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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 β-lactamases in members of the family *Enterobacteriaceae* has become a worldwide problem (3, 4, 6, 7, 11–13, 16).

Most extended-spectrum β-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 β-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 β-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 β-lactamase (9). An *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 β-lactamase with a pI of 9.0 that was transferred by conjugation into *E. coli* XL1-Blue MRF′Kan using kanamycin (25 μg/ml) and cefotaxime (2 μg/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 β-lactamase with a pI of 9.0. Plasmid DNA was digested with KpnI and ligated to the plasmid vector pBGS18$^-$ (25); afterwards, the ligation mixture was introduced into *E. coli* TG1 cells by transformation with $CaCl_2$, 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 β-lactamase activity band from the *E. coli* transformants cofocused with the β-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 β-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

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TABLE 1. MICs of β-lactams for the JC19325 clinical strain, *E. coli* XL1(pMC-1), *E. coli* TG1, *E. coli* TG1(pMC-2), and *E. coli* TG1(pMC-3)

Antibiotics[a]	MIC (μg/ml) for:				
	JC19325 (produces CTX-M-32)	XL1(pMC-1[b]) (produces CTX-M-32)	TG1	TG1(pMC-2[c]) (produces CTX-M-32)	TG1(pMC-3[d]) (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

[a] Clavulanate was used at 4 μg/ml.
[b] Transconjugant harboring CTX-M-32.
[c] Transformant harboring CTX-M-32 β-lactamase gene.
[d] Transformant harboring CTX-M-32mut or CTX-M-1 β-lactamase gene.

was the inverted repeat right (IRR) sequence of IS*Ecp1B* 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 IS*Ecp1B* from the ATG site of the $bla_{CTX\text{-}M\text{-}32}$ gene; moreover, this IRR provided −35 and −10 promoter sequences, thus probably contributing to the expression of the $bla_{CTX\text{-}M\text{-}32}$ gene. (iii) Third, this IRR was downstream of a *tnpA* gene that encoded the transposase of IS*5*. Figure 1 shows the 2,326-bp sequence of the original 4-kpb KpnI fragment.

To purify the CTX-M enzyme, the $bla_{CTX\text{-}M\text{-}32}$ 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 β-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 (≥99% pure) (Fig. 2).

For kinetic experiments, CTX-M-32 β-lactamase was used at a 1,800 μM concentration. The β-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 μM, 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$^-$ 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-1 β-lactamase 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 Pro-to-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 β-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 (μM)	K_{cat} (s^{-1})	K_{cat}/K_m (μM^{-1})·(s^{-1})
Ampicillin	8 ± 2.6	3 ± 1.1	0.4
Cephalothin	211 ± 0.5	928 ± 16.4	4.4
Cefuroxime	261 ± 0.2	162 ± 11.6	0.6
Cefotaxime	322 ± 2.7	320 ± 10.4	1
Ceftazidime	271 ± 0.2	0.91 ± 0.5	0.003
Cefepime	1,287 ± 4.1	43 ± 8.8	0.03
Aztreonam	31 ± 0.7	1 ± 0.4	0.03

