A Novel CTX-M β-Lactamase (CTX-M-8) in Cefotaxime-Resistant Enterobacteriaceae Isolated in Brazil

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To estimate the diversity of extended-spectrum β-lactamases in Brazil, 18 strains from different species of the family Enterobacteriaceae exhibiting a positive double-disk synergy test were collected by a clinical laboratory from several hospitals in Rio de Janeiro, Brazil, in 1996 and 1997. Four strains (Proteus mirabilis, Enterobacter cloacae, Enterobacter aerogenes, and Citrobacter amalonaticus) hybridized with a 550-bp CTX-M probe. The P. mirabilis strain produced a CTX-M-2 enzyme. The E. cloacae, E. aerogenes, and C. amalonaticus isolates harbored a bla gene which was identified by cloning and sequencing as a blaCTX-M gene. E. coli HB101 transconjugants and the E. coli DH5α transformant harboring a recombinant plasmid produced a CTX-M β-lactamase with an isoelectric point of 7.6 conferring a resistance phenotype characterized by a higher level of resistance to cefotaxime than to ceftazidime, as observed with the other CTX-M enzymes. The deduced protein sequence showed a novel Ambler class A CTX-M enzyme, named CTX-M-8, which had 83 to 88% identity with the previously described CTX-M enzymes. The phylogenetic study of the CTX-M family including CTX-M-8 revealed four CTX-M types, CTX-M-8 being the first member of a new phylum of CTX-M enzymes. The evolutionary distances between the four types of CTX-M were large, suggesting that the four clusters branched off early from a distant unknown enzyme and that intermediate enzymes probably existed.

Shortly after the introduction of the broad-spectrum cephalosporins such as cefotaxime, aztreonam, and ceftazidime, extended-spectrum β-lactamases (ESBLs) were isolated, first in Europe (24, 40) and then worldwide. According to the structural classification of Ambler et al. (1) and the latest functional scheme of Bush et al., these ESBLs are generally class A enzymes of the 2be group, arising subsequently to a few amino acid substitutions, from the common plasmid-mediated TEM and SHV-1 β-lactamases (12, 20).

The CTX-M β-lactamases, a new family in class A ESBLs, were characterized at the beginning of the 1990s in the first reports of the MEN-1 (CTX-M-1) enzyme (4, 6). In contrast to TEM and SHV type cefotaxime-hydrolyzing ESBLs, CTX-Ms are much more active against cefotaxime than against ceftazidime. The amino acid residues critical for their extended-spectrum activity are distinct from those of TEM- and SHV-1-derived ESBLs (4, 14–16, 18, 27).

The growing CTX-M family comprises nine members: CTX-M-1 (MEN-1) (4, 6), CTX-M-2 (5), Toho-1 (19), CTX-M-3 (17), CTX-M-4 (16), CTX-M-5 (11), Toho-2 (27), CTX-M-7 (15) (previously designated CTX-M-5), and CTX-M-6 (15). They have high homology with the class A chromosomally encoded β-lactamases of Proteus vulgaris (31), Serratia fonticola (30), Citrobacter diversus (32), and Klebsiella oxytoca (2, 33) and plasmid-mediated β-lactamase SFO-1 (28). However, there is no clear direct phylogenic connection between CTX-M enzymes and these β-lactamases (7).

First described in Europe, CTX-M-producing strains have been characterized in November 1996 from blood. Toho-2 and CTX-M-3, produced by three different strains of the family Enterobacteriaceae, were characterized in hospitals of Rio de Janeiro in 1996 and 1997. In this report, we describe a novel CTX-M type enzyme, designated CTX-M-8, produced by three different strains of the family Enterobacteriaceae.

MATERIALS AND METHODS

Clinical strains. Table 1 shows the clinical strains and plasmids used in this study. Clinical strains Rio-1, Rio-2, and Rio-3, which produced a novel β-lactamase, were isolated from patients hospitalized in intensive care units of three private hospitals of Rio de Janeiro, Brazil. Enterobacter cloacae Rio-1 was isolated in November 1996 from blood. Citrobacter amalonaticus Rio-2 and Enterobacter aerogenes Rio-3 were isolated in March 1997 from a surgical wound and blood, respectively. CTX-M-1-producing E. coli MEN (4), CTX-M-2-producing P. mirabilis Rio-4, and TEM-1-producing E. coli TR4 (38) were used as reference strains.

