## High-Level Resistance to Ceftazidime Conferred by a Novel Enzyme, CTX-M-32, Derived from CTX-M-1 through a Single Asp240-Gly Substitution

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A clinical strain of *Escherichia coli* isolated from pleural liquid with high levels of resistance to cefotaxime, ceftazidime, and aztreonam harbors a novel CTX-M gene ( $bla_{CTX-M-32}$ ) whose amino acid sequence differs from that of CTX-M-1 by a single Asp240-Gly substitution. Moreover, by site-directed mutagenesis we demonstrated that this replacement is a key event in ceftazidime hydrolysis

The emergence of plasmid-mediated extended-spectrum  $\beta$ -lactamases in members of the family *Enterobacteriaceae* has become a worldwide problem (3, 4, 6, 7, 11–13, 16).

Most extended-spectrum  $\beta$ -lactamases are derivatives of TEM-1, TEM-2, or SHV-1 enzymes; however, there are an increasing number of reports that describe the worldwide emergence of  $\beta$ -lactamases belonging to other families, such as CTX-M and/or OXA derivatives (8).

The family of CTX-M enzymes is grouped on the basis of similarities in amino acid sequences into four major phylogenetic trees (6): the CTX-M-1 group (CTX-M-1 or MEN-1, CTX-M-3, CTX-M-10, CTX-M-12, CTX-M-15, and now CTX-M-32), the CTX-M-2 group (CTX-M-2, CTX-M-4, CTX-M-5, CTX-M-6, CTX-M-7, CTX-20, and Toho-1), the CTX-M-8 group, and the CTX-M-9 group (CTX-M-9, CTX-M-13, CTX-M-14, CTX-M-16, CTX-M-18, CTX-M-19, CTX-M-21, and Toho-2). The designation CTX-M refers to a potent activity against cefotaxime and having only a remnant of activity toward ceftazidime.

Here we report the molecular characterization of a new CTX-M  $\beta$ -lactamase derived from CTX-M-1 through a single Asp240-Gly substitution, CTX-M-32. In addition, we report experimental data showing that substitution of this amino acid is itself sufficient to confer hydrolytic activity against ceftazidime.

Patterns of antibiotic susceptibility shown by the clinical strain *E. coli* JC19325, as well as its transconjugant and transformants, are shown in Table 1. The MICs were determined by E-test and interpreted according to the method of the National Committee for Clinical Laboratory Standards (18). The clinical strain JC19325 showed a high level of resistance to cefotaxime, ceftazidime, and aztreonam (MICs of >256 µg/ml), cefoxitin (MIC of >256 µg/ml), and cefepime (MIC of 64 µg/ml). Moreover, clavulanic acid acted synergistically with amoxicillin, cefotaxime, and ceftazidime (E-test; ABBiodisk, Solna, Sweden), thus indicating the presence of a class A  $\beta$ -lactamase (9). An

\* Corresponding author. Mailing address: Servicio de Microbiologia, Complejo Hospitalario Universitario Juan Canalejo, C/Xubias de Arriba s/n, 15006 La Coruña, Spain. Phone: 981-178000, ext. 21144. Fax: 981-178216. E-mail: germanbou@canalejo.org. *Escherichia coli* TG1 transformant harboring the pMC-2 plasmid showed higher MICs of the affected antibiotics, probably due to more copies of the *bla* gene.

Isoelectric focusing was performed using polyacrylamide gels containing Ampholine, within a pH range of 3.5 to 9.5, as previously described (17). The clinical isolate produced one enzyme with a pI of 9.0.

In the present study the *E. coli* XL1-Blue MRF'Kan strain (Stratagene Europe, Amsterdam, The Netherlands) was used in the conjugation experiments.

The clinical strain JC19325 had one plasmid which harbored a  $\beta$ -lactamase with a pI of 9.0 that was transferred by conjugation into *E. coli* XL1-Blue MRF'Kan using kanamycin (25  $\mu$ g/ml) and cefotaxime (2  $\mu$ g/ml) as selective antibiotics. A few of the transconjugants which grew harbored an identical plasmid of approximately 15 kb, which was named pMC-1.

Plasmid DNA was isolated by the alkaline lysis method (23) from the transconjugant that produced a single  $\beta$ -lactamase with a pI of 9.0. Plasmid DNA was digested with KpnI and ligated to the plasmid vector pBGS18<sup>-</sup> (25); afterwards, the ligation mixture was introduced into *E. coli* TG1 cells by transformation with CaCl<sub>2</sub>, and transformants were detected on Luria-Bertani agar plates with cefotaxime (2 µg/ml) and kanamycin (25 µg/ml). The resulting plasmid, designated pMC-2, carried a *bla*-producing insert of size circa 4 kb. Double-stranded templates were subjected to nucleotide sequencing by using the method of Sanger et al. (23, 23a).

