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

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24 Extended-spectrum β -lactamases (ESBLs) of the CTX-M-type are increasingly reported
25 worldwide, with more than 90 known variants. Clinical isolate *Escherichia coli* Bre-1
26 was isolated in 2009 and displayed an unusual ESBL phenotype: made of a synergy
27 image between expanded-cephalosporins and clavulanic acid disks, and susceptibility to
28 penicillins. *E. coli* Bre-1 harbored a novel CTX-M-encoding gene, designated *bla*_{CTX-M-}
29 ₉₃. CTX-M-93 differed from CTX-M-27 by only a single L169Q substitution. As
30 compared to CTX-M-27, CTX-M-93 conferred higher MICs of ceftazidime to *E. coli*
31 (MIC, 8 versus 1.5 μ g/mL) and decreased MICs for other expanded-cephalosporins
32 (MIC cefotaxime, 1 versus 32 μ g/mL) and for penicillins (MIC ticarcillin, 0.5 versus
33 >256 μ g/mL). Comparison of enzymatic properties revealed that L169Q substitution
34 lead to decreased K_m for ceftazidime (25.5 versus 330 μ M), but decreased hydrolytic
35 activity against good substrates, such as cefotaxime (k_{cat} , 0.6 versus 113 s^{-1}), probably
36 owing to the alteration of omega loop positioning during the catalytic process. *Bla*_{CTX-M-}
37 ₉₃ gene was surrounded by *ISEcp1* and *IS903* elements and inserted onto a 150-kb non
38 self-transferable IncF type plasmid. *E. coli* Bre-1 belongs to the phylogroup D, and is of
39 MLST type 624, a ST-type found only in rare spanish CTX-M-14-producing *E. coli*
40 isolates.
41 We have characterized a novel CTX-M-variant, CTX-M-93, lacking significant
42 penicillin hydrolysis, but with increased ceftazidime hydrolysis.

43 A widely accepted definition of extended-spectrum β -lactamases (ESBLs) is: molecular
44 Ambler class A or functional class 2be β -lactamases capable of conferring bacterial
45 resistance to the penicillins, first-, second-, and third-generation cephalosporins, and
46 aztreonam (but not to cephamycins or carbapenems) with hydrolysis rates of at least
47 10% of that for benzylpenicillin and activity inhibited by β -lactamase inhibitors such as
48 clavulanic acid (1,7,23). ESBL-producing *Enterobacteriaceae* are mostly associated
49 with urinary tract infections, but may also cause significant bloodstream-associated
50 infections, that result in increased hospital costs, length of stay, and patient mortality
51 (11,16). Plasmid-encoded ESBLs of the CTX-M-type are reported increasingly
52 worldwide in Gram-negative rods and account now for most of the ESBLs found in
53 *Enterobacteriaceae* (11,16). CTX-Ms form a rapidly growing family that comprises
54 currently up to 90 variants that are divided into five groups according to amino-acid
55 sequence identity (CTX-M-1, -2, -8, -9 and -25 groups) with different groups prevalent
56 in different countries (16,24).

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

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

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

77

78 **Materials and methods**

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

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

88 **Nucleic acid extractions, PCR and DNA sequencing.** Whole-cell DNAs were
89 extracted using QIAamp DNA Mini Kit (Qiagen, Les Ulis, France). The *bla*_{CTX-M},
90 *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.](http://www.ncbi.nlm.nih.gov)
98 [ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)).

99 **β -Lactamase gene cloning.** *bla*_{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 μ g/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*_{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 plasmid pM-27 coding for CTX-M-27 β -

