

## Novel Cefotaximase (CTX-M-16) with Increased Catalytic Efficiency Due to Substitution Asp-240→Gly

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Three clinical strains (*Escherichia coli* Rio-6, *E. coli* Rio-7, and *Enterobacter cloacae* Rio-9) collected in 1996 and 1999 from hospitals in Rio de Janeiro (Brazil) were resistant to broad-spectrum cephalosporins and gave a positive double-disk synergy test. Two *bla*<sub>CTX-M</sub> genes encoding  $\beta$ -lactamases of pl 7.9 and 8.2 were implicated in this resistance: the *bla*<sub>CTX-M-9</sub> gene observed in *E. coli* Rio-7 and *E. cloacae* Rio-9 and a novel CTX-M-encoding gene, designated *bla*<sub>CTX-M-16</sub>, observed in *E. coli* strain Rio-6. The deduced amino acid sequence of CTX-M-16 differed from CTX-M-9 only by the substitution Asp-240→Gly. The CTX-M-16-producing *E. coli* transformant exhibited the same level of resistance to cefotaxime (MIC, 16  $\mu$ g/ml) but had a higher MIC of ceftazidime (MIC, 8 versus 1  $\mu$ g/ml) than the CTX-M-9-producing transformant. Enzymatic studies revealed that CTX-M-16 had a 13-fold higher affinity for aztreonam and a 7.5-fold higher  $k_{cat}$  for ceftazidime than CTX-M-9, thereby showing that the residue in position 240 can modulate the enzymatic properties of CTX-M enzymes. The two *bla*<sub>CTX-M-9</sub> genes and the *bla*<sub>CTX-M-16</sub> gene were located on different plasmids, suggesting the presence of mobile elements associated with CTX-M-encoding genes. CTX-M-2 and CTX-M-8 enzymes were found in Brazil in 1996, and two other CTX-M  $\beta$ -lactamases, CTX-M-9 and CTX-M-16, were subsequently observed. These reports are evidence of the diversity of CTX-M-type extended-spectrum  $\beta$ -lactamases in Brazil.

The first extended-spectrum  $\beta$ -lactamase (ESBL) of the CTX-M type (MEN-1, CTX-M-1) was reported at the beginning of the 1990s (3, 5). Initially found in Europe, CTX-M-producing strains have now been observed over a wide geographic area including the Near East (6), Far East (20, 25, 37), South America (4, 6; M. Galas, F. Pasteran, R. Melano, A. Petroni, G. Lopez, A. Corso, A. Rossi, and WHONET Collaborative Group, Abstr. 38th Intersci. Conf. Antimicrob. Agents Chemother., abstr. E-109, p. 201, 1998), and Europe (3, 5, 14–17, 33). CTX-M enzymes have been observed in different species of the family *Enterobacteriaceae*: *Escherichia coli* (3, 5, 20, 25, 37), *Salmonella enterica* serovar Typhimurium (4, 15, 16), *Klebsiella pneumoniae* (6), *Proteus mirabilis* (6, 9), *Citrobacter freundii* (6, 17), *Citrobacter amalonaticus* (9), *Enterobacter aerogenes* (9), and *Enterobacter cloacae* (9), and in the species *Vibrio cholerae* E1 Tor (M. Galas, A. Petroni, R. Melano, A. Corso, M. Rodriguez, M. L. Cacace, A. Bru, and A. Rossi, Abstr. 38th Intersci. Conf. Antimicrob. Agents Chemother. abstr. C-174, p. 119, 1998).

The CTX-M enzymes form a rapidly growing family that comprises 11 enzymes, of which 8 have been described in the last 3 years. The CTX-M enzymes have been subclassified by amino acid sequence similarities into four groups (9). The first group contains enzymes CTX-M-1 (MEN-1) (3, 5) and CTX-M-3 (17); the second group contains the enzymes CTX-M-2 (6), Toho-1 (20), CTX-M-4 (14, 16), CTX-M-5 (11), CTX-M-6 (15), and CTX-M-7 (15) (previously designated CTX-M-5);

and the third and fourth groups contain CTX-M-8 (9) and Toho-2, respectively (25). *E. coli* strains producing CTX-M-9 and CTX-M-10, which are related to Toho-2 and CTX-M-3, respectively, were recently reported in Spain (33; A. Oliver, J. C. Pérez-Díaz, T. M. Coque, F. Baquero, and R. Cantón, Abstr. 40th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 1480, p. 99, 2000).

The CTX-M-type enzymes are much more active against cefotaxime than against ceftazidime and aztreonam. The amino acid residues critical for their extended-spectrum activity are partially known. Ser-237 and Arg-276 seem to be involved in the cefotaxime-hydrolyzing activity of CTX-M enzymes, but mutagenesis experiments of these residues led to only slight changes in their catalytic activities (14, 16). The extended-spectrum activity of CTX-M-type  $\beta$ -lactamases seems therefore to be an “intrinsic” enzymatic property of these ESBLs and not the result of a few point mutations (14–16, 19).

To estimate the diversity of ESBLs in Brazil, clinical strains that exhibited ESBL phenotypes in different species were collected from hospitals in Rio de Janeiro in 1996 and 1999. We report three CTX-M-producing strains isolated in Brazil: two producing CTX-M-9 and one producing a novel Asp-240→Gly variant of CTX-M-9, designated CTX-M-16.

### MATERIALS AND METHODS

**Clinical strains.** Table 1 shows the clinical strains and the plasmids used in this study. Three clinical strains were isolated from three different patients hospitalized in 1996 (Rio-6 and Rio-7) and in 1999 (Rio-9) of distinct private hospitals of Rio de Janeiro, Brazil. *E. coli* strain MEN producing CTX-M-1 (3) was used to synthesize the CTX-M probe.