During isoelectric focusing, the pI 9.0  $\beta$ -lactamase activity band from the *E. coli* transformants cofocused with the  $\beta$ -lactamase activity band from the clinical strain JC19325. Nucleotide sequencing of the KpnI insert revealed some interesting features, including (i) a new *bla* gene. This new *bla* gene was 876 bp long, initiated with an ATG codon, and ended with a TGA codon (291 amino acids long). The initiation codon was preceded by a Shine-Dalgarno ribosome-binding sequence, AAGGAA. The EMBL and Swiss-Prot database searches for this open reading frame revealed similarities to CTX-M  $\beta$ -lactamases. The deduced amino acid sequence had the closest homology (99%) with the CTX-M-1 enzyme (2, 3), from which it differed by the single amino acid substitution Asp240-Gly (Ambler numbering) (1). (ii) The second interesting feature

	MIC (µg/ml) for:										
Antibiotics <sup>a</sup>	JC19325 (produces CTX-M-32)	XL1(pMC-1 <sup>b</sup> ) (produces CTX-M-32)	TG1	TG1(pMC-2 <sup>c</sup> ) (produces CTX-M-32)	TG1(pMC-3 <sup>d</sup> ) (produces CTX-M-1)						
Amoxicillin	>256	>256	3	>256	>256						
Amoxicillin + clavulanate	12	6	2	4	8						
Piperacillin	>256	>256	0.38	>256	>256						
Cephalothin	>256	>256	3	>256	>256						
Cefuroxime	>256	>256	1.5	>256	>256						
Cefoxitin	>256	3	2	2	2						
Cefotaxime	>256	>256	0.02	>256	>256						
Cefotaxime + clavulanate	>1	0.03	0.02	0.03	0.06						
Ceftazidime	128	96	0.06	>256	6						
Ceftazidime + clavulanate	>4	0.25	0.06	0.19	0.25						
Cefepime	64	16	0.02	64	48						
Aztreonam	>256	>256	0.03	>256	48						
Imipenem	0.19	0.25	0.12	0.19	0.19						
Meropenem	0.047	0.03	0.008	0.02	0.02						

FABLE 1.	MICs of $\beta$ -lactams for the JC19325	clinical strain, E. coli	XL1(pMC-1), E.	coli TG1, E. col	i TG1(pMC-2), and
		E. coli TG1(pMC-	-3)		

<sup>a</sup> Clavulanate was used at 4 µg/ml.

<sup>b</sup> Transconjugant harboring CTX-M-32.

<sup>c</sup> Transformant harboring CTX-M-32 β-lactamase gene.

 $^{d}$  Transformant harboring CTX-M-32mut or CTX-M-1  $\beta$ -lactamase gene.

was the inverted repeat right (IRR) sequence of ISEcp1B 80 bp upstream of the ATG start codon of CTX-M-32. No putative promoter sequences were found in the 80-bp sequence that separated the IRR of ISEcp1B from the ATG site of the  $bla_{CTX-M-32}$  gene; moreover, this IRR provided -35 and -10promoter sequences, thus probably contributing to the expression of the  $bla_{CTX-M-32}$  gene. (iii) Third, this IRR was downstream of a *tnpA* gene that encoded the transposase of IS5. Figure 1 shows the 2,326-bp sequence of the original 4-kpb KpnI fragment.

To purify the CTX-M enzyme, the  $bla_{CTX-M-32}$  gene was cloned in the pGEX-6P-1 vector, which allowed a fusion protein between glutathione S-transferase (GST) and the CTX-M enzyme. The  $\beta$ -lactamase was purified to homogeneity following the manufacturer's directions for the GST gene fusion system (Amersham Pharmacia Biotech, Europe GmbH). The purified protein appeared on sodium dodecyl sulfate-polyacrylamide gel electrophoresis as a band of 28 kDa ( $\geq$ 99% pure) (Fig. 2).

For kinetic experiments, CTX-M-32  $\beta$ -lactamase was used at a 1,800  $\mu$ M concentration. The  $\beta$ -lactamase showed a hydrolytic profile similar to that expected for a molecular class A CTX-M enzyme (6), with the  $K_m$  for ampicillin lower than the  $K_m$  for cefalothin, a  $K_m$  for cefotaxime of <500  $\mu$ M, and a clear hydrolytic activity towards cefotaxime. Moreover, moderate hydrolytic activity was detected against ceftazidime, as ceftazidime MICs suggested (Table 2).

The CTX-M-32 enzyme is derived from CTX-M-1 by a single amino acid replacement, Asp240-Gly. To confirm the importance of the Asp-Gly substitution in the hydrolysis of ceftazidime, we replaced the Gly240 with Asp in CTX-M-32 by using site-directed mutagenesis as previously described (14). The CTX-M-32mut or CTX-M-1 gene was then cloned into the pBGS18<sup>-</sup> vector, yielding the pMC-3 plasmid. The mutagenesis was confirmed by nucleotide sequencing. The MICs for *E. coli* TG1 harboring pMC-2 and pMC-3 are shown in Table 1. The MICs of ceftazidime corresponding to *E. coli* 

TG1 harboring CTX-M-1 β-lactamase were clearly lower than those corresponding to *E. coli* TG1 carrying CTX-M-32. To confirm this result, the substrate profile of the CTX-M-32. To tamase was determined with the enzyme purified as mentioned above for CTX-M-32 (GST gene fusion system) (Fig. 2). For kinetic experiments, CTX-M-1 β-lactamase was used at a 1,670 µM concentration.  $K_{cat}/K_m$  (in micromolar per second) values for ceftazidime and cefotaxime were 0.0001 and 1.5; therefore, a lower catalytic efficiency with respect to ceftazidime was detected with CTX-M-1, according to the differences in ceftazidime MICs between CTX-M-32 and CTX-M-1 enzymes (Table 1).