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

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

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

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

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

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

145 **Overexpression and β -Lactamase purification.** A two liter culture of *E. coli* BL21
146 (pET-M93) was IPTG induced (1 mg/ml) as previously described (20). The β -lactamase
147 extract was obtained after sonification, as described previously (20), was dialyzed
148 overnight against 50 mM DEA buffer (pH 9.7) and was then loaded onto a
149 preequilibrated Q-Sepharose column (Amersham Pharmacia Biotech) in 50 mM DEA
150 buffer (pH 9.7). The β -lactamase activities, as determined qualitatively for each fraction
151 using nitrocefin hydrolysis (Oxoid), were detected in the flow through. The fractions
152 containing the highest β -lactamase activity were pooled and dialyzed overnight against
153 50 mM sodium phosphate buffer (pH 7.0). It was filtrated onto a polyethersulfone
154 membrane (Vivaspin 50,000 MW, Sartorius Stedim, Aubagne, France) that removes
155 molecules with a molecular weight higher than 50 kDa, prior to a 10-fold concentration
156 (Vivaspin 10,000 MW, Sartorius Stedim) as described by the manufacturer. The protein
157 content was measured using the Bio-Rad DC protein assay, and the specific activities of
158 the crude extract and of the purified β -lactamase were determined as previously
159 reported (20) with 100 μ M of cephalothin as the substrate. One unit of enzyme activity

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

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

168 Kinetic parameters (maximal velocity, V_{max} and Michaelis-Menten constant,
169 K_m) were determined by using the Eadie-Hoffstee linearization ($V = V_{\text{max}} - V K_m /$
170 $[S]$) of the Michaelis-Menten equation ($V = V_{\text{max}} [S] / (K_m + [S])$) as previously
171 described (12,25). K_{cat} values were determined by dividing the V_{max} ($\mu\text{M s}^{-1}$) for
172 each substrate by the concentration of purified enzyme.

173 Various concentrations of clavulanic acid and tazobactam were preincubated with
174 the enzyme for 3 min at 30°C before testing the rate of cephalothin (100 μM)
175 hydrolysis. The 50% inhibitory concentrations (IC_{50}) of these inhibitors were
176 determined as the concentration of these inhibitors that inhibited hydrolytic activity by
177 50%.

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

181

182 **Results**

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

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

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

200 **Genetic environment and support of the *bla*_{CTX-M-93} gene.** Analysis of the sequences
201 surrounding *bla*_{CTX-M-93} gene identified two insertion sequences in the same orientation
202 named *ISEcp1* and *IS903* and located respectively upstream and downstream of the
203 gene.

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

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

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

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

218 Kinetic parameters of purified CTX-M-93 β -lactamase revealed activity against
219 restricted and expanded-spectrum cephalosporins (Table 3). The enzyme showed the
220 highest affinity for penicillin G (K_m value of $22.5 \mu\text{M}$) and ceftazidime (K_m value of
221 $25.5 \mu\text{M}$), and the highest level of activity against ceftazidime (k_{cat} value of 2.5 s^{-1}) and
222 cephalothin (k_{cat} value of 1.8 s^{-1}). Interestingly, catalytic efficiency for ceftazidime
223 (k_{cat}/K_m of $0.1 \mu\text{M}^{-1} \text{ s}^{-1}$) is better than for cefotaxime (k_{cat}/K_m of $0.009 \mu\text{M}^{-1} \text{ s}^{-1}$) and
224 also higher than for CTX-M-27. However, the other catalytic efficiencies of CTX-M-93
225 are lower than those of CTX-M-27. As expected, CTX-M-93 had no detectable
226 hydrolysis for amoxicillin, ticarcillin and piperacillin. Determinations of IC₅₀
227 performed with cephalothin ($100 \mu\text{M}$) as substrate showed that CTX-M-93 is inhibited
228 by amoxicillin (IC₅₀ of $16 \mu\text{M}$) and ticarcillin (IC₅₀ of $0.11 \mu\text{M}$).

229 Surprisingly, CTX-M-93 was weakly inhibited in vitro by β -lactam inhibitors
230 (IC₅₀ of $140 \mu\text{M}$ for tazobactam and $870 \mu\text{M}$ for clavulanic acid) although MICs of
231 cephalosporins were significantly lowered by the addition of β -lactam inhibitors (Table

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

236 **Site-directed mutagenesis and effects for MICs.** The leucine residue at position 169
237 located in the omega-loop is fully conserved among CTX-M enzymes (data not shown).
238 To investigate the role of the glutamine residue of CTX-M-93, pM-27 plasmid was
239 generated coding for CTX-M-27 β -lactamase, which differed from CTX-M-93 by the
240 Gln169Leu substitution. *E. coli* TOP10 (pM-27), compared to *E. coli* TOP10 (pM-93),
241 showed higher MICs of amoxicillin (> 256 vs 4 μ g/ml), ticarcillin (> 256 vs 0.5 μ g/ml)
242 and piperacillin (48 vs 12 μ g/ml) but lower MICs of ceftazidime (1.5 vs 8 μ g/ml) (Table
243 2).