**Mating-out assays.** Direct transfers of plasmids harboring *bla* genes were performed by mating donor strains with in vitro-obtained rifampin- or nalidixic acid-resistant mutants of *E. coli* HB101 (34) as recipient strains, at 37°C on solid

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TABLE 1. Clinical strains from Rio de Janeiro (Brazil) and recombinant plasmids used in the study

Strain or plasmid	Relevant genotype or phenotype	$\beta$ -Lactamase pI(s)
<b>Strains</b>		
<i>E. coli</i> Rio-6	Clinical strain harboring ESBL resistance phenotype (1996) <sup>a</sup>	5.4, 8.2
<i>E. coli</i> Rio-7	Clinical strain harboring ESBL resistance phenotype (1996) <sup>a</sup>	5.4, 6.3, 7.9
<i>E. cloacae</i> Rio-9	Clinical strain harboring ESBL resistance phenotype (1999) <sup>a</sup>	5.4, 6.3, 7.9, >8
<i>E. coli</i> DH5 $\alpha$	<i>supE44 hsdS20</i> (r <sub>B</sub> <sup>-</sup> m <sub>B</sub> <sup>-</sup> ) <i>recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44 <math>\Delta</math>lacU169</i> ( $\phi$ 80 <i>lacZDM15</i> ) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	
<i>E. coli</i> HB101		
<b>Plasmids</b>		
pRio-6	Natural plasmid of 70 kb from <i>E. coli</i> Rio-6 containing <i>bla</i> <sub>CTX-M-16</sub>	5.4, 8.2
pRio-7	Natural plasmid of 98 kb from <i>E. coli</i> Rio-7 containing <i>bla</i> <sub>CTX-M-9</sub>	Not obtained
pRio-9	Natural plasmid of 180 kb from <i>E. cloacae</i> Rio-9 containing <i>bla</i> <sub>CTX-M-9</sub>	5.4, 7.9
pCPRio-6	Recombinant pBK-CMV plasmid containing 0.9-kb fragment with <i>bla</i> <sub>CTX-M-16</sub>	8.2
pCPRio-7	Recombinant pBK-CMV plasmid containing 0.9-kb fragment with <i>bla</i> <sub>CTX-M-9</sub>	7.9
pBK-CMV	Phagemid vector kanamycin resistance phenotype	

<sup>a</sup> Year isolated is shown in parentheses.

Mueller-Hinton medium. Transconjugants were selected on Mueller-Hinton agar containing rifampin (300  $\mu$ g/ml) or nalidixic acid (150  $\mu$ g/ml) and cefotaxime (2  $\mu$ g/ml).

**Susceptibility to  $\beta$ -lactams.** MICs were determined by a dilution method on Mueller-Hinton agar (Sanofi Diagnostics Pasteur, Marnes la Coquette, France) with an inoculum of 10<sup>4</sup> CFU per spot. Antibiotics were provided as powders by SmithKline Beecham Pharmaceuticals (clavulanate), Lederle Laboratories (tazobactam), Eli Lilly, Paris, France (cephalothin), Roussel-Uclaf (cefotaxime, cefpirome), Glaxo Wellcome Research and Development (ceftazidime), and Bristol-Myers Squibb (aztreonam).

Detection of ESBLs was performed with the standard double-disk synergy tests as described previously (21). Antibiotic disks for agar tests were obtained from Sanofi Diagnostics Pasteur.

**Isoelectric focusing.** Isoelectric focusing was performed with polyacrylamide gels containing ampholines with a pH range of 3.5 to 10 as previously described (8). Visualization of  $\beta$ -lactamase activity was carried out with an agarose overlay containing 6% (wt/vol) potassium iodide, 0.6% (wt/vol) iodine, and 0.6% (wt/vol)  $\beta$ -lactam substrate: penicillin G to show overall  $\beta$ -lactamase content or cefotaxime to show the cefotaxime-hydrolyzing  $\beta$ -lactamases.  $\beta$ -lactamases of known pIs were used as standards: TEM-1 (pI 5.4), TEM-3 (pI 6.3), TEM-24 (pI 6.5), SHV-1 (pI 7.6), P99 (pI 7.8), and SHV-5 (pI 8.2).

**$\beta$ -Lactamase preparation.** CTX-M-producing *E. coli* DH5 $\alpha$  (pCIRio-6 and pCIRio-7) were grown in 6 liters of brain heart infusion broth containing cefotaxime at 2  $\mu$ g/ml for 18 h at 37°C. The bacteria collected by centrifugation were suspended with 20 mM morpholineethane sulfonic acid (MES)-NaOH (pH 6.0) and disrupted by ultrasonic treatment (four times for 30 s, each time at 20 W). After centrifugation (10,000  $\times$  g for 10 min at 4°C), nucleic acids were precipitated by addition of 0.2 M (7% [vol/vol]) spermine and centrifugation at 48,000  $\times$  g for 60 min at 4°C. The clarified supernatant was dialyzed overnight against 20 mM MES-NaOH (pH 6.0). The CTX-M purification was carried out as previously described (8) by ion-exchange chromatography with an SP Sepharose column (Amersham Pharmacia Biotech) and gel-filtration chromatography with a Superose 12 column (Amersham Pharmacia Biotech). The total protein concentration was estimated by the Bio-Rad protein assay (Bio-Rad, Richmond, Calif.), with bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) used as a standard.

The purity of CTX-M extracts was estimated as previously described (8) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and staining with Coomassie blue R-250 (Sigma Chemical Co.). The renaturation of proteins and the detection of  $\beta$ -lactamase activity were performed as previously described (8) with renaturation buffer {Tris-HCl (100 mM), Triton X-100 (2% [vol/vol]), pH 7.0} and 0.5 mM nitrocephin (Oxoid, Paris, France) in 100 mM phosphate buffer (pH 7.0), respectively.