Three different enzymes, CTX-M-15, -16, -19 and, recently, CTX-M-27 have been reported to be associated with ceftazidime hydrolysis (4, 5, 20, 21). The amino acid changes associated with the phenotype of ceftazidime hydrolysis were a Proto-Ser substitution at position 167 in CTX-M-19 with respect to CTX-M-18 (20) and an Asp-to-Gly substitution at position 240 in CTX-M-16 with respect to CTX-M-9 (4) and in CTX-M-27 with respect to CTX-M-14 (5). In agreement with these previous results, we also report that the Asp240 substitution is a key factor in the evolution of CTX-M  $\beta$ -lactamases, as it increases their hydrolytic activity toward ceftazidime.

Regarding the CTX-M enzymes, to our knowledge only six different enzymes have been published in the group 1 CTX-M

TABLE 2. Substrate profile of β-lactamase CTX-M-32

Antibiotic	$K_m (\mu M)$	$K_{\rm cat}$ (s <sup>-1</sup> )	$\frac{K_{\text{cat}}/K_m}{(\mu M^{-1}) \cdot (s^{-1})}$
Ampicillin	$8 \pm 2.6$	$3 \pm 1.1$	0.4
Cephalothin	$211 \pm 0.5$	$928 \pm 16.4$	4.4
Cefuroxime	$261 \pm 0.2$	$162 \pm 11.6$	0.6
Cefotaxime	$322 \pm 2.7$	$320 \pm 10.4$	1
Ceftazidime	$271 \pm 0.2$	$0.91 \pm 0.5$	0.003
Cefepime	$1,287 \pm 4.1$	$43 \pm 8.8$	0.03
Aztreonam	31 ± 0.7	$1 \pm 0.4$	0.03

GGT ACC KpnI GTC AGG GAG AAC TCA TCT CGG GGC AAG TTT CGT GCT TAG ATG CTT TCA 48 GCA CTT ATC TCT TCC GCA TTT AGC TAC CGG GCA GTG CCA TTG GCA TGA 96 CAA CGG TCC CA<u>C CCC CAA ACA GGT TGC CCC</u> ACC CCT CCC TGA AAT CCC 144 CAG TTT TTA GTG AGA TCT CTC CCA CTG ACG TAT CAT TTG GTC CGC CCG 192 Η S R Е W 0 R Ι Μ Q D Α R AAA CAG GTT GGC CAG CGT GAA TAA CAT CGC TTG GTT ATC CAG GTT TTT 240 F ਜ T. Ν А τ. Т L Μ А L Ν D Ν ĸ 0 CAG CAG CCC GTA TCT GTC TTT CGC GAA GCC CCT GAA CTG CCG CTT GAT 288 Τ. Τ. G R Υ R Κ А F G F Q R K Ι GAT GCG AAA CGG GTG CTC CCC CTTGGC ACG GAT GCT GGC TTT GTA CAT 336 Т F R Ρ Η Ε G К Α R Ι S Α K Μ Y TTTTCC GAT GTT GGC CGT GAT GTTCTT GCG CGG ATG CTG CTT CAA GGT 384 G Τ Т Κ N T Α Ν Κ R Ρ Η Q Κ  $\mathbf{L}$ Т TTTTGC CTT GCC GGG ACG CTC GGÇ GAT CAG CCA CAC ATC CTC GTC CGC 432 Κ Κ G Ρ E А R Α Т L W D v D D Α GGC CAG CTC CTC GCG CTG TGG CGC TCC TTG GTA GCC GGC ATC GGC TGA 480 Ε Α L Е Ε R Q Ρ Δ G Q Y G А D Α GAC AAA TTG CTC CTC TCC ATG AAG CAG ATT ACC CAA CTG ATT GAG GTC 528 S F V 0 E E G H T. L Ν G  $\mathbf{L}$ Q Ν L CTC GTT ATG GGC CGC GGT GGT GAC TAG GCT GTG CTŢ GGT CAG GCC 576 ACT D Ħ Ε Ν Т Α A Т v L S Η Т L G S GGC ATC GAC ACC AAT GTG GGC CTT CAT GCC AAA GTG CCA TCG ATT GCC 624 к Α D V G Т Η A Κ М G F Η W R Ν TTT CTT GGT CTG ATG CAT CTC CGG ATC GCG TTG CTG CTC TTT GTT CTT 672 G К Κ Τ Q Η Μ Ε D R Ρ O 0 E K Ν GGT AGA GCT GGG TGC CTC AAT GAT GGT GGC ATC CAC CAA AGT TTG GCC 720 Κ Т S S Ρ Ε Ι T Т A V Α D L т G GCC TGC TTC GGC CAG CCA GCG ATT GAT GGT GGT CAT CAT GAC CTT GAA 768 Q Т М Μ v G А Ε A L W R Ν Т K Ι TTG ACG CAA GGC CAG TTG ATG CTG CTC GAG CAG GTG GCG GAA ATT CAT 816 F R L 0 Α L Q Н O Е Η R F L L Ν GAT GGT GGT GCG ATC CGG CAG GGC GCT ATC CAG GGA TAA TCG GGC AAA 864 Μ Ι Т Т R D Ρ L А S D S L R А L CAG GCG CAT GGA GGC GAT TTC GTA CAG GGC ATC TTC CAT GGC ACC GTC 912 F  $\mathbf{L}$ R М S Α Ι Е Υ L Ά D Е М Α G

FIG. 1. Nucleotide sequence of a 2,326-bp DNA fragment of the pMC-2 plasmid. The deduced amino acid sequence is indicated in single-letter code below the nucleotide sequence. Stop codons are indicated by asterisks. The -35 and -10 promoter sequences of the  $bla_{CTX-M-32}$  gene and the IRR sequence of IS*Ecp1* are underlined and indicated by bold letters, as is the +1 position of the transcriptional start of the  $bla_{CTX-M-32}$  gene (10). The CTX-M-32 and transposase of IS5 proteins are indicated by arrows. Bold amino acids are those conserved in class A  $\beta$ -lactamases (15). Oligonucleotides used for sequencing are indicated by arrows, and KpnI restriction sites delimiting the 4-kbp insert are also underlined.

