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CTX-M-93, a CTX-M Variant Lacking Penicillin Hydrolytic Activity▽

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Extended-spectrum β-lactamases (ESBLs) of the CTX-M type are increasingly being reported worldwide, with more than 90 known variants. Clinical *Escherichia coli* isolate Bre-1 was isolated in 2009 and displayed an unusual ESBL phenotype, made of a synergy image between expanded cephalosporins and clavulanic acid discs 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. Compared to CTX-M-27, CTX-M-93 conferred higher MICs of ceftazidime for *E. coli* (MIC of 8 versus 1.5 μg/ml) and decreased MICs of other expanded-cephalosporins (MIC of cefotaxime of 1 versus 32 μg/ml) and penicillins (MIC of ticarcillin of 0.5 versus >256 μg/ml). A comparison of enzymatic properties revealed that the L169Q substitution led to a decreased K_m for ceftazidime (25.5 versus 330 μM) but decreased hydrolytic activity against good substrates, such as cefotaxime (k_{cat} of 0.6 versus 113 s^{-1}), probably owing to the alteration of the omega loop positioning during the catalytic process. The $bla_{\text{CTX-M-93}}$ gene was surrounded by the IS*Ecp1* and IS*903* elements and inserted onto a 150-kb non-self-transferrable IncF-type plasmid. *E. coli* Bre-1 belongs to phylogroup D and is of multilocus sequence type (MLST) 624, a sequence 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 as follows: molecular Ambler class A or functional class 2be β-lactamases capable of conferring bacterial resistance to the penicillins, narrow-spectrum, expanded-spectrum, and broad-spectrum 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 associated mostly with urinary tract infections but may also cause significant bloodstream-associated infections that result in increased hospital costs, lengths of stay, and patient mortality (11, 16). Plasmid-encoded ESBLs of the CTX-M type are increasingly being reported worldwide in Gram-negative rods and now account for most of the ESBLs found in the *Enterobacteriaceae* (11, 16). CTX-Ms form a rapidly growing family that currently comprises up to 90 variants that are divided into five groups according to amino acid sequence identity (the CTX-M-1, -2, -8, -9, and -25 groups), with different groups being prevalent in different countries (16, 24).

β-Lactamases of the CTX-M types are structurally related to the naturally produced β-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 against ceftazidime and aztreonam (31). The cefotaxime-hydrolyzing activity of CTX-M enzymes is related to the flexibility of the β3 strand and omega loop and 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 recently been reported: the P167S mutant of CTX-M-18 (also called CTX-M-14), designated CTX-M-19 (29); the P167Q mutant of CTX-M-3, designated CTX-M-54 (2); the P167T mutant of 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 β-lactamases produced by an *Escherichia coli* clinical isolate displaying *in vitro* susceptibility to penicillins and resistance to broad-spectrum cephalosporins that is reversed by the addition of clavulanic acid. 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 β-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* clinical strain Bre-1 was isolated from a urinary tract infection.

Bacterial identification was performed by using the API 20E system (bioMérieux, Marcy-l'Etoile, France). Antibiograms were determined with the disc dif-

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TABLE 1. Strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Relevant genotype, description, or sequence	Source or reference
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 harboring 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 that contains a 0.9-kb fragment encoding CTX-M-93 (pI 8.9)	This study
pM-93NB	Recombinant pPCRScript plasmid that contains a 0.9-kb fragment encoding CTX-M-93 with NdeI and BamHI restriction sites	This study
pM-27	Recombinant pPCRScript plasmid that contains a 0.9-kb fragment encoding CTX-M-27 (Gln169Leu)	This study
pM-93-Q169M	Recombinant pPCRScript plasmid that contains a 0.9-kb fragment encoding a modified CTX-M-93 protein with a Gln169Met substitution	This study
pM-27-L169C	Recombinant pPCRScript plasmid that 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 that contains a 0.9-kb fragment encoding CTX-M-93	This study
Primers[a]		
TEM-α	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

[a] Primers for Rep typing were reported previously (8). Primers for MLST typing were from the MLST databases at the University College Cork (http://mlst.ucc.ie/mlst/dbs/Ecoli) (37).

fusion method on Mueller-Hinton agar (Bio-Rad, 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 37°C for 18 h. MICs of β-lactams were determined by using the Etest technique (bioMérieux).