Mating-out assays. Direct transfers of plasmids carrying resistance genes were performed by mating donor strains with in vitro-obtained rifampin- or nalidixic acid-resistant mutants of E. coli HB101 (35) as the recipient strain at 37°C in solid and liquid Mueller-Hinton medium. Transconjugants were selected on Mueller-Hinton agar containing rifampin (300 μg/ml) or nalidixic acid (150 μg/ml) and cefotaxime (2 μg/ml). The transconjugants E. coli TR-Rio-2 and TR-Rio-3 were obtained from clinical strains of C. amalonaticus Rio-2 and of E. aerogenes Rio-3, respectively.
SUSCEPTIBILITY OF β-LACTAMS. MICs were determined by a dilution method on Mueller-Hinton agar (Sanofi Diagnostics Pasteur, Marnes la Coquette, France) with an inoculum of 104 CFU per spot. Antibiotics were provided as powders by SmithKline Beecham Pharmaceuticals (amoxicillin, ticarcillin, and clavulanate), Lederle Laboratories (piperacillin, tazobactam), Eli Lilly, Paris, France (cephalothin), Roussel-Uclaf (cefotaxime, cefpirome), Glaxo Wellcome Research and Development (ceftazidime), Bristol-Myers Squibb (aztreonam, cefepime), Glaxo (ceftazidime), Piramal (piperacillin, tazobactam), and Merck Sharp & Dohme (imipenem).

RESULTS

Characterization of clinical isolates. The clinical isolates Rio-1, Rio-2, and Rio-3 (Table 1) exhibited a resistance to broad-spectrum cephalosporins and a positive double-disk synergy test and produced β-lactamases of PI 7.6 and 5.4. In addition, the Enterobacter strains Rio-1 and Rio-3 harbored β-lactamases with alkaline PI values consistent with cephalosporinase production.

PCR and DNA sequencing identified the β-lactamase of PI 5.4 as TEM-1 penicillinase. The enzyme of PI 7.6 was not of the SHV type, and no PCR products were obtained with the CTX-M-1 and CTX-M-2 type-specific primers CTX-MA and CTX-MB. In contrast, CTX-M type genes were detected in the three strains by hybridization with a CTX-M type gene probe, suggesting the presence of new CTX-M type genes in these strains.

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. A hydropathic plot was obtained by the method of Nielsen et al. (29). Multiple sequence alignment and pairwise comparisons of sequences were carried out with the help of the software Clustal W, version 1.74 (43). Nine class A CTX-M enzymes were compared to CTX-M-8: CTX-M-1, CTX-M-2, Toho-1, CTX-M-3, CTX-M-4, CTX-M-5, CTX-M-6, CTX-M-7, and Toho-2. A dendrogram was derived from the protein multiple-sequence alignment by the parsimony method using the phylogenetic package PAUP (phylogenetic analysis using parsimony), version 3.0 (42).

**TABLE 1. Strains and plasmids used in the study**

| Strain or plasmid          | Relevant genotype or phenotype (place and yr of isolation) | Source or reference |
|---------------------------|----------------------------------------------------------|---------------------|
| **E. cloacae Rio-1**      | Broad-spectrum cephalosporin resistant (Rio de Janeiro, Brazil, 1996) | This study          |
| **E. aerogenes Rio-2**    | Broad-spectrum cephalosporin resistant (Rio de Janeiro, Brazil, 1997) | This study          |
| **E. coli MEN**           | CTX-M-1-producing E. coli                                 | This study          |
| **E. coli TR4**           | TEM-1-producing E. coli                                   | 4                   |
| **E. coli HB101**         | conC1446 supE44 hsdR52 (F lacI1 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mit-1) | 38                  |
| **E. coli DH15a**         | supE44 Δac3169 (F80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 | 35                  |
| **Plasmids**              |                                                          |                     |
| pRio-2                    | Natural plasmid from **E. aerogenes** Rio-2 containing blalCTX-M-8** and bla**TEM-1**-resistance phenotype: ESBL, amikacin, gentamicin, kanamycin, netilmicin, tobramycin | This study          |
| pRio-3                    | Natural plasmid from **E. aerogenes** Rio-3 containing blalCTX-M-8** and bla**TEM-1**-resistance phenotype: ESBL, amikacin, gentamicin, kanamycin, netilmicin, tobramycin | This study          |
| pC1Rio-2                  | Recombinant plasmid containing a 10-kb EcoRI fragment with blalCTX-M-8 | This study          |

**FIG. 1.** Map of the EcoRI CTX-M-8-encoding insert of pC1Rio-2. Arrows indicate the strategy used to establish the nucleotide sequence.
Transfer of β-lactam resistance. Transconjugants TrRio-2 and TrRio-3 were only obtained from C. amalonaticus Rio-2 and E. aerogenes Rio-3 strains. They produced cefotaxime-hydrolyzing β-lactamase of pl 7.6, associated with the TEM-1 penicillinase. The CTX-M gene was carried by large plasmids (≥75 kb), which also harbored aminoglycoside resistance genes (Table 1).