**Determination of  $\beta$ -lactamase kinetic constants.** The kinetic constants  $K_m$  and  $K_{cat}$  of the  $\beta$ -lactamases were obtained by computerized microacidimetric method as previously described (24). The concentrations of the inhibitors (clavulanate and tazobactam) required to inhibit enzyme activity by 50% (IC<sub>50</sub>s) were determined as described previously with penicillin G (7). The specific activities, IC<sub>50</sub>, and  $K_i$  values were determined with penicillin G (200 mM) as the reporter substrate. The kinetic constants were determined three times. The variation coefficients had a maximum of 25%, except with the CTX-M-9 enzyme for aztreonam and ceftazidime, for which the maximum was 40%.

**PCR of CTX-M genes.** The detection of CTX-M-1, CTX-M-2, and CTX-M-9-like-encoding genes and the synthesis of CTX-M probe were performed with the primers CTX-MA (5'-CGCTTTGCGATGTGCAG-3') and CTX-MB (5'-ACCGGATATCGTTGGT-3') (temperature of annealing, 54°C), which correspond to conserved regions of CTX-M-type genes. An internal fragment of 550 pb was amplified from positions 264 to 814 (*bla*<sub>CTX-M-1</sub> numbering). The complete open reading frames of *bla*<sub>CTX-M-9</sub>-like genes were amplified with the primers CTX-M-9A (5'-CTGATGTAACACGGATTGAC-3') and CTX-M-9C (5'-AGCGCCCCATTATTGAGAG-3'), which were located in the flanked sequences of *bla*<sub>CTX-M-9</sub> (temperature of annealing, 54°C).

**Plasmid extraction and hybridization.** Plasmid DNAs were extracted by the method of Kado and Liu (22). The plasmid size was determined by comparison with plasmids Rsa (39 kb), TP114 (61 kb), pCFF04 (85 kb), and pCFF14 (180 kb) (7).

DNA probes used for hybridization were PCR products obtained with the primers CTX-MA and CTX-MB. Labeling was performed by random priming with a 2,4-dinitrophenol DNA labeling kit purchased from Appligene Oncor (Illkirch, France). Hybridization and revelation were performed with a DNP-DNA chemiluminescence detection kit (Appligene Oncor) according to the manufacturer's recommendations for DNA extracts denatured and immobilized on Nytran Filters.

**$\beta$ -Lactamase gene cloning.** The recombinant DNA manipulations were performed as described by Sambrook et al. (34). T4 DNA ligase was purchased from Boehringer, Mannheim, Germany. The CTX-M-encoding sequence was cloned as follows. PCR products, which were obtained with proofreading *Taq* polymerase *Tfu* (Appligene Oncor) and the primers CTX-M-9A and CTX-M-9C, were ligated in the *Sma*I site of the phagemid pBK-CMV (Stratagene, La Jolla, Calif.). *E. coli* DH5 $\alpha$  (34) was transformed by electroporation. The transformants harboring the recombinant CTX-M-encoding plasmids were selected on Mueller-Hinton agar supplemented with 2  $\mu$ g of cefotaxime per ml.

**DNA sequencing.** The sequences were determined by direct sequencing of PCR products, performed by the dideoxy chain termination procedure of Sanger et al. (35) on an ABI 1377 automatic sequencer using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit with AmpliTaq DNA polymerase FS (Perkin-Elmer/Applied Biosystems, Foster City, Calif.).

**Computer analysis.** The nucleotide sequence and the deduced protein sequence were analyzed with the software available over the Internet at the National Center for Biotechnology Information. Hydrophobic blotting was performed using the method of Nielsen et al. (27). Multiple sequence alignment and pairwise comparisons of sequences were carried out with the help of ClustalW Software (version 1.74) (36). Twelve class A CTX-M enzymes were compared to CTX-M-16: CTX-M-1, CTX-M-2, Toho-1, CTX-M-3, CTX-M-4, CTX-M-5, CTX-M-6, CTX-M-7, CTX-M-8, CTX-M-9, CTX-M-10, and Toho-2. Phylogenetic analysis was performed by the neighbor-joining method using PHYLIP (Phylogeny Inference Package, version 3.5c) from a distance matrix, which was carried out with the Dayhoff PAM matrix (13).

**Nucleotide sequence accession number.** The *bla*<sub>CTX-M-16</sub> nucleotide sequence data appear in the GenBank nucleotide sequence database under accession number AY029068.