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GCT	CAG	GTT	GTA	CCA	ATG	CTG	CAT	GCA	GTG	AAT	ACG	CAG	CAT	GGT	CTC	960
D	S	L	Ν	Y	W	Н	Q	М	С	Η	Ι	R	L	М	т	
CAG	CGG	ATA	GGG	CCG	TCG	GCC	ATT	GCC	CGC	CTT	GGG	ATA	AAA	CGG	CTC	1008
Е	L	Ρ	Y	Ρ	R	R	G	N	G	А	К	Ρ	Y	F	Р	
GAT	GAC	AGC	GGT	CAT	ATT	CTG	CCA	TGG	CAG	AAT	CTG	CTC	CAT	GCG	GGA	1056
Е	I	v	А	Т	М	N	Q	W	Ρ	$\mathbf{L}$	I	Q	Е	М	R	
GAG	GAA	AAT	CTC	TTT	TCG	GGT	CTG	ACG	GCG	CTT	AGT	GCT	GAA	TTC	ACT	1104
S	$\mathbf{L}$	F	I	E	К	R	т	Q	R	R	К	т	S	F	Е	
ATC	GGC	GAA	GGT	GAG	TTG	ATG	GCT	CAT	GAT	GAT	CCC	TCT	GGG	ATG	GCT	1152
S	D	А	F	т	$\mathbf{r}$	Q	Н	S	М	◄	]	CS5				
CCG	GAT	GAA	TAT	GAT	GAT	CTC	ATA	TCA	GGA	ACT	TGT	TCG	CAC	CTT	CCT	1200
TAA	GTA	TCA	TTG	CAG	CAA	AGA	TGA	AAT	CAA	TGA	TTT	ATC	AAA	AAT	GA <u>T</u>	1248
TGA	AAG	GTG	$\mathbf{GTT}$	GTA	AAT	AAT	GT <u>T</u>	ACA	<b>AT</b> G	$\mathbf{T}\mathbf{G}\mathbf{T}$	GAG	<u>AA</u> G	CAG	TCT	AAA	1296
-35	5							-10				+1				
TTC	TTC	GTG	AAA	TAG	TGA	TTT	TTG	AAG	CTA	ATA	AAA	A <u>ac</u>	ACA	CGT	GGA	1344
													IRR	ISE	cpl	
ATT	TAG	GTT	AGA	CTA	TAA	ATA	GAA	AAA	GGC	$\mathbf{GTT}$	TTG	ACA	GAC	TAT	TCA	1392
IRR	ISE	<i>cp1</i>														
TGT	TGT	TGT	TAA	TTC	GTC	TCT	TCC	AGA	ATA	AGG	AAT	CCC	ATG	GTT	AAA	1440
								стх-	M-32	_	,-	->	М	v	ĸ	
AAA	TCA	CTG	CGT	∋CAG	TTC	ACG	CTG	<b>CTX -</b> ATG	<b>M-32</b> GCG	ACG	GCA	ACC	M GTC	V ACG	K CTG	1488
AAA K	TCA S	CTG L	CGT R	<sub>&gt;</sub> CAG Q	TTC F	ACG T	CTG L	CTX- ATG M	<b>M-32</b> GCG A	ACG T	GCA A	ACC T	M GTC V	V ACG T	K CTG L	1488
<u>AAA</u> K TTG	TCA S TTA	CTG L GGA	CGT R AGT	<pre></pre>	TTC F CCG	ACG T CTG	CTG L TAT	CTX- ATG M GCG	M-32 GCG A CAA	ACG T ACG	GCA A GCG	ACC T GAC	M GTC V GTA	V ACG T CAG	K CTG L CAA	1488 1536
AAA K TTG L	TCA S TTA L	CTG L GGA G	CGT R AGT S	<pre></pre>	TTC F CCG P	ACG T CTG L	CTG L TAT Y	CTX - ATG M GCG A	<b>M-32</b> GCG A CAA Q	ACG T ACG T	GCA A GCG A	ACC T GAC D	M GTC V GTA V	V ACG T CAG Q	K CTG L CAA Q	1488 1536
AAA K TTG L AAA	TCA S TTA L CTT	CTG L GGA G GCC	CGT R AGT S GAA	>CAG Q GTG V TTA	TTC F CCG P GAG	ACG T CTG L CGG	CTG L TAT Y CAG	CTX- ATG M GCG A TCG	M-32 GCG A CAA Q GGA	ACG T ACG T GGA	GCA A GCG A AGA	ACC T GAC D CTG	M GTC V GTA V GGT	V ACG T CAG Q GTG	K CTG L CAA Q GCA	1488 1536 1584
AAA K TTG L AAA K	TCA S TTA L CTT L	CTG L GGA G GCC A	CGT R AGT S GAA E	>CAG Q GTG V TTA L	TTC F CCG P GAG E	ACG T CTG L CGG R	CTG L TAT Y CAG Q	CTX- ATG M GCG A TCG S	M-32 GCG A CAA Q GGA G	ACG T ACG T GGA G	GCA A GCG A AGA R	ACC T GAC D CTG L	M GTC V GTA V GGT G	V ACG T CAG Q GTG V	K CTG L CAA Q GCA A	1488 1536 1584
AAA K TTG L AAA K TTG	TCA S TTA L CTT L ATT	CTG L GGA G GCC A AAC	CGT R AGT S GAA E ACA	CAG Q GTG V TTA L GCA	TTC F CCG P GAG E GAT	ACG T CTG L CGG R AAT	CTG L TAT Y CAG Q TCG	CTX- ATG M GCG A TCG S CAA	M-32 GCG A CAA Q GGA G ATA	ACG T ACG T GGA G CTT	GCA A GCG A AGA R TAT	ACC T GAC D CTG L ÇGT	M GTC V GTA V GGT G	V ACG T CAG Q GTG V GAT	K CTG L CAA Q GCA A GAG	1488 1536 1584 1632
AAA K TTG L AAA K TTG L	TCA S TTA L CTT L ATT I	CTG L GGA GCC A AAC N	CGT R AGT S GAA E ACA T	>CAG Q GTG V TTA L GCA A	TTC F CCG P GAG E GAT D	ACG T CTG L CGG R AAT N	CTG L TAT Y CAG Q TCG S	CTX- ATG M GCG A TCG S CAA Q	M-32 GCG A CAA Q GGA G ATA I	ACG T ACG T GGA G CTT L	GCA A GCG A AGA R TAT Y	ACC T GAC D CTG L ÇGT R	M GTC V GTA V GGT G GCT A	V ACG T CAG Q GTG V GAT D	K CTG L CAA Q GCA A GAG E	1488 1536 1584 1632
AAA K TTG L AAA K TTG L Ç <u>GC</u>	TCA S TTA L CTT L ATT I TTT	CTG L GGA G CC A AAC N GCG	CGT R AGT S GAA E ACA T ATG	>CAG Q GTG V TTA L GCA A TGC	TTC F CCG P GAG GAT D AGC	ACG T CTG CGG R AAT N ACC	CTG L TAT Y CAG Q TCG S AGT	CTX- ATG M GCG A TCG S CAA Q AAA	M-32 GCG A CAA Q GGA G ATA I GTG	ACG T ACG T GGA G CTT L ATG	GCA A GCG A AGA R TAT Y GCC	ACC T GAC D CTG L ÇGT R GTG	M GTC V GTA GGT G GCT A GCC	V ACG T CAG GTG V GAT D GCG	K CTG L CAA Q GCA A GAG E GTG	1488 1536 1584 1632 1680
AAA K TTG L AAA K TTG L C <u>GC</u> R	TCA S TTA L CTT L ATT I TTT F	CTG L GGA GCC A AAC N GCG A	CGT R AGT S GAA E ACA T ATG M	<pre>CAG Q GTG V TTA L GCA A TGC C</pre>	TTC F CCG P GAG E GAT D AGC <b>S</b>	ACG T CTG CGG R AAT N ACC <b>T</b>	CTG L TAT Y CAG Q TCG S AGT <b>S</b>	CTX - ATG M GCG A TCG S CAA Q AAA K	M-32 GCG A CAA Q GGA G ATA I GTG V	ACG T ACG GGA G CTT L ATG M	GCA A GCG A AGA R TAT Y GCC A	ACC T GAC D CTG L ÇGT R GTG V	M GTC V GTA V GGT GCT A GCC A	V ACG T Q GTG V GTG D GCG A	K CTG L CAA Q GCA A GAG GAG GTG V	1488 1536 1584 1632 1680