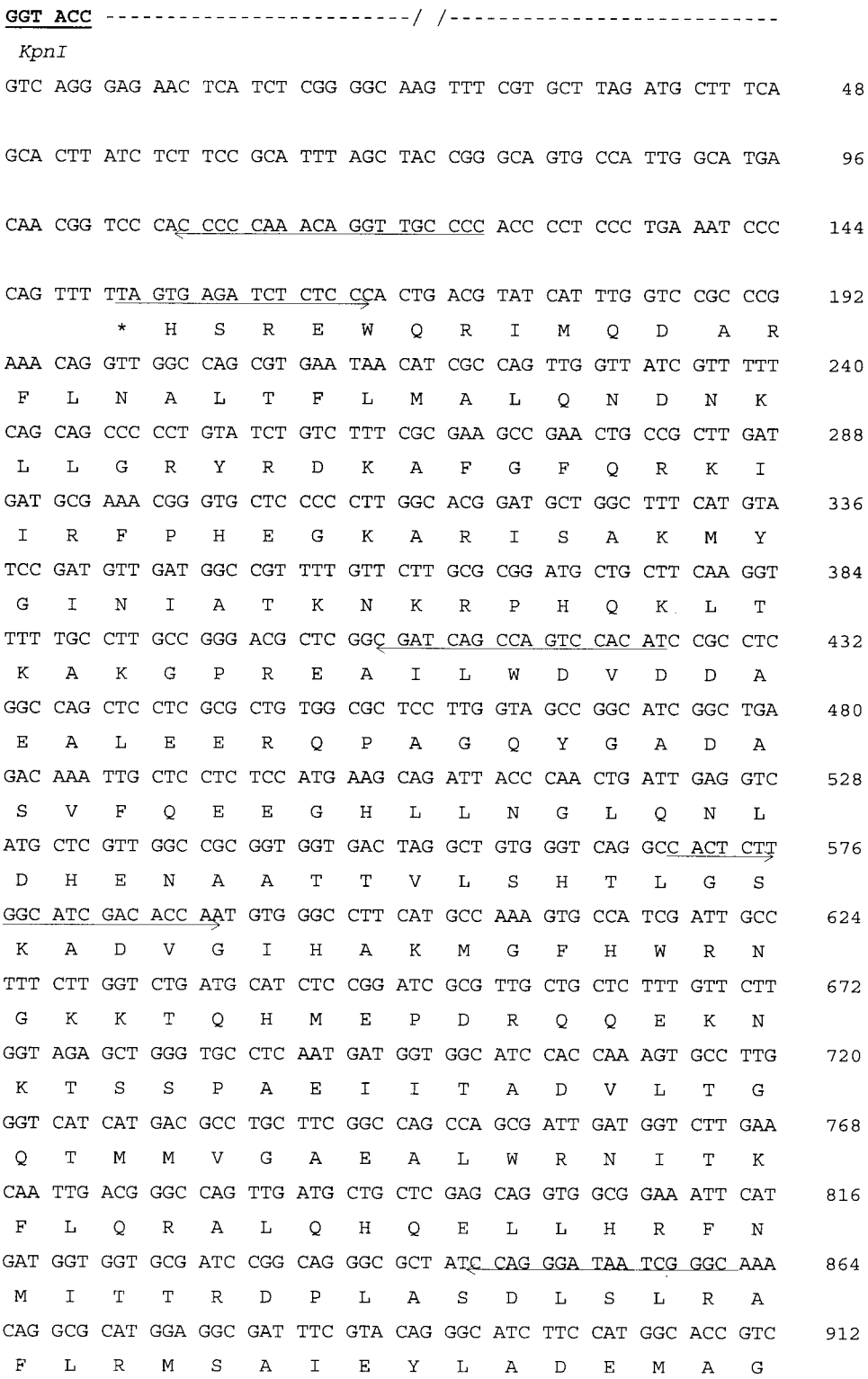

FIG. 1. Nucleotide sequence of a 2,326-bp DNA fragment of the pMC-2 plasmid. The deduced amino acid sequence is indicated in single-letter code below the nucleotide sequence. Stop codons are indicated by asterisks. The −35 and −10 promoter sequences of the $bla_{CTX\text{-}M\text{-}32}$ gene and the IRR sequence of IS*Ecp1* are underlined and indicated by bold letters, as is the +1 position of the transcriptional start of the $bla_{CTX\text{-}M\text{-}32}$ gene (10). The CTX-M-32 and transposase of IS*5* proteins are indicated by arrows. Bold amino acids are those conserved in class A β-lactamases (15). Oligonucleotides used for sequencing are indicated by arrows, and KpnI restriction sites delimiting the 4-kbp insert are also underlined.