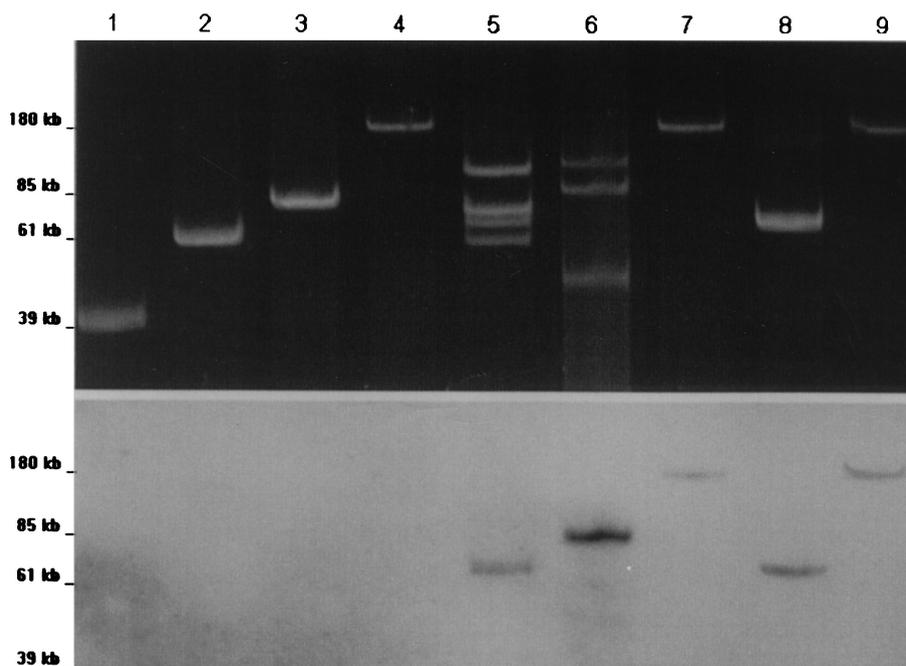


FIG. 1. (A) Agarose (0.7%) electrophoresis of plasmid DNA from the three clinical strains, Rio-6, Rio-7, and Rio-9, and the *E. coli* transconjugants. (B) Hybridization of plasmid content with a CTX-M probe. Lanes: 1 to 4, reference plasmids RSa (39 kb), TP114 (61 kb), pCFF04 (85 kb), and pCFF14 (180 kb); 5, *E. coli* Rio-6; 6, *E. coli* Rio-7; 7, *E. cloacae* Rio-9; 8, *E. coli* DH5 $\alpha$ (pRio-6); 9, *E. coli* DH5 $\alpha$ (pRio-9).

## RESULTS

**Characterization of clinical isolates.** The clinical isolates Rio-6, Rio-7, and Rio-9 exhibited resistance to broad-spectrum cephalosporins (MICs: cefotaxime, 16 to 128  $\mu$ g/ml; ceftazidime, 32 to 256  $\mu$ g/ml; aztreonam, 64 to 128  $\mu$ g/ml) and a positive double-disk synergy test. Isoelectric point determination with penicillin G as substrate revealed the presence of two to three different  $\beta$ -lactamases per strain (Table 1), but using cefotaxime as substrate, only one enzyme, of pI 7.9 in strains Rio-7 and Rio-9 or of pI 8.2 in strain Rio-6, showed a strong cefotaxime-hydrolyzing activity. The three strains exhibited a positive amplification with the primers CTX-MA and CTX-MB, which were designated from conserved sequences of *bla*<sub>CTX-M</sub> genes. These results suggested the presence of two distinct *bla*<sub>CTX-M</sub> genes which encoded enzymes of pI 8.2 in strain Rio-6 and of pI 7.9 in strains Rio-7 and Rio-9.

**Plasmid content and transfer of  $\beta$ -lactam resistance.** Transconjugants were obtained from *E. coli* Rio-6 and *E. cloacae* Rio-9. They produced cefotaxime-hydrolyzing  $\beta$ -lactamases of alkaline pI (8.2 or 7.9) and associated with a  $\beta$ -lactamase of pI 5.4, which was identified by PCR and sequencing as the TEM-1 penicillinase.

The plasmid contents of the clinical strains are shown in Fig. 1 as is their hybridization with CTX-M probe. *E. coli* Rio-6 contained four plasmids of 118, 78, 70, and 61 kb. Only the plasmid of 70 kb, designated pRio-6, was observed in the transconjugant of strain Rio-6 and hybridized with CTX-M probe. *E. cloacae* Rio-9 contained one plasmid of 180 kb, designated pRio-9, which was observed in the transconjugant of strain Rio-9 and hybridized with the CTX-M probe. *E. coli* Rio-7 contained three plasmids (134, 98, and 48 kb); among

them the plasmid of 98 kb, designated pRio-7, hybridized with the CTX-M probe.

**DNA sequencing.** DNA sequencing of PCR products obtained with primers CTX-M-9A and CTX-M-9C revealed that strains Rio-7 and Rio-9 contained the gene *bla*<sub>CTX-M-9</sub>, previously reported in an *E. coli* strain isolated in Spain (33), whereas strain Rio-6 harbored a new gene, designated *bla*<sub>CTX-M-16</sub>, which differed from *bla*<sub>CTX-M-9</sub> by the mutation A $\rightarrow$ G at position 725.

The amino acid sequence deduced for *bla*<sub>CTX-M-16</sub> differed from that of CTX-M-9 by the substitution Asp $\rightarrow$ Gly in position 240 (Fig. 2). The strain Rio-6 thus produced a novel CTX-M-type enzyme, which was designated CTX-M-16.

On the basis of amino acid sequence alignments (Fig. 2) with CTX-M signal peptide sequences previously determined by direct amino acid sequencing (4, 29) and from hydropathy plots, it is likely that the signal peptide of CTX-M-16 and CTX-M-9 comprise 28 amino acids. Thus, the putative mature enzymes CTX-M-9 and CTX-M-16 consist of 263 amino acid residues with calculated molecular masses of 28,945 and 27,887 Da, respectively.

A dendrogram was constructed on the basis of peptide alignment (Fig. 3). Bootstrapping gave a high degree of resolution for internal nodes (superior to 50% majority-consensus confidence). The dendrogram showed four major branches, of which one comprised the enzymes Toho-2, CTX-M-9, and CTX-M-16.