AAA K TTG L AAA K TTG L <u>ÇGC</u> R CTG	TCA S TTA L CTT L ATT I TTT F AAG	CTG GGA GCC A AAC N GCG A AAA	CGT R AGT S GAA E ACA T ATG M AGT	CAG Q GTG V TTA L GCA A TGC C GAA	TTC F CCG P GAG E GAT D AGC <b>S</b> AGC	ACG T CTG CGG R AAT N ACC <b>T</b> GAA	CTG L TAT Y CAG Q TCG S AGT <b>S</b> CCG	CTX- ATG M GCG A TCG S CAA Q AAA K AAT	M-32 GCG A CAA Q GGA G ATA I GTG V CTG	ACG T ACG T GGA G CTT L ATG M TTA	GCA A GCG A AGA R TAT Y GCC A AAT	ACC T GAC D CTG L QGT R GTG V CAG	M GTC V GTA V GGT G GCT A GCC A CGA	V ACG T CAG GTG GTG GAT GCG A GTT	K CTG L CAA Q GCA A GAG GTG V GAG	1488 1536 1584 1632 1680 1728
AAA K TTG L AAA K TTG L <u>ÇGC</u> R CTG L	TCA S TTA L CTT L ATT I TTT F AAG K	CTG L GGA GCC A AAC N <u>GCG</u> A AAA K	CGT R AGT S GAA E ACA T ATG M AGT S	CAG Q GTG V TTA L GCA A TGC C GAA E	TTC F CCG P GAG E GAT D AGC <b>S</b>	ACG T CTG CGG R AAT N ACC T GAA E	CTG L TAT Y CAG Q TCG S AGT S CCG P	CTX- ATG M GCG A TCG S CAA Q AAA X AAT N	M-32 GCG A CAA Q GGA G ATA I GTG V CTG L	ACG T ACG GGA G CTT L ATG M TTA L	GCA A GCG A AGA R TAT Y GCC A AAT N	ACC T GAC D CTG CTG CTG QGT R GTG V CAG Q	M GTC V GTA V GGT GC GC A GCC A CGA	V ACG T CAG GTG GTG GCG A GTT V	K CTG L CAA Q GCA A GAG GTG V GAG E	1488 1536 1584 1632 1680 1728
AAA K TTG L AAA K TTG L C <u>GC</u> R CTG L ATC	TCA S TTA L CTT L ATT F AAG K AAA	CTG GGA GCC A AAC AAC AAA K AAA	CGT R AGT S GAA E ACA T ATG M AGT S TCT	CAG Q GTG V TTA L GCA A TGC C GAA E GAC	TTC F CCG P GAG E GAT D AGC <b>S</b> TTG	ACG T CTG CGG R AAT ACC T GAA E GTT	CTG L TAT Y CAG Q TCG S AGT <b>S</b> CCG P AAC	CTX- ATG M GCG A TCG S CAA Q AAA K AAA K AAT N TAT	M-32 GCG A CAA Q GGA GGA ATA I GTG V CTG L AAT	ACG T ACG GGA G CTT L ATG M TTA L CCG	GCA A GCG A AGA R TAT Y GCC A AAT N ATT	ACC T GAC D CTG CTG CTG Q GTG V CAG Q GCG	M GTC V GTA GGT GGT GCC A GCC A CGA R GAA	V ACG T CAG GTG V GAT GTT Q C GTT V AAG	K CTG L CAA Q GCA A GAG GTG V GAG E CAC	1488 1536 1584 1632 1680 1728 1776
AAA K TTG AAA K TTG L Ç <u>GC</u> R CTG L ATC I	TCA S TTA L CTT I TTT F AAG K AAA K	CTG G GGA GCC A AAC N GCG A AAA K AAA K	CGT R AGT S GAA E ACA T ATG AGT S TCT S	CAG Q GTG V TTA GCA A TGC C GAA E GAC D	TTC F CCG P GAG E GAT D AGC <b>S</b> TTG L	ACG T CTG CGG R AAT N ACC <b>T</b> GAA E GTT V	CTG L TAT Y CAG CAG S AGT S CCG P AAC N	CTX- ATG M GCG A TCG S CAA Q AAA K AAA K AAT N TAT Y	M-32 GCG A CAA Q GGA GGA ATA I GTG V CTG L AAT N	ACG T ACG GGA G CTT L ATG M TTA L CCG P	GCA A GCG A AGA R TAT Y GCC A AAT N ATT I	ACC T GAC D CTG CTG CTG CGT CAG CAG Q GCG A	M GTC V GTA V GGT GCT A GCC A CGA R GAA E	V ACG T CAG GTG GTG GCT A GTT V AAG K	K CTG L CAA Q GCA A GAG GTG V GAG E CAC H	1488 1536 1584 1632 1680 1728 1776
AAA K TTG L AAA K TTG L CTG CTG L ATC I GTC	TCA S TTA L CTT L ATT F AAG K AAA K GAT	CTG GGA GCC A AAC M GCG A AAA K AAA K GGG	CGT R AGT S GAA E ACA T ATG AGT S TCT S ACG	CAG Q GTG V TTA L GCA A TGC C GAA E GAC D ATG	TTC F CCG P GAG E GAT D AGC S TG S TTG L TCA	ACG T CTG CGG R AAT N ACC <b>T</b> GAA E GTT V CTG	CTG L TAT Y CAG Q TCG S AGT CCG P AAC N GCT	CTX- ATG M GCG A TCG S CAA Q AAA Q AAA K AAT N TAT Y GAG	M-32 GCG A CAA Q GGA G ATA I GTG CTG L AAT N CTT	ACG T ACG GGA G CTT L ATG M TTA L CCG P AGC	GCA A GCG A AGA TAT Y GCC A AAT N ATT I GCG	ACC T GAC D CTG CTG CTG GTG GTG CAG GCG A GCC	M GTC V GTA GGT GGT A GCC A CGA R GAA E GCG	V ACG T CAG GTG V GAT GCG A GTT V AAG K CTA	K CTG L CAA Q GCA A GAG GTG V GAG E CAC H CAG	1488 1536 1584 1632 1680 1728 1776 1824
AAA K TTG L AAA K TTG L QGC R CTG ATC I GTC V	TCA S TTA L CTT I TTT F AAG K AAA K GAT D	CTG GGA GCC A AAC AAC AAA AAA K AAAA K GGG G	CGT R AGT S GAA E ACA T ATG M AGT S TCT S ACG T T	CAG Q GTG V TTA GCA A TGC C GAA E GAC D ATG M	TTC F CCG P GAG E GAT D AGC S TTG L TCA S	ACG T CTG CGG R AAT ACC T GAA E GTT V CTG L	CTG L TAT Y CAG CCG S AGT S CCG P AAC N GCT A	CTX- ATG M GCG A TCG S CAA Q AAA K AAT N TAT Y GAG E	M-32 GCG A CAA Q GGA G ATA I GTG V CTG L AAT N CTT L	ACG T ACG GGA G CTT L ATG M TTA L CCG P AGC S	GCA A GCG A AGA R TAT Y GCC A AAT N ATT I GCG A	ACC T GAC D CTG CTG CTG GTG V CAG GCG A GCC A	M GTC V GTA GGT GGT A GCC A CGA R GAA E GCA A	V ACG T Q GTG GAT GCG A GTT V AAG K CTA	K CTG L CAA Q GCA A GAG GAG CAC H CAG Q	1488 1536 1584 1632 1680 1728 1776 1824