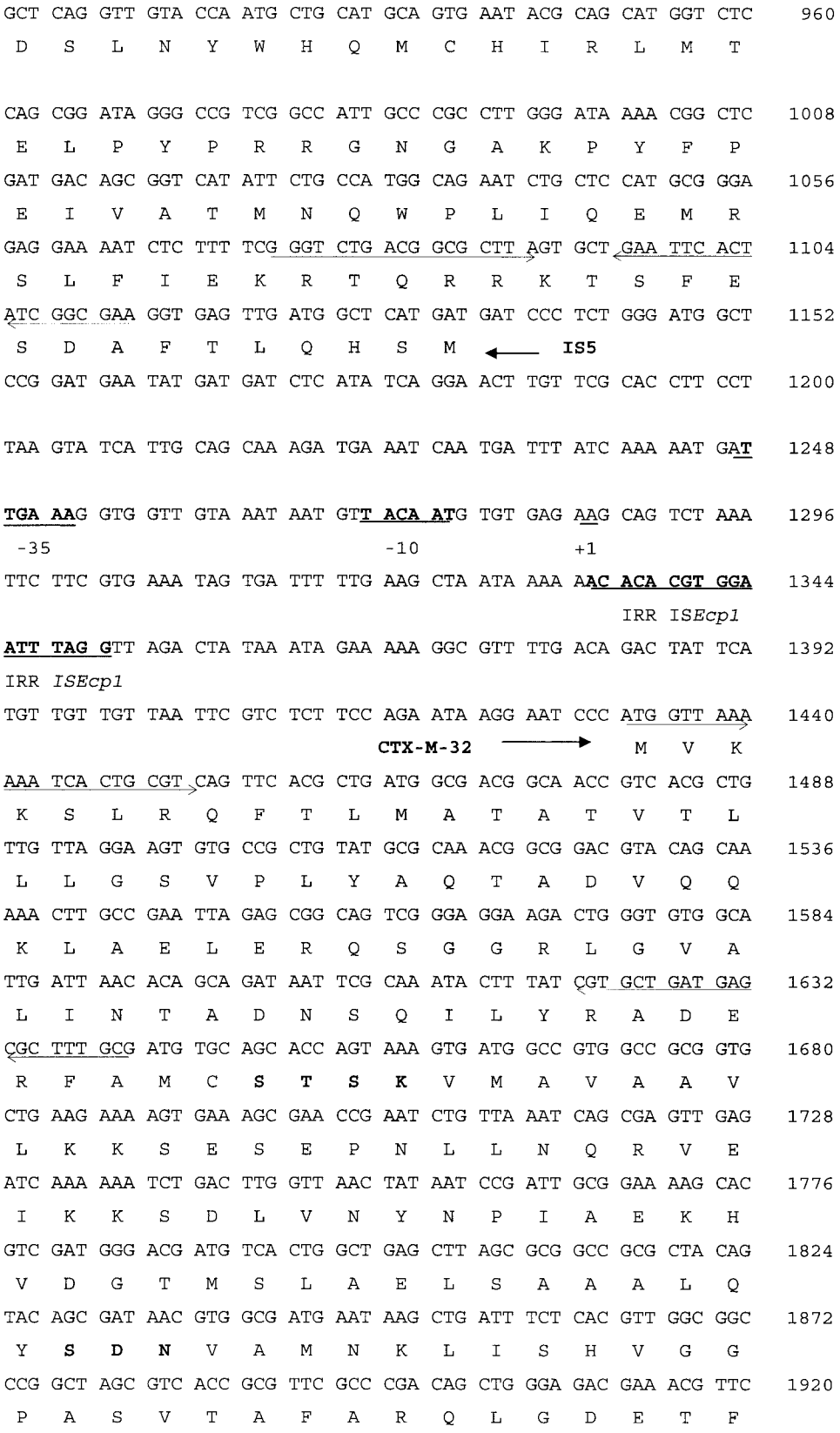

FIG. 1—*Continued*.