Nucleic acid extractions, PCR, and DNA sequencing. Whole-cell DNAs were extracted by using the QIAamp DNA minikit (Qiagen, Les Ulis, France). bla_{CTX-M}, bla_{SHV}, bla_{TEM}, and minor ESBL genes (23) were searched for and characterized as described previously (24). PCR experiments were performed with an ABI 2700 thermocycler (Applied Biosystems, Les Ulis, France) by using laboratory-designed primers (Table 1).

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

β-Lactamase gene cloning. $bla_{CTX\text{-}M\text{-}93}$ was amplified by using primers CTX-M-ATG and CTX-M-stop (Table 1) and cloned directly into the PCR-Script Cam cloning kit (Stratagene, Agilent Technologies, Massy, France) according to instructions provided by the manufacturer, resulting in pM-93. The transformants harboring pM-93 were selected on Mueller-Hinton agar supplemented with 1 μg/ml ceftazidime. In order to overexpress CTX-M-93, an 893-bp PCR-generated fragment containing NdeI and BamHI restriction sites, respectively (Table 1), was cloned into the pPCRscript Cam+ plasmid, according to the manufacturer's instructions, using primers CTX-M-NdeI and CTX-M-BamHI, resulting in plasmid pM-93NB. The insert containing $bla_{CTX\text{-}M\text{-}93}$ of pM-93NB was removed with the NdeI and BamHI restriction enzymes and cloned into the NdeI/BamHI-restricted pET-9a expression vector (Stratagene, Amsterdam, Netherlands), resulting in pET-M-93, by using standard techniques (32).

Site-directed mutagenesis. Recombinant plasmid pM-93 was used as a template for site-directed mutagenesis according to instructions provided by the manufacturer (QuikChange site-directed mutagenesis kit; Stratagene). Primers CTX-M-93-Q169L-A and CTX-M-93-Q169L-B (Table 1) were used to generate recombinant plasmid 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 glutamine residue at position 169 was replaced with a methionine 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 the TLA-2 ESBL (15).

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

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

Phylogenetic grouping. The *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 previously 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 for members of the *Enterobacteriaceae* was performed as described previously (8). Genetic structures surrounding the $bla_{CTX\text{-}M\text{-}93}$ gene were determined as previously described (19, 24, 30). Primers specific for the known genetic environment of group 9 CTX-M variants were used (Table 1).

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 isoelectrofocusing (IEF) as previously described (20, 25).

Overexpression and β-lactamase purification. A 2-liter culture of *E. coli* BL21(pET-M93) was induced by isopropyl-β-D-thiogalactopyranoside (IPTG) (1 mg/ml) as previously described (20). The β-lactamase extract was obtained after sonication as described previously (20), was dialyzed overnight against 50 mM diethanolamine (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 β-lactamase activities, as determined qualitatively for each fraction using nitrocefin hydrolysis (Oxoid), were detected in the flowthrough fraction. The fractions containing the highest β-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 molecular weight [MW]; Sartorius Stedim, Aubagne, France) that removes molecules with a molecular mass higher than 50 kDa, prior to a 10-fold concentration (Vivaspin, 10,000 MW; Sartorius Stedim), according to instructions provided by the manufacturer. The protein content was measured by using the Bio-Rad DC protein assay, and the specific activities of the crude extract and of the purified β-lactamase were determined as previously reported (20), with 100 μM cephalothin as the substrate. One unit of enzyme activity was defined as the amount of enzyme that hydrolyzes 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 with 100 mM sodium phosphate (pH 7.0). The rates of hydrolysis were determined with an Ultrospec 2000 spectrophotometer (Amersham Pharmacia Biotech). The wavelengths and absorption coefficients of β-lactams were previously reported (25).

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

Various concentrations of clavulanic acid and tazobactam were preincubated with the enzyme for 3 min at 30°C before the rate of cephalothin (100 μM) hydrolysis was tested. The 50% inhibitory concentrations (IC_{50}s) 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 deposited in the GenBank nucleotide database under accession number HQ166709.

RESULTS

Characterization of *E. coli* clinical isolate Bre-1 and β-lactam susceptibility. *E. coli* Bre-1 was isolated in the urine specimens of a 61-year-old patient hospitalized at the University Hospital of Brest, Brest, France, in June 2009. *E. coli* Bre-1 exhibited resistance to narrow- and broad-spectrum cephalosporins (MICs of cephalothin of 16 μg/ml, cefotaxime of 6 μg/ml, and ceftazidime of 8 μg/ml), with synergy images between ceftazidime and coamoxiclav (clavulanic acid plus amoxicillin), suggesting the presence of an ESBL. However, *E. coli* Bre-1 showed low MICs of 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 being ST624.