AAA K TTG L AAA TTG L CTG CTG ATC I ATC I GTC V TAC	TCA S TTA L CTT I TTT F AAG K AAA K GAT D AGC	CTG GGA GCC A AAC M GCG A AAA K AAA K GGG G GAT	CGT R AGT S GAA E ACA T ATG AGT S TCT S ACG T AAC	>CAG Q GTG V TTA L GCA A TGC C GAA E GAC D ATG M GTG	TTC F CCG P GAG GAT D AGC S TG L TCA S GCG	ACG T CTG CGG R AAT N ACC T GAA E GAA E GTT V CTG L ATG	CTG L TAT Y CAG CCG S AGT S CCG P AAC N GCT A AAT	CTX- ATG M GCG A TCG S CAA Q AAA X AAA K AAAT N TAAT Y GAG E AAG	M-32 GCG A CAA Q GGA G ATA I GTG CTG AAT N CTT L CTG	ACG T ACG GGA G CTT L ATG M TTA L CCG P AGC S ATT	GCA A GCG A AGA TAT Y GCC A AAT N ATT I GCG A TCT	ACC T GAC D CTG CTG CTG CTG CAG GCG A GCG A GCC A CAC	M GTC V GTA V GGT GGT A GCC A CGA R GAA E GCG A GTT	V ACG T CAG GTG D GCG A GTT V AAG K CTA L GGC	K CTG L CAA Q GCA A GAG GTG V GAG CAC H CAG Q GGC	1488 1536 1584 1632 1680 1728 1776 1824 1872
AAA K TTG L AAA K TTG L CTG CTG L ATC I GTC V TAC Y	TCA S TTA L CTT I ATT F AAG K AAA K GAT D AGC <b>S</b>	CTG GGA GCC A AAC AAA AAA K AAA K GGG GAT D	CGT R AGT S GAA E ACA T ATG ATG S TCT S ACG T AACC N	CAG Q GTG V TTA L GCA A TGC C GAA E GAC D ATG M GTG V	TTC F CCG P GAG GAT D AGC S TTG L TCA S GCG A	ACG T CGG R AAT ACC T GAA E GTT CTG L ATG M	CTG L TAT Y CAG Q TCG S AGT S CCG P AAC N GCT A AAT N	CTX- ATG M GCG A TCG S CAA CAA Q AAA K AAT N TAT Y GAG E AAG K	M-32 GCG A CAA Q GGA GGA ATA I GTG CTG L AAT N CTT L CTG L	ACG T ACG GGA G CTT L ATG M TTA L CCG P AGC S ATT I	GCA A GCG A AGA R TAT Y GCC A AAT N ATT I GCG A TCT S	ACC T GAC D CTG CTG CTG CAG CAG CAG CAG A GCC A CAC H	M GTC V GTA GGT GGT A GCC A CGA R GAA E GCG A GTT V	V ACG T CAG GTG V GAT GCG A GTT V AAG K CTA GGC G	K CTG L CAA Q GCA A GAG CAC H CAG GGC GGC G	1488 1536 1584 1632 1680 1728 1776 1824 1872
AAA K TTG AAA K TTG L QGC R CTG ATC I ATC I GTC V TAC Y CCG	TCA S TTA L CTT I TTT F AAG K AAA K GAT D AGC S GCT	CTG GGA GCC A AAC N GCG A AAA K AAA K GGG G GAT D AGC	CGT R AGT S GAA E ACA T ACA M AGT S CT S ACG T C T AAC N GTC	CAG Q GTG V TTA GCA A TGC C GAA E GAC D ATG M GTG V ACC	TTC F CCG P GAG E GAT D AGC S TTG L TCA S GCG A GCG	ACG T CTG CGG R AAT ACC T GAA E GTT V CTG L ATG M TTC	CTG L TAT Y CAG Q TCG S AGT S CCG P AAC N GCT A AAT N GCC	CTX- ATG M GCG A TCG S CAA Q AAA K AAA K AAA N TAT Y GAG E AAG K CGA	M-32 GCG A CAA Q GGA G ATA I GTG V CTG L CTG L CTG L CAG	ACG T GGA G CTT L ATG M TTA L CCG P AGC S ATT I CTG	GCA A GCG A AGA R TAT Y GCC A AAT AAT I GCG A TCT S GGA	ACC T GAC D CTG CTG CTG CAG GCG A GCG A GCC A CAC H GAC	M GTC V GTA C GGT GGT C GA C GA C GA C GA C GA	V ACG T Q GTG GAT GCG A GTT AAG K CTA GCC G C ACG	K CTG L CAA Q GCA A GAG CAG CAC H CAG CAG Q GGC G TTC	1488 1536 1584 1632 1680 1728 1776 1824 1872 1920
AAA K TTG L AAA TTG CGC R CTG ATC I ATC I GTC V TAC Y CCG P	TCA S TTA L CTT I TTT F AAG K AAA K GAT D AGC S GCT A	CTG GGA GCC A AAC M GCG A AAA K AAA K GGG G GAT D AGC S	CGT R AGT S GAA E ACA T ATG ATG S TCT S ACG T AACG T AACC N GTC V	CAG Q GTG V TTA L GCA A TGC C GAA E GAC D ATG M GTG V ACC T	TTC F CCG P GAG C GAT D AGC S TTG L TCA S GCG A GCG A	ACG T CTG CGG R AAT N ACC T GAA E GAA E GAA E GTT V CTG L ATG M TTC F	CTG L TAT Y CAG S TCG S AGT S CCG P AAC N GCT A AAT N GCC A	CTX- ATG M GCG A TCG S CAA Q AAA Q AAA K AAT N TAT Y GAG E AAG K CGA R	M-32 GCG A CAA Q GGA G ATA I GTG CTG L AAT N CTT L CTG L CTG Q	ACG T ACG GGA G CTT L ATG ATG L CCG P AGC S ATT I CTG L	GCA A GCG A AGA TAT Y GCC A AAT N ATT I GCG A TCT S GGA G	ACC T GAC D CTG CTG CTG CTG CTG CAG GCG A GCC A GCC A CAC A CAC A CAC	M GTC V GTA V GGT GGT A GCC A CGA R GAA E GCG A GTT V CAA E	V ACG T CAG GTG D GCG A GTT V AAG CTA L GCC G G C A C T	K CTG L CAA Q GCA A GAG CAC CAC H CAG Q GGC G GC TTC F	1488 1536 1584 1632 1680 1728 1776 1824 1872 1920