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CGT CTC GAC CGT ACC GAG CCG ACG TTA AAC ACC GCC ATT CCG GGC GAT   1968
 R   L   D   R   T   E   P   T   L   N   T   A   I   P   G   D
CCG CGT GAT ACC ACT TCA CCT CGG GCA ATG GCG CAA ACT CTG CGT AAT   2016
 P   R   D   T   T   S   P   R   A   M   A   Q   T   L   R   N
CTG ACG CTG GGT AAA GCA TTG GGT GAC AGC CAA CGG GCG CAG CTG GTG   2064
 L   T   L   G   K   A   L   G   D   S   Q   R   A   Q   L   V
ACA TGG ATG AAA GGC AAT ACC ACC GGT GCA GCG AGC ATT CAG GCT GGA   2112
 T   W   M   K   G   N   T   T   G   A   A   S   I   Q   A   G
CTG CCT GCT TCC TGG GTT GTG GGG GAT AAA ACC GGC AGC GGT GGC TAT   2160
 L   P   A   S   W   V   V   G   D   K   T   G   S   G   G   Y
GGC ACC ACC AAC GAT ATC GCG GTG ATC TGG CCA AAA GAT CGT GCG CCG   2208
 G   T   T   N   D   I   A   V   I   W   P   K   D   R   A   P
CTG ATT CTG GTC ACT TAC TTC ACC CAG CCT CAA CCT AAG GCA GAA AGC   2230
 L   I   L   V   T   Y   F   T   Q   P   Q   P   K   A   E   S
CGT CGC GAT GTA TTA GCG TCG GCG GCT AAA ATC GTC ACC AAC GGT TTG   2278
 R   R   D   V   L   A   S   A   A   K   I   V   T   N   G   L
TAA TAG CGG AAA CGG GTG GCC GGT AAC CTG CTG TGT CAG GTA CCA TTC   2326
 *                                              KpnI
```

FIG. 1—*Continued*.

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 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 β-lactamase in broad-spectrum hydrolysis of antibiotics such as ceftazidime.

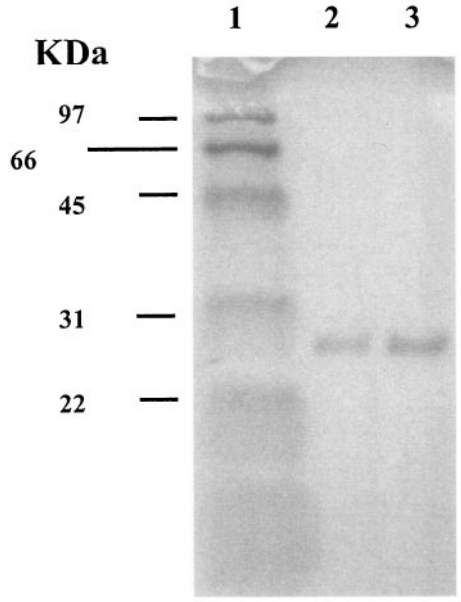

FIG. 2. Electrophoresis analysis of CTX-M-32 and CTX-M-1 purified extracts in a sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis gel stained with Coomassie brilliant blue R-250. Lanes: 1, protein molecular markers; 2, purified CTX-M-1 protein used in kinetic experiments; 3, purified CTX-M-32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

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 β-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 β-lactamase is AJ557142.

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REFERENCES

1. **Ambler, R. P., A. F. W. Coulson, J. M. Frere, J. M. Ghuysen, B. Joris, M. Forsman, et al.** 1991. A standard numbering scheme for the class A β-lactamases. Biochem. J. **276:**269–270.
2. **Barthélémy, M., J. Péduzzi, H. Bernard, C. Tancrède, and R. Labia.** 1992. Close amino acid sequence relationship between the new plasmid-mediated extended-spectrum β-lactamase MEN-1 and chromosomally encoded enzymes of *Klebsiella oxytoca*. Biochim. Biophys. Acta **1122:**15–22.
3. **Bauernfeind, A., I. Stemplinger, R. Jungwirth, S. Ernst, and J. M. Casellas.** 1996. Sequences of beta-lactamase genes encoding CTX-M-1 (MEN-1) and CTX-M-2 and relationship of their amino acid sequences with those of other beta-lactamases. Antimicrob. Agents Chemother. **40:**509–513.
4. **Bonnet, R., C. Dutour, J. L. Samapaio, C. Chanal, D. Sirot, R. Labia, C. D Champs, and J. Sirot.** 2001. Novel cefotaximase (CTX-M-16) with increased catalytic efficiency due to substitution Asp-240-Gly. Antimicrob. Agents Chemother. **45:**2269–2275.
5. **Bonnet, R., C. Recule, R. Baraduc, C. Chanal, D. Sirot, C. De Champs, and J. Sirot.** 2003. Effect of D240G substitution in a novel ESBL CTX-M-27. J. Antimicrob. Chemother. **52:**29–35.
6. **Bonnet, R., J. L. Sampaio, R. Labia, C. De Champs, D. Sirot, C. Chanal, and J. Sirot.** 2000. A novel CTX-M β-lactamase (CTX-M-8) in cefotaxime-resistant *Enterobacteriaceae* isolated in Brazil. Antimicrob. Agents Chemother. **44:**1936–1942.

7. **Bou, G., M. Cartelle, M. Tomas, D. Canle, F. Molina, R. Moure, J. M. Eiros, and A. Guerrero.** 2002. Identification and broad dissemination of the CTX-M-14 beta-lactamase in different *Escherichia coli* strains in the northwest area of Spain. J. Clin. Microbiol. **40:**4030–4036.
8. **Bradford, P. A.** 2001. Extended-spectrum β-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistant threat. Clin. Microbiol. Rev. **14:**933–951.