Resistance gene determination. Preliminary PCR amplification experiments with primers designed to amplify internal fragments of ESBL genes (Table 1) gave positive results for $bla_{CTX\text{-}M}$. External primers were used to amplify the $bla_{CTX\text{-}M}$ 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 $bla_{CTX\text{-}M\text{-}93}$ gene. Analysis of the sequences surrounding the $bla_{CTX\text{-}M\text{-}93}$ gene identified two insertion sequences in the same orientation, named IS*Ecp1* and IS*903*, located upstream and downstream of the gene, respectively.

Analysis of the plasmid content of *E. coli* Bre-1 identified a single 150-kb plasmid named pNBRE-1. The electrotransformation of this plasmid into reference strain *E. coli* TOP10 indicated that 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 recipient strain *E. coli* J53.

Biochemical properties of CTX-M-93. IEF analysis of β-lactamase extracts of *E. coli* Bre-1, *E. coli* TOP10(pNBRE-1), and

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

Drug[b]	MIC (μg/ml)						
	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-Q169 M)	*E. coli* TOP10(pM-27-L169C)	*E. coli* TOP10
Amoxicillin	6	4	4	>256	96	8	4
Amoxicillin + CLA	4	4	4	4	4	4	4
Ticarcillin	4	2	2	>256	256	16	2
Ticarcillin + CLA	2	2	2	4	4	4	2
Piperacillin	8	24	8	48	24	3	1.5
Piperacillin + TZB	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	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.

clone *E. coli* TOP10(pM-93) 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 the purified CTX-M-93 β-lactamase revealed activity against restricted- and expanded-spectrum cephalosporins (Table 3). The enzyme showed the highest affinities for penicillin G (K_m value of 22.5 μM) and ceftazidime (K_m value of 25.5 μM) and the highest levels of activity against ceftazidime (k_{cat} value of 2.5 s^{-1}) and cephalothin (k_{cat} value of 1.8 s^{-1}). Interestingly, the catalytic efficiency for ceftazidime (k_{cat}/K_m of 0.1 μM^{-1} s^{-1}) is better than that for cefotaxime (k_{cat}/K_m of 0.009 μM^{-1} s^{-1}) and also higher than that of 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 IC_{50}s performed with cephalothin (100 μM) as a substrate showed that CTX-M-93 is inhibited by amoxicillin (IC_{50} of 16 μM) and ticarcillin (IC_{50} of 0.11 μM).

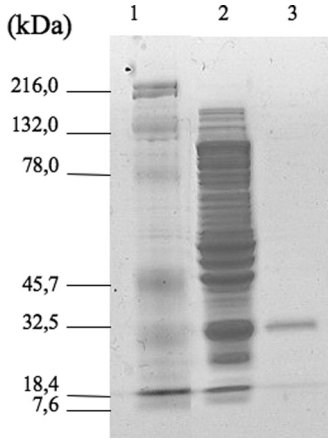

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of CTX-M-93 purification. Lanes: 1, protein molecular mass reference; 2, clarified extract of the overexpressed CTX-M-93 β-lactamase; 3, purified extract of the CTX-M-93 β-lactamase.

Surprisingly, CTX-M-93 was weakly inhibited *in vitro* by β-lactam inhibitors (IC_{50}s 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 for disc diffusion antibiograms between ceftazidime and coamoxiclav is in fact the result of an inhibition of CTX-M-93 by amoxicillin and not clavulanic acid. Indeed, amoxicillin discs alone gave the same synergy images with ceftazidime as coamoxiclav (data not shown).

Site-directed mutagenesis and effects of 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, plasmid pM-27, coding for CTX-M-27 β-lactamase, was generated, 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 versus 4 μg/ml), ticarcillin (>256 versus 0.5 μg/ml), and piperacillin (48 versus 12 μg/ml) but lower MICs of ceftazidime (1.5 versus 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 plasmids 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 those for *E. coli*

TABLE 3. Kinetic parameters of the purified β-lactamase CTX-M-93 compared to those of CTX-M-27[a]

Substrate	CTX-M-93			CTX-M-27[b]		
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (μM^{-1} s^{-1})	Mean k_{cat} (s^{-1}) ± SD	Mean K_m (μM) ± SD	k_{cat}/K_m (μM^{-1} s^{-1})
Penicillin G	0.07	22.5	0.003	11 ± 0.5	6 ± 0.2	1.8
Amoxicillin	ND	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	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

[a] Data are means from three independent experiments. Standard deviations were within 15%. ND, not determinable (the initial rate of hydrolysis was lower than 0.001 $\mu M^{-1} \cdot s^{-1}$); NT, not tested.
[b] Results were described previously by Bonnet et al. (4).