CGT	CTC	GAC	CGT	ACC	GAG	CCG	ACG	TTA	AAC	ACC	GCC	ATT	CCG	GGC	GAT	1968
R	$\mathbf{L}$	D	R	т	Е	Ρ	Т	$\mathbf{L}$	N	т	А	I	Ρ	G	D	
CCG	CGT	GAT	ACC	ACT	TCA	CCT	CGG	GCA	ATG	GCG	CAA	ACT	CTG	CGT	AAT	2016
Ρ	R	D	Т	т	S	Ρ	R	А	М	А	Q	т	L	R	N	
CTG	ACG	CTG	GGT	AAA	GCA	TTG	GGT	GAC	AGC	CAA	CGG	GCG	CAG	CTG	GTG	2064
L	Т	$\mathbf{L}$	G	к	А	$\mathbf{L}$	G	D	S	Q	R	А	Q	$\mathbf{L}$	v	
ACA	TGG	ATG	AAA	GGC	AAT	ACC	ACC	GGT	GCA	GCG	AGC	ATT	CAG	GCT	GGA	2112
Т	W	М	к	G	Ν	Т	т	G	A	А	S	I	Q	А	G	
CTG	CCT	GCT	TCC	TGG	$\mathbf{GTT}$	GTG	GGG	GAT	AAA	ACC	GGC	AGC	GGT	GGC	TAT	2160
Г	Ρ	А	S	W	V	v	G	D	ĸ	т	G	S	G	G	Y	
GGC	ACC	ACC	AAC	GAT	ATC	GCG	GTG	ATC	TGG	CCA	AAA	GAT	CGT	GCG	CCG	2208
G	т	т	Ν	D	I	А	v	I	W	Ρ	К	D	R	А	Ρ	
CTG	ATT	CTG	GTC	ACT	TAC	TTC	ACC	CAG	CCT	CAA	CCT	AAG	GCA	GAA	AGÇ	2230
$\mathbf{L}$	I	$\mathbf{L}$	v	Т	Y	F	Т	Q	P	Q	Ρ	K	A	Е	S	
CGT	CGC	GAT	GTA	TTA	GCG	TCG	GCG	GCT	AAA	ATC	GTC	ACC	AAC	GGT	TTG	2278
R	R	D	v	L	А	S	А	А	К	I	V	т	Ν	G	г	
<u>TAA</u>	TAG	CGG	AAA	CGG	GTG	GCC	GGT	AAC	CTG	CTG	TGT	CA <b>G</b>	GTA	CCA	TTC	2326
*													Kpn	Ι		
							FIG.	1—C	ontinu	ied.						