244 Analysis of amino acid residues found at Ambler position 169 among class A
245 ESBLs (Fig. 2) revealed a leucine or a methionine residue for most ESBLs. Only CTX-
246 M-93 and TLA-2 differed at position 169, with a glutamine and a cysteine, respectively.
247 Recombinant plasmid pM-93-Q169M and pM-27-L169C were generated to evaluate the
248 role of these methionine and cysteine residues at position 169. MICs for recombinant
249 strains *E. coli* TOP10(pM-93-Q169M) and *E. coli* TOP10(pM-27-L169C) were
250 compared to *E. coli* TOP10(pM-93) and *E. coli* TOP10(pM-27) respectively (Table 2).
251 MICs of *E. coli* TOP10 (pM-93-Q169M) were higher for amoxicillin (96 vs 4 mg/l),
252 ticarcillin (256 vs 2 μ g/ml) and piperacillin (24 vs 12 μ g/ml) than MICs of *E. coli*
253 TOP10(pM93), whereas MICs of ceftazidime were identical. MICs of *E. coli*
254 TOP10(pM-27-L169C) were lower for amoxicillin (8 vs > 256 μ g/ml), ticarcillin (16 vs
255 >256 μ g/ml) and piperacillin (3 vs 48 μ g/ml) than MICs of *E. coli* TOP10(pM-27), so as

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

257 **Discussion**

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

266 The genetic environment of *bla*_{CTX-M-93} gene, made of the insertion sequences
267 *ISEcp1* and *IS903* upstream and downstream, respectively, was similar to the genetic
268 environment previously described for *bla*_{CTX-M-14} (19,24). *ISEcp1* insertion sequences
269 have been involved in the mobilization of *bla*_{CTX-M} genes and in providing the promoter
270 for expression of these genes (30,31). *Bla*_{CTX-M-93} was inserted on a non self-transferable
271 150-kb plasmid of broad spectrum IncF type. It was isolated from a clinical strain of *E.*
272 *coli* ST624, an ST found only in rare Spanish CTX-M-14 producing *E. coli* isolates
273 (36).

274 Amino acids of the omega loop play a major role in the enzyme substrate
275 profiling particularly for cephalosporins (2,13,17,27,29,34). Substitutions in the omega
276 loop might affect the expression, the stability and the activity of the enzymes. Position
277 169 is well-conserved among ESBLs, most of them showing a leucine or a methionine
278 residue (Fig. 2). No natural class A β-lactamase have a glutamine residue at position
279 169 as CTX-M-93 does. Site-directed mutagenesis experiments showed that the

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

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

303

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430 1136-1151.

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

Strains, plasmids, primers	Relevant genotype and produced β -lactamase (pI)	Source
Strains		
<i>E. coli</i> Bre-1	Clinical <i>E. coli</i> strain producing CTX-M-93 (pI 8.9)	This study
<i>E. coli</i> Top10	SupE44 hsdS20 (r_B^- , m_B^-) recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1	Stratagene
<i>E. coli</i> J53	Azide resistant <i>E. coli</i> strain	22
<i>E. coli</i> 50192	<i>E. coli</i> NTCC 50192 harbouring 154-, 66-, 48-, and 7-kb plasmids	22
Plasmids		
pNBre-1	natural plasmid encoding CTX-M-93	This study
pPCRScrip	recombinant pPCRScrip plasmid	Stratagene
pET-9a	recombinant pET-9a plasmid	Stratagene
pM-93	recombinant pPCRScrip plasmid which contains a 0.9-kb fragment encoding CTX-M-93 (pI 8.9)	This study
pM-93NB	recombinant pPCRScrip plasmid which contains a 0.9-kb fragment encoding CTX-M-93 with NdeI and BamHI restriction sites	This study
pM-27	recombinant pPCRScrip plasmid which contains a 0.9-kb fragment encoding CTX-M-27 (Gln169Leu)	This study
pM-93-Q169M	recombinant pPCRScrip 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-ATGCGTTATWTTTCGCCTGTGT-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 - ACGCTTTTGCAAATTTTCGGC-3	This study
SFO-A	5 - TTACGTCAAACCACCCTGATGG-3	This study
SFO-B	5 - TTCTGCATTCTGCTGTGGCTG-3	This study
BES-A	5 - ATGTGGCAGTGGCTTGAAA-3	This study
BES-B	5 -TTATCTTGCAGTACCAGTCG -3	This study
BEL-1A	5-CGACAATGCCGCAGCTAACC -3	26
BEL-1B	5-CAGAAGCAATTAATAACGCC -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- GAATGGCGGTATTCAGCGTAGGTTTCAG -3	This study
CTX-M-93-Q169L-B	5-CTGAACCTACGCTGAATACCGCCATTC-3	This study
CTX-M-93-Q169M-A	5- GAATGGCGGTATTCATCGTAGGTTTCAG -3	This study
CTX-M-93-Q169M-B	5-CTGAACCTACGATGAATACCGCCATTC-3	This study
CTX-M-27-L169C-A	5-GAATGGCGGTATTACACGTAGGTTTCAG-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.