**Cloning of the  $\beta$ -lactamase gene.** The coding sequences of CTX-M-9 and CTX-M-16, obtained by PCR with primers CTX-M-9A and CTX-M-9B, were cloned downstream of the *LacZ* promoter of plasmid pBK-CMV. The obtained recom-

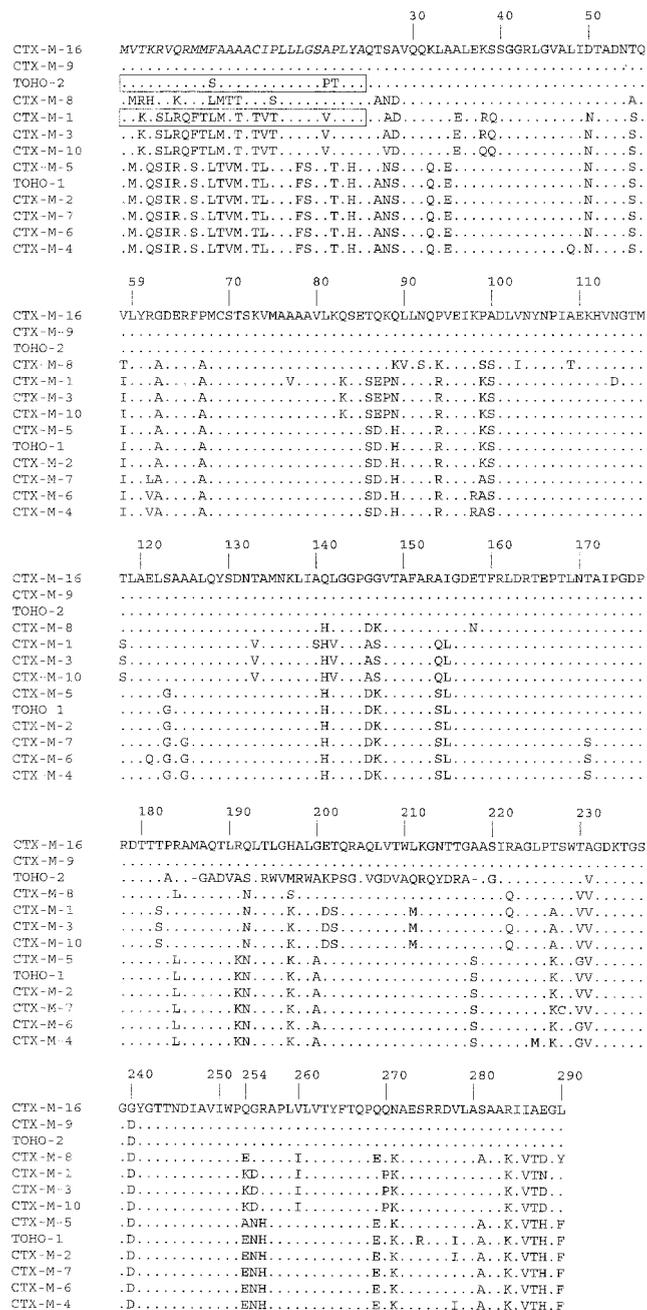


FIG. 2. Alignments of the CTX-M-16 amino acid sequence with those of CTX-M-1, CTX-M-2, CTX-M-3, CTX-M-4, CTX-M-5, CTX-M-6, CTX-M-7, CTX-M-8, CTX-M-9, Toho-1, and Toho-2. Dots indicate amino acids identical to those of CTX-M-16. Italic letters represent the peptide signal of CTX-M-16, as determined by hydropathy plotting. The peptide signals determined by amino acid sequencing are boxed. The amino acids are numbered according to the standard numbering scheme for the class A  $\beta$ -lactamases of Ambler et al. (1).

binant plasmids were designated pCPRio-6 and pCPRio-7 and contained a 0.9-kb insert encoding the enzymes CTX-M-16 and CTX-M-9, respectively.

**$\beta$ -Lactam susceptibility.** MICs of  $\beta$ -lactams for the *E. coli* DH5 $\alpha$  transformants producing CTX-M-9 (pCPRio-7) and CTX-M-16 (pCPRio-6) are listed in Table 2. These CTX-M-producing strains exhibited a high level of resistance to amino- and carboxypenicillins (MICs, >2,048  $\mu$ g/ml), piperacillin

(MIC, 256  $\mu$ g/ml), and cephalothin and cefuroxime (MICs, 512  $\mu$ g/ml). The similar level of resistance to cefotaxime (MIC, 16  $\mu$ g/ml) and aztreonam (MICs, 4 to 8  $\mu$ g/ml) was observed with CTX-M-16 and CTX-M-9 producers, while the MICs of ceftazidime were higher for CTX-M-16 producers than for CTX-M-9 producers (MICs, 8 versus 1  $\mu$ g/ml).

Clavulanate and tazobactam restored partially or totally the activities of the  $\beta$ -lactams. All strains were susceptible to associations of clavulanate or tazobactam and broad-spectrum cephalosporins (MICs, 0.06 to 0.25  $\mu$ g/ml).

**Biochemical properties of the  $\beta$ -lactamase CTX-M-16.** The purified proteins appeared on SDS-polyacrylamide gels as a band of 28.6 kDa for CTX-M-16 (Fig. 4) and CTX-M-9 (data not shown). The specific activities of purified ( $\geq 97\%$  pure) CTX-M-16 and CTX-M-9 were 140 and 615  $\mu$ mol  $\cdot$  min $^{-1} \cdot$  mg of protein $^{-1}$ , respectively, with 200 mM penicillin G as the substrate.