TOP10(pM-93) and *E. coli* TOP10(pM-27), respectively (Table 2). MICs for *E. coli* TOP10(pM-93-Q169M) were higher for amoxicillin (96 versus 4 mg/liter), ticarcillin (256 versus 2 μg/ml), and piperacillin (24 versus 12 μg/ml) than MICs for *E. coli* TOP10(pM93), whereas MICs of ceftazidime were identical. MICs for *E. coli* TOP10(pM-27-L169C) were lower for amoxicillin (8 versus >256 μg/ml), ticarcillin (16 versus >256 μg/ml), piperacillin (3 versus 48 μg/ml), and ceftazidime (0.38 versus 1.5 μg/ml) than those for *E. coli* TOP10(pM-27).

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 an ESBL profile to *E. coli* Bre-1, 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 by only two substitutions (Asp240Gly and Leu169Gln) (4). CTX-M-27 is rarely isolated compared to CTX-M15 or CTX-M-14, but epidemic situations have also been described (6, 16, 36).

The genetic environment of the $bla_{CTX\text{-}M\text{-}93}$ gene, made of the insertion sequences IS*Ecp1* and IS*903* upstream and downstream, respectively, was similar to the genetic environment previously described for $bla_{CTX\text{-}M\text{-}14}$ (19, 24). IS*Ecp1* insertion sequences have been involved in the mobilization of $bla_{CTX\text{-}M}$ genes and in providing the promoter for the expression of these genes (30, 31). $bla_{CTX\text{-}M\text{-}93}$ was inserted on a non-self-transferrable 150-kb plasmid of the broad-spectrum IncF type. It was isolated from a clinical strain of *E. coli* of 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 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, with most of them showing a leucine or a methionine residue (Fig. 2). No natural class A β-lactamases 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 the weak activity of CTX-M-93 against penicillins. Interestingly, the Leu169Cys substitution also conferred a weak activity against these substrates. The Cys residue at position 169 was found in TLA-2 (15), an Ambler class A ESBL identified in a wastewater treatment plant, which also has a weak activity against amino-, carboxy-, and ureidopenicillins. As for CTX-M-93, TLA-2 was weakly inhibited *in vitro* by clavulanic acid and tazobactam, although the MICs of cephalosporins were lowered by their addition. A random-mutagenesis technique applied to CTX-M-9 (13) to investigate substitutions that increased activity against ceftazidime selected novel amino acid substitutions. A mutant with a Leu169Gln substitution was

TABLE 4. Inhibition profile of CTX-M-93 compared with that of CTX-M-27

β-Lactamase	IC_{50} (μM)	
	Clavulanic acid	Tazobactam
CTX-M-93	870	140
CTX-27[a]	0.020	0.007

[a] Results were described previously by Bonnet et al. (4).

```
                                    169
CTX-M      ASVTAFARQL GDETFRLDRT EPTLNT-AIP GDPRDTTSPR
SFO-1      AKVTEYARTI GDKTFRLDRT EPTLNT-AIP SDKRDTTSPL
KPC-1      AGLTAFMRSI GDTTFRLDRW ELELNS-AIP GDARDTSSPR
BES-1      QALNRFVQGL GDPAFRLDRI EPHLNS-AEP GDVRDTTTPL
TEM-1      KELTAFLHNM GDHVTRLDRW EPELNE-AIP NDERDTTMPA
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 VQYTNWTTPD
CME-1      KTVQKLMDVN 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
```

FIG. 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.

selected and resulted in an increased activity against ceftazidime but a concomitant compromised activity against penicillins. A previously reported *in vitro* mutagenesis experiment with the TEM-1 enzyme (35) showed a similar behavior against ceftazidime and penicillins for mutants with the Leu169Pro substitution. According to these different studies, position 169 in the omega loop of class A β-lactamases may be implicated in the enzyme's activity against amino-, carboxy-, and ureidopenicillins and also against ceftazidime. It was shown previously 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 the 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 also raised the question of whether penicillins could be options for treating urinary tract infections associated with such a CTX-M variant.

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