Cloning of the β-lactamase gene. The genes carried by pRio-2 were cloned in plasmid pACYC184. Transformant C1Rio-2 contained recombinant plasmid pC1Rio-2 of 14 kb, producing only the CTX-M type β-lactamase of pl 7.6. The restriction map of the insert and hybridization with CTX-M type gene probe localized gene blaCTX-M close to the cloning site of pACYC184 (Fig 1).

β-Lactam susceptibility. MICs of β-lactams for C. amalonaticus Rio-2, its E. coli HB101 transconjugant TrRio-2 harboring plasmid pRio-2, and E. coli DH5α harboring recombinant plasmid pC1Rio-2 are listed in Table 2. These CTX-M-producing strains exhibited a high level of resistance to amoxicillin (MICs > 2,048 μg/ml), ticarcillin (MICs > 2,048 μg/ml), and cephalothin (MICs ≥ 1,024 μg/ml) and a low level of resistance to cefotaxime (MICs, 8 to 32 μg/ml). Unlike those for the C. amalonaticus Rio-2 isolate, MICs for E. coli transconjugant TrRio-2 and transformant C1Rio-2 of cefotaxime were 8- to 16-fold higher than those of ceftazidime and 2- to 4-fold higher than those of aztreonam. The same results were observed with strains Rio-1, Rio-3, and TrRio-3 (data not shown). MICs of cefepime and ceftirixone, even when they were weak, were appreciably higher than those obtained with TEM-1-producing E. coli (2 to 16 μg/ml versus 0.12 μg/ml). In contrast, the activities of imipenem and cefoxitin were not affected.

Clavulanate restored partially or totally the activities of the β-lactams. All strains were susceptible to associations of clavulanate and broad-spectrum cephalosporins (MICs, 0.06 to 1 μg/ml).

Kinetic parameters. The substrate and inhibition profiles of CTX-M-8 are shown in Table 3. The best affinities were observed with penicillins and cefuroxime (Km, 11 to 19 μM) and led to good catalytic efficiency. However, the best substrate of CTX-M-8 was cephalothin. The catalytic activity of the enzyme against cephalothin was 10-fold higher than that against penicillins despite the fact that cephalothin had higher Km values (Km, 87 μM).

Likewise, CTX-M-8 had activity against cefotaxime, cefepime, and ceftiraxone. The greater catalytic efficiency of CTX-M-8 against cefotaxime than against cefepime, and to lesser extent cephalothin, was due to the higher Km values for the last two substrates (cefepime Km, 990 μM; cephalothin Km, 1,200 μM; cefotaxime Km, 74 μM). In contrast, the catalytic activities for ceftazidime (relative Vmax ≤ 1%) and aztreonam (relative Vmax, 9%) were at least 49-fold and 5-fold lower, respectively, than that for cefotaxime (relative Vmax, 49%). Aztreonam and ceftazidime were poor substrates of CTX-M-8. Imipenem and cefoxitin were not affected by CTX-M-8.

### Table 2. β-Lactam MICs for the CTX-M-8-producing C. amalonaticus and its E. coli derivatives

| β-Lactam(s) | C. amalonaticus Rio-2 (pRio-2) | E. coli HB101 TrRio-2 (pRio-2) | E. coli DH5α C1Rio-2 (pC1Rio-2) | E. coli HB101 TR4 (pTR4) | E. coli DH5α |
|-------------|-------------------------------|-------------------------------|--------------------------------|--------------------------|-------------|
| Amoxicillin | >2,048 | >2,048 | >2,048 | >2,048 | 2 |
| Amoxicillin + ca | 64 | 16 | 32 | 16 | 1 |
| Ticarcillin | >2,048 | >2,048 | >2,048 | >2,048 | 2 |
| Cefotaxime | >1,024 | 1,024 | >1,024 | 256 | 4 |
| Cefoxitin | 4 | 2 | 4 | 2 | 2 |
| Cefotaxime + ca | 0.25 | 0.06 | 0.06 | 0.06 | 0.06 |
| Aztreonam | 64 | 4 | 8 | 0.25 | 0.12 |
| Cefepime | 8 | 2 | 4 | 0.12 | 0.06 |
| Cefepime + ca | 0.06 | 0.06 | 0.06 | 0.06 | 0.06 |
| Cefpirome | 16 | 4 | 8 | 0.12 | 0.06 |
| Cefpirome + ca | 0.12 | 0.06 | 0.06 | 0.06 | 0.06 |
| Imipenem | 0.12 | 0.25 | 0.25 | 0.12 | 0.06 |