The substrates of CTX-M-9 and CTX-M-16 are shown in Table 3. Common enzymatic features included the following: better affinities were found for penicillins ( $K_m$ , 6 to 35  $\mu$ M) than for cephalosporins, except for CTX-M-16 and aztreonam ( $K_m$  17  $\mu$ M); cephalothin was the best substrate ( $K_{cat}$  for ceph-

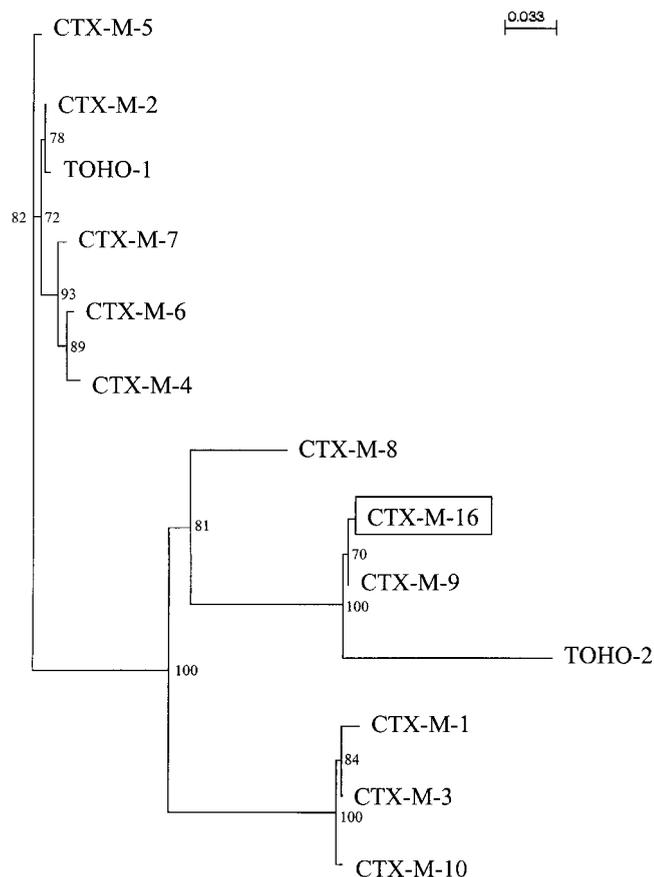


FIG. 3. Dendrogram of CTX-M family. Branch lengths are scaled according to the amino acid changes. The scale indicates the distance measured for protein sequences based on the Dayhoff PAM matrix (13). The percentages at the branch points refer to the number of times a particular nod was found in 100 bootstrap replications. The distance along the vertical axis has no significance.

TABLE 2.  $\beta$ -Lactam MICs for CTX-M-9- and CTX-M-16-producing *E. coli* DH5 $\alpha$ 

$\beta$ -Lactam	MIC ( $\mu$ g/ml) for <i>E. coli</i> DH5 $\alpha$ with:		
	PCPRio-6 <sup>a</sup> CTX-M-16	pCPRio-7 <sup>b</sup> CTX-M-9	pBK-CMV
Amoxicillin	>2,048	>2,048	2
Amoxicillin + CLA <sup>c</sup>	8	8	2
Ticarcillin	>2,048	>2,048	2
Ticarcillin + CLA <sup>c</sup>	16	16	2
Piperacillin	256	256	2
Piperacillin + TZB <sup>d</sup>	2	2	2
Cephalothin	512	512	4
Cefuroxime	512	512	<2
Cefotaxime	16	16	0.06
Cefotaxime + CLA <sup>c</sup>	0.06	0.06	0.06
Cefotaxime + TZB <sup>c</sup>	0.06	0.06	0.06
Cefotaxime + SUL <sup>c</sup>	0.50	2	0.06
Aztreonam	8	4	0.12
Aztreonam + CLA <sup>c</sup>	0.06	0.06	0.06
Aztreonam + TZB <sup>c</sup>	0.06	0.06	0.12
Aztreonam + SUL <sup>c</sup>	0.25	0.50	0.12
Ceftazidime	8	1	0.12
Ceftazidime + CLA <sup>c</sup>	0.25	0.25	0.12
Ceftazidime + TZB <sup>c</sup>	0.25	0.25	0.12
Ceftazidime + SUL <sup>c</sup>	0.50	0.50	0.12
Cefpirome	2	2	0.06
Cefpirome + CLA	0.06	0.06	0.06
Cefpirome + TZB <sup>c</sup>	0.06	0.06	0.06
Cefpirome + SUL <sup>c</sup>	0.25	0.50	0.06

<sup>a</sup> *E. coli* DH5 $\alpha$  harboring recombinant plasmid pCPRio-6, which encodes  $\beta$ -lactamase CTX-M-16.

<sup>b</sup> *E. coli* DH5 $\alpha$  harboring recombinant plasmid pCPRio-7, which encodes  $\beta$ -lactamase CTX-M-9.

<sup>c</sup> CLA, clavulanate; TZB, tazobactam; SUL, sulbactam at fixed concentration of 2  $\mu$ g/ml.

<sup>d</sup> TZB at fixed concentration of 4  $\mu$ g/ml, as in the commercially available combination with piperacillin.

alothin 10- to 43-fold higher than that for penicillin); and a higher  $k_{cat}$  for cefotaxime (450 to 1400 s<sup>-1</sup>) was found than for ceftazidime (2 to 15 s<sup>-1</sup>) and aztreonam (10 to 3 s<sup>-1</sup>).

However, CTX-M-16 had globally higher affinities (lower  $K_m$  values) than CTX-M-9 for  $\beta$ -lactams, particularly for aztreonam, since the binding of this substrate was 13-fold higher for CTX-M-16 than for CTX-M-9. Conversely, CTX-M-9 had higher  $K_{cat}$  values than CTX-M-16 with all substrates except cefuroxime and particularly for cefotaxime and ceftazidime, for which  $k_{cat}$  values were higher for CTX-M-16 than for CTX-M-9.

CTX-M-9 and CTX-M-16 were susceptible to tazobactam (IC<sub>50</sub>s, 0.036 and 0.030  $\mu$ M, respectively), clavulanate (IC<sub>50</sub>s, 0.007 and 0.008  $\mu$ M, respectively), and to a lesser extent to sulbactam (IC<sub>50</sub>s, 3.0 and 4.5  $\mu$ M, respectively).