enzymes: CTX-M-1, -3, -10, -12, -15, and -32 (3, 16, 19, 20). Among these, only CTX-M-15 and -32 showed more efficient ceftazidime hydrolysis than their parental enzymes, CTX-M-3 and CTX-M-1, respectively. The two former enzymes share the same amino acid substitution, although CTX-M-15 differs from

![](_page_4_Figure_4.jpeg)

FIG. 2. Electrophoresis analysis of CTX-M-32 and CTX-M-1 purified extracts in a sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis gel stained with Coomassie brilliant blue R-250. Lanes: 1, protein molecular markers; 2, purified CTX-M-1 protein used in kinetic experiments; 3, purified CTX-M-32 protein used in kinetic experiments.

CTX-M-32 in four additional amino acid changes. In terms of evolution, CTX-M-32 is probably an ancestor between CTX-M-1 and CTX-M-15 and constitutes a step forward in the evolution of  $\beta$ -lactamase in broad-spectrum hydrolysis of antibiotics such as ceftazidime.

In summary, we report the genetic and biochemical characterization of a new CTX-M enzyme, CTX-M-32. This is the fourth report of a CTX-M  $\beta$ -lactamase isolation in Spain, as CTX-M-9, CTX-M-10, and CTX-M-14 have previously been isolated in this country (7, 19, 22, 24).

Nucleotide sequence accession number. The GenBank accession number for the CTX-M-32  $\beta$ -lactamase is AJ557142.

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