	<i>E. coli</i> Bre-1 ^a (CTX-M-93)	<i>E. coli</i> TOP10 (pNBRE-1)	<i>E. coli</i> TOP10 (pM-93)	<i>E. coli</i> TOP10 (pM-27)	<i>E. coli</i> TOP10 (pM-93-Q169M)	<i>E. coli</i> TOP10 (pM-27-L169C)	<i>E. coli</i> 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 μ g/ml ; TZB, tazobactam at a fixed concentration of 4 μ g/ml.

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

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

^c NT, not tested

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

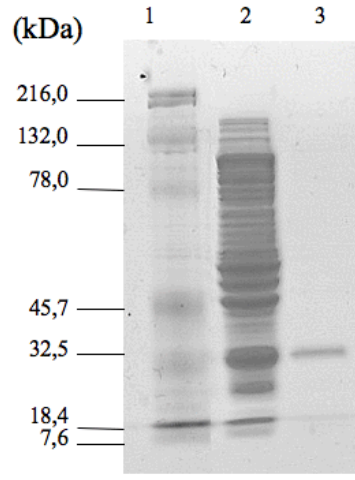
β -lactamase	IC50 (μ M)	
	Clavulanic acid	Tazobactam
CTX-M-93	870	140
CTX-27 ^a	0.020	0.007

^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



169

CTX-M	ASVTAFARQL	GDETFRLDRT	EPTLNT-AIP	GDPRDTSPL
SFO-1	AKVTEYARTI	GDKTFRLDRT	EPTLNT-AIP	SDKRDTSPL
KPC-1	AGLTAFMRSI	GDTTFRLDRW	ELELNS-AIP	GDARDTSSPR
BES-1	QALNRFVQGL	GDPAFRLDRI	EPHLNS-AEP	GDVRDTTTPL
TEM-1	KELTAFLNHM	GDHVTRLDRW	EPELNE-AIP	NDERDTTTPA
SHV	AGLTAFLRQI	GDNVTRLDRW	ETELNE-ALP	GDARDTTTPA
GES-1	AAMTQYFRKI	GDSVSRLDRK	EPEMGD-NTP	GDLRDTTTPI
BEL-1	AAMTQYFRKI	GDSVSRLDRK	EPEMGD-NTP	GDLRDTTTPI
TLA-1	NKVHNFISKL	GVKNISIKAT	EEEMHK-AWN	VQYTNWTTTPD
CME-1	KTVQKLMDEV	GIKNFQIKYN	EEEMHKNDVK	TLYANYTTTA
CGA-1	QVVQKFMDSK	GVKGFQIKYN	EEDMHK-DWN	VQYENYSTTK
VEB-1	DSVQKFLNAN	HFTDISIKAN	EEQMHK-DWN	TQYQNWATPT
PER-1	AALHDYIQSM	GIKETAVVAN	EAQMHA-DDQ	VQYQNWTSMK
TLA-2	EAVKRYIISK	GISDFDIRAT	EKECHE-SWN	VQYSNWSTPV
CTX-M-93	GGVTAFARAI	GDETFRLDRT	EPTQNT-AIP	GDPRDTTTPR