## DISCUSSION

The starting point of this work was the observation of three clinical strains isolated in Brazil, *E. coli* Rio-6 (isolated from urine of a patient hospitalized in a home care unit) and *E. coli* Rio-7 and *E. cloacae* Rio-9 (isolated from tracheal aspirations of patients hospitalized in an intensive care unit), that exhibited resistance to broad-spectrum cephalosporins with a positive double-disk synergy test. Two different CTX-M-encoding genes were implicated in this resistance phenotype: the novel CTX-M-encoding gene *bla*<sub>CTX-M-16</sub>, which was related to *bla*<sub>CTX-M-9</sub> and was observed in *E. coli* strain Rio-6, and the *bla*<sub>CTX-M-9</sub> gene (33) in strains *E. coli* Rio-7 and *E. cloacae* Rio-9.

The *bla*<sub>CTX-M-16</sub> gene is probably the result of a point mutation of the *bla*<sub>CTX-M-9</sub> gene, because the two genes were

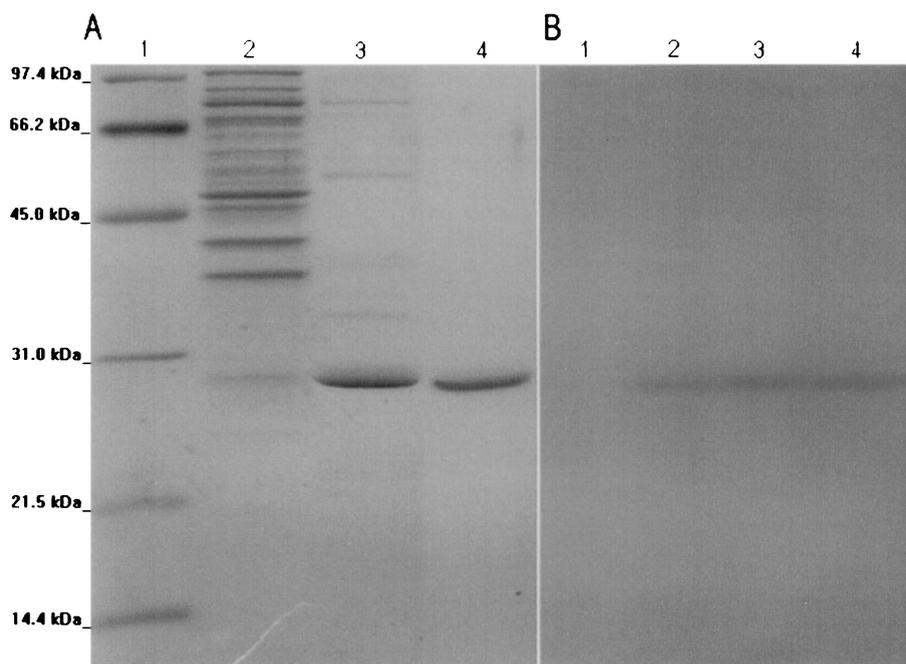


FIG. 4. Electrophoresis analysis of CTX-M-9 and CTX-M-16 purified extracts. (A) SDS-PAGE stained with Coomassie brilliant blue R-250. (B) Zymogram detection of  $\beta$ -lactamase activity with nitrocefin after renaturation treatment and SDS-PAGE. Lanes: 1, protein molecular mass reference; 2, clarified extract of  $\beta$ -lactamase CTX-M-16; 3, fraction with  $\beta$ -lactamase activity eluted from ionic exchange column; 4, purified extract of  $\beta$ -lactamase CTX-M-16.

TABLE 3. Substrate profiles of  $\beta$ -lactamases CTX-M-9 and CTX-M-16

Substrate	CTX-M-9			CTX-M-16		
	$K_{cat}$ ( $s^{-1}$ )	$K_m$ ( $\mu M$ )	$K_{cat}/K_m$ ( $\mu M^{-1} \cdot s^{-1}$ )	$K_{cat}$ ( $s^{-1}$ )	$K_m$ ( $\mu M$ )	$K_{cat}/K_m$ ( $\mu M^{-1} \cdot s^{-1}$ )
Penicillin G	295	2.5	11.8	65	6	10.8
Amoxicillin	90	20	4.5	40	10	4.0
Ticarcillin	60	35	1.7	10	13	0.8
Piperacillin	110	20	5.5	45	8	5.6
Cephalothin	3,000	150	20.0	2,800	83	33.7
Cefuroxime	350	50	7.0	500	45	11.0
Cefotaxime	450	120	3.7	1,400	150	9.3
Cefpirome	950	800	1.2	840	520	1.6
Aztreonam	10	220	0.04	3	17	0.2
Ceftazidime	2	600 <sup>a</sup>	0.003	15	350	0.04

<sup>a</sup>  $K_m$  values were determined as  $K_i$  by substrate competition with penicillin G.

characterized in the same geographic area and differed by one nucleotide only.  $bla_{CTX-M-16}$  and the closely related gene  $bla_{Toho-2}$  were observed at almost the same time, in Spain and Japan, respectively (25, 33). The concurrent emergence of these  $bla_{CTX-M-9}$ -type genes in two geographic areas widely separated could be explained by their transfer from widespread environmental strains, as previously observed with the gene  $bla_{SFO-1}$ , which was probably transferred from *Serratia fonticola* to *E. cloacae* (26). The most closely related enzymes to CTX-M genes are the chromosome-encoded enzymes of *Klebsiella oxytoca* (2, 32), *Serratia fonticola* (28), *Citrobacter diversus* (30), and *Proteus vulgaris* (29), but there is no direct phylogenetic connection between these  $\beta$ -lactamases and CTX-M enzymes. In addition,  $bla_{CTX-M-2}$ -like genes have been observed in the chromosome of *K. ascorbata* (A. Philippon, Abstr. 39<sup>th</sup> Intersci. Conf. Antimicrob. Agents Chemother., abstr 2044a, 1999), which therefore is a potential reservoir for a CTX-M-encoding gene. However, the CTX-M-9 and CTX-M-16 enzymes are distantly related to the CTX-M-2-like  $\beta$ -lactamases and hence could originate from variant genes present in the same species or in related species. The two  $bla_{CTX-M-9}$  genes and the  $bla_{CTX-M-16}$  gene, like  $bla_{CTX-M-8}$  and  $bla_{CTX-M-3}$  (9, 17), were located on different plasmids. This suggests that mobile elements associated with CTX-M-encoding genes are present and that they could be involved in gene transfers.