9. **Bush, K., G. A. Jacoby, and A. A. Medeiros.** 1995. A functional classification scheme for β-lactamases and its correlation with molecular structure. Antimicrob. Agents Chemother. **39:**1211–1233.
10. **Cao, V., T. Lambert, and P. Courvalin.** 2002. ColE1-like plasmid pIP843 of *Klebsiella pneumoniae* encoding extended-spectrum β-lactamase CTX-M-17. Antimicrob. Agents Chemother. **46:**1212–1217.
11. **Chanawong, A., F. H. M'Zali, J. Heritage, J. H. Xiong, and P. M. Hawkey.** 2002. Three cefotaximases, CTX-M-9, CTX-M-13, and CTX-M-14, among *Enterobacteriaceae* in the People's Republic of China. Antimicrob. Agents Chemother. **46:**630–637.
12. **Coque, T. M., A. Oliver, J. C. Pérez-Díaz, F. Baquero, and R. Cantón.** 2002. Genes encoding TEM-4, SHV-2, and CTX-M-10 extended-spectrum β-lactamases are carried by multiple *Klebsiella pneumoniae* clones in a single hospital (Madrid, 1989 to 2000). Antimicrob. Agents Chemother. **46:**500–510.
13. **Dutour, C., R. Bonnet, H. Marchandin, M. Boye, C. Chanal, D. Sirot, and J. Sirot.** 2002. CTX-M-1, CTX-M-3, and CTX-M-14 β-lactamases from *Enterobacteriaceae* isolated in France. Antimicrob. Agents Chemother. **46:**534–537.
14. **Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease.** 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77:**51–59.
15. **Joris, B., P. Ledent, O. Dideberg, E. Fonze, J. Lamotte-Brasseur, J. A. Kelly, J. M. Ghuysen, and J. M. Frere.** 1991. Comparison of the sequences of class A β-lactamases and of the secondary structure elements of penicillin-recognizing proteins. Antimicrob. Agents Chemother. **35:**2294–2301.
16. **Kariruki, S., J. E. Corkill, G. Revathi, R. Musoke, and C. A. Hart.** 2001. Molecular characterization of a novel plasmid-encoded cefotaximase (CTX-M-12) found in clinical *Klebsiella pneumoniae* isolates from Kenya. Antimicrob. Agents Chemother. **45:**2141–2143.
17. **Matthew, M., A. M. Harris, M. J. Marshall, and G. W. Ross.** 1975. The use of analytical isoelectric focusing for detection and identification of β-lactamases. J. Gen. Microbiol. **88:**169–178.
18. **National Committee for Clinical Laboratory Standards.** 2003. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A6. National Committee for Clinical Laboratory Standards, Wayne, Pa.
19. **Oliver, A., J. C. Pérez-Díaz, T. M. Coque, F. Baquero, and R. Cantón.** 2001. Nucleotide sequence and characterization of a novel cefotaxime-hydrolyzing β-lactamase (CTX-M-10) isolated in Spain. Antimicrob. Agents Chemother. **45:**616–620.
20. **Poirel, L., M. Gniadkowski, and P. Nordmann.** 2002. Biochemical analysis of the ceftazidime-hydrolysing extended-spectrum beta-lactamase CTX-M-15 and of its structurally related beta-lactamase CTX-M-3. J. Antimicrob. Chemother. **50:**1031–1034.
21. **Poirel, L., T. Naas, I. Le Thomas, A. Karim, E. Bingen, and P. Nordmann.** 2001. CTX-M-type extended-spectrum beta-lactamase that hydrolyzes ceftazidime through a single amino acid substitution in the omega loop. Antimicrob. Agents Chemother. **45:**3355–3361.
22. **Sabate, M., R. Tarrago, F. Navarro, E. Miro, C. Verges, J. Barbe, and G. Prats.** 2000. Cloning and sequence of the gene encoding a novel cefotaxime-hydrolyzing beta-lactamase (CTX-M-9) from *Escherichia coli* in Spain. Antimicrob. Agents Chemother. **44:**1970–1973.
23. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

23a. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.

24. **Simarro, E., F. Navarro, J. Ruiz, E. Miro, J. Gomez, and B. Mirelis.** 2000. *Salmonella enterica* serovar Virchow with CTX-M-like beta-lactamase in Spain. J. Clin. Microbiol. **38:**4676–4678.
25. **Spratt, B., P. J. Hedge, T. S. Heesen, A. Edelman, and J. K. Broome-Smith.** 1986. Kanamycin-resistant vectors that are analogues of plasmids pUC8, pUC9, pEMBL8, and pEMBL9. Gene **41:**337–342.