - ATGTGGCAGTGGCTTGGAAA-3	This study
BES-B	5 -TTATCTTGCAGTACCAGTCG -3	This study
BEL-1A	5-CGACAATGCCGCAGCTAACC -3	26
BEL-1B	5-CAGAAGCAATTAATAACGCCC -3	26

VEB-1A	5 – CGACTTCCATTTCCCGATGC -3	21
VEB-1B	5 - GGACTCTGCAACAAATACGC-3	21
PER-A	5 - ATGAATGTCATTATAAAAGC-3	21
PER-B	5 - AATTTGGGCTTAGGGCAGAA-3	21
GES-1A	5 - ATGCGCTTCATTCACGCAC-3	28
GES-1B	5 - CTATTTGTCCGTGCTCAGG-3	28
CTX-M-ATG	5-ATGGTGACAAAGAGAGTGCA-3	This study
CTX-M-Stop	5-TTACAGCCCTTCGGCGATGA-3	This study
CTX-M-NdeI	5-AAAACATATGGTGACAAAGAGAGTGCA-3	This study
CTX-M-BamHI	5-AAAAGGATCCTTACAGCCCTTCGGCGATGA-3	This study
ISEcp prom+	5-TGCTCTGTGGATAACTTGC-3	24
IS903B1	5-GGCTTTGTTGAATAAATCAG-3	24
CTX-M-93-Q169L-A	5- GAATGGCGGTATTCAGCGTAGGTTCAG -3	This study
CTX-M-93-Q169L-B	5-CTGAACCTACGCTGAATACCGCCATTC-3	This study
CTX-M-93-Q169M-A	5- GAATGGCGGTATTCATCGTAGGTTCAG -3	This study
CTX-M-93-Q169M-B	5-CTGAACCTACGATGAATACCGCCATTC-3	This study
CTX-M-27-L169C-A	5-GAATGGCGGTATTACACGTAGGTTCAG-3	This study
CTX-M-27-L169C-B	5-CTGAACCTACGTGTAATACCGCCATTC-3	This study
Rep typing	primers for rep typing	8
MLST typing	primers for MLST typing were from http ://mlst.ucc.ie/mlst/dbs/Ecoli	37

Table 2. MICs for clinical strain *E. coli* Bre-1 and the corresponding transformants and recombinant clones.

	E. coli Bre-1[a] (CTX-M-93)	*E. coli* TOP10 (pNBRE-1)	*E. coli* TOP10 (pM-93)	*E. coli* TOP10 (pM-27)	*E. coli* TOP10 (pM-93-Q169M)	*E. coli* TOP10 (pM-27-L169C)	*E. coli* TOP10
Amoxicillin	6	4	4	>256	96	8	4
Amoxicillin + CLA[b]	4	4	4	4	4	4	4
Ticarcillin	4	2	2	>256	256	16	2
Ticarcillin + CLA[b]	2	2	2	4	4	4	2
Piperacillin	8	24	8	48	24	3	1.5
Piperacillin + TZB[b]	1	2	1	1	1	1	1
Cefalothin	16	1	1	>256	16	0.5	0.5
Cefuroxime	1	0.25	0.125	>256	>256	6	0.25
Cefixime	>256	128	6	0.5	6	0.75	0.25
Cefoxitin	4	0.25	1	1	0.5	1	0.25
Cefotaxime	6	3	1	32	12	0.75	0.25

Ceftazidime	8	16	8	1.5	4	0.38	0.125
Ceftazidime + CLA[b]	0.25	0.5	0.25	0.125	0.125	0.125	0.125
Cefpirome	3	1.5	0.5	2	1.5	0.12	0.032
Cefepime	0.75	0.38	0.125	0.5	0.38	0.47	0.023
Imipenem	0.19	0.19	0.19	0.19	0.19	0.19	0.19

[a] *E. coli* BRE-1 produced β-lactamase CTX-M-93.
[b] CLA, clavulanic acid at a fixed concentration of 2 μg/ml ; TZB, tazobactam at a fixed concentration of 4 μg/ml.

Table 3. Kinetic parameters of the purified ß-lactamases CTX-M-93 compared to CTX-M-27

	CTX-M-93			CTX-M-27[a]		
Substrate	k_{cat} (s^{-1})	K_m (µM)	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)	k_{cat} (s^{-1})	K_m (µM)	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)
Penicillin G	0.07	22.5	0.003	11 ± 0.5	6 ± 0.2	1.8
Amoxicillin	ND[b]	ND	ND	5 ± 1.0	10 ± 0.4	0.5
Ticarcillin	ND	ND	ND	1 ± 0.3	13 ± 0.3	0.07
Piperacillin	ND	ND	ND	9 ± 0.5	8 ± 0.3	1.1
Cefalothin	1.8	33	0.06	232 ± 30.0	83 ± 1.0	2.8
Cephaloridine	1.4	51	0.03	NT[c]	NT	NT
Cefuroxime	0.23	41.23	0.008	79 ± 4.0	45 ± 2.0	1.75
Cefotaxime	0.6	67	0.009	113 ±15.0	150 ± 8.0	0.75
Ceftazidime	2.5	25.5	0.1	3 ± 0.3	330 ± 22.0	0.009
Aztreonam	ND	ND	ND	0.4 ± 0.2	17 ± 1.0	0.02

Data are means from three independent experiments. Standard deviations were within 15%.

[a] Results were from Bonnet et al. (4)

[b] ND, not determinable (the initial rate of hydrolysis was lower than 0.001 $\mu M^{-1}.s^{-1}$).

[c] NT, not tested

Table 4: Inhibition profile of CTX-M-93 compared with those of CTX-M-27

β-lactamase	IC50 (μM)	
	Clavulanic acid	Tazobactam
CTX-M-93	870	140
CTX-27[a]	0.020	0.007

[a] Results were from Bonnet et al. (4)

Legends to Figures

Figure 1: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of CTX-M-93 purification. Lanes: 1, protein molecular mass reference; 2, clarified extract of the overexpressed CTX-M-93 β-lactamase; 3, purified extract of CTX-M-93 β-lactamase.

Figure 2: Alignment of the amino acid sequences of ESBLs around Ambler position 169 in the Omega loop. The amino acids of the omega loop are underlined