The phylogenetic relations between the enzymes reported in the CTX-M family show four types of CTX-M enzymes—CTX-M-1 type, which includes CTX-M-1, CTX-M-3, and CTX-M-10; CTX-M-2 type, which includes CTX-M-2, Toho-1, CTX-M-4, CTX-M-5, CTX-M-6, and CTX-M-7; CTX-M-8 type, which has only one member; and CTX-M-9 (or Toho-2) type, which includes Toho-2, CTX-M-9, and the novel enzyme CTX-M-16.

The catalytic properties of CTX-M-9 and CTX-M-16 have similarities with those of previously reported CTX-M enzymes, such as higher catalytic activity against cefotaxime than against ceftazidime and aztreonam. The enzymes CTX-M-9 and CTX-M-16 include the amino acid residues Ser-237, Phe-160, Gly-232, and Arg-276, which are thought to play a part in the cefotaxime-hydrolyzing activity of CTX-M enzymes (3, 14–16, 19, 20, 25). In addition, the CTX-M-9 and CTX-M-16  $\beta$ -lac-

tamases are susceptible to inhibitors, and in particular to tazobactam, as reported elsewhere (9, 15, 16, 25).

The residue in position 240 is not conserved among  $\beta$ -lactamases of class A. At least three types of residue are observed at this position: (i) acid residues (Glu or Asp) in TEM and SHV-1 penicillinases, as well as in CTX-M ESBLs and their closely related enzymes; (ii) a Lys residue in some SHV- and TEM-type ESBLs; (iii) a Gly residue in carboxypenicillinases and in the ESBLs PER, VEB-1, and BES-1 (8, 31). CTX-M-16 is the first CTX-M-type ESBL which harbors the substitution Asp-240→Gly. This substitution increased the binding of aztreonam (13-fold), as well as the  $k_{cat}$  against ceftazidime (7.5-fold) and to a lesser extent that against cefotaxime (3-fold). The cefotaximase BES-1, which harbors the same Gly residue in position 240, exhibits the same behavior against aztreonam, cefotaxime, and ceftazidime as does CTX-M-16 (8). The ESBL PER-1, which has a good catalytic efficiency against cefotaxime and ceftazidime, also contains a Gly-240 residue. Bouthors et al. (10) showed that the substitution Gly-240→Glu of PER-1 causes a reduction of affinity for aztreonam (around fourfold) and a decrease in catalytic activity against cefotaxime and ceftazidime (around twofold and threefold, respectively).

Residues Lys-240 and Arg-240 are known to play a major role in the extended-spectrum activity of TEM- and SHV-type ESBLs. They lead to an increase in hydrolyzing activities against ceftazidime and aztreonam but are not involved in cefotaxime-hydrolyzing activity. Lys and Arg are positively-charged residues which can form an electrostatic bond with the carboxylic acid group on oximino substituents of aztreonam and ceftazidime (23) and induce a rotation of the aminothiazole-oxime group (18). They could therefore remove the obstruction caused by the bulky *t*-carbon group of the oxime and allow hydrogen bonding of the acylamide NH to the  $\beta$ 3-strand of the enzyme (18). Thus, Lys and Arg in position 240 favor carboxy oximino  $\beta$ -lactam bonding and hydrolyzing. Residue Gly-240, which has no side chain, is not able to form electrostatic interactions with  $\beta$ -lactams. However, the absence of a side chain in position 240 could facilitate the positioning of the aminothiazole-oxime group of cefotaxime, aztreonam, and ceftazidime in the catalytic pocket.

In contrast with cefotaxime- and ceftazidime-hydrolyzing activities, aztreonam-hydrolyzing activity was not modified by the residue Gly-240. The sulfonic acid group of aztreonam may form weaker interactions with the side chains of residues Ser-130, Lys-234, Thr-235, and Arg-276 of the CTX-M  $\beta$ -lactamases than does the functionally equivalent carboxylate group of cefotaxime and ceftazidime (12). Thus, the weak interaction of aztreonam with CTX-M-16 may alter its positioning in its catalytic pocket, resulting in a weak aztreonam-hydrolyzing activity. It is likely therefore that aztreonam, unlike cefotaxime and ceftazidime, requires the electrostatic interaction mediated by the positively-charged residues in position 240 for correct positioning and, hence, for hydrolysis. However, the Asp-240→Lys substitution would require two mutations in the corresponding codon and thus is a more improbable spontaneous genetic event than the single mutation observed in the CTX-M-16 enzyme.

Since the first report of MEN-1 (CTX-M-1) a decade ago (3, 5), a great variety of CTX-M enzymes have been observed. In

a previous study, we reported CTX-M-2- and CTX-M-8-producing *Enterobacteriaceae* isolated in Brazil (9), and in this work we report CTX-M-9- and CTX-M-16-producing *Enterobacteriaceae*. These findings show the spread, diversity, and implantation of CTX-M enzymes in Brazil. CTX-M-2 was first characterized in Argentina, and Galas et al. reported that the predominant ESBL in the country is CTX-M-2 (M. Galas et al., 38th ICAAC, abstr. E-109). Like Eastern Europe and Japan (14–17, 37), South America is an important source of CTX-M-producing bacteria.

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