### Table 3. Substrate profile of CTX-M-8 β-lactamase

| Substrate | Relative Vmax | Km (μM) | Relative Vmax/Km |
|-----------|---------------|---------|------------------|
| Benzylopenicillin | 100 | 11 | 100 |
| Amoxicillin | 37 | 12 | 34 |
| Ticarcillin | 12 | 14 | 10 |
| Piperacillin | 49 | 19 | 29 |
| Cephalothin | 1,070 | 87 | 137 |
| Cefuroxime | 25 | 12 | 23 |
| Cefotaxime | 49 | 74 | 7 |
| Cefpirome | 300 | 1,200 | 3 |
| Cefepime | 96 | 990 | 1 |
| Aztreonam | 9 | 800 | 0.12 |
| Ceftazidime | ≥1 | >500 | >0.02 |
| Cefoxitin | ND | 5 | ND |
| Imipenem | ND | 1 | ND |

* Values are percentages of the Vmax for benzylpenicillin.

* Values are percentages of the relative Vmax/Km ratio for benzylpenicillin.

* ND, no catalytic activity detected.
CTX-M-8 was susceptible to tazobactam (IC₅₀ 0.010 μM), clavulanate (IC₅₀ 0.036 μM), and sulbactam (IC₅₀ 4.0 μM).

DNA sequencing. The nucleotide sequence and the deduced amino acid sequence are given in Fig. 2. There was an open reading frame of 873 nucleotides. This coding region had 73 to 75% identity with the previously described CTX-M-type genes.

The initiation codon sequence was preceded by putative 235 (TTGAGA) and 210 (TTTTTT) consensus sequences. A terminator hairpin loop was detected 10 nucleotides from the stop codon (Fig. 2). Sequencing of CTX-M type genes of strains Rio-1 and Rio-3 confirmed that these strains harbored the same blaCTX-M gene.

The precursor amino acid sequence deduced from the nucleotide sequence consisted of 291 amino acid residues. On the basis of alignments with the CTX-M peptide sequence previously determined by direct amino acid sequencing (4, 27) (Fig. 3) and hydropathy plots, it is likely that the signal peptide comprised 28 amino acids. Thus, the putative mature CTX-M type enzyme consisted of 263 amino acid residues with a calculated isoelectric point of 7.72 and a calculated molecular weight of 28,039. The four structural elements characteristic of class A β-lactamases were found: S-X-X-K at positions 70 to 73, S-D-N at positions 130 to 132, E at position 166, and K-T-G at positions 234 to 236 (Fig. 2).

Homology with other β-lactamases. The sequence of the mature form of the CTX-M type enzyme has less than 37% amino acid identity with the sequences of TEM-1 and SHV-1 but 75 to 77% amino acid identity with the sequences of class A β-lactamases of P. vulgaris R0104 (31), S. fonticola CUV (30), C. diversus UL27 (32), K. oxytoca E23004 (2), and K. oxytoca D488 (33). The previously described CTX-M β-lactamases have 83 to 88% amino acid identity with this novel enzyme, which was designated CTX-M-8.

The peptide sequence alignment shown in Fig. 3 displays the conserved regions and the amino acid substitutions observed in different CTX-M enzymes. Excluding positions 185 to 219 of Toho-2, whose sequences have been discussed elsewhere (25), seven conserved regions were observed. Still excluding positions 186 to 218 of Toho-2, the alignments of CTX-M enzymes showed 72 polymorphic positions, of which 5 were reported solely in CTX-M-8 (positions 92, 103, 109, 158, and 197). Twelve amino acid residues of CTX-M-8 had not previously been observed in the other CTX-Ms (Fig. 3).

Phylogenetic analysis. A dendrogram (Fig. 4) was constructed on the basis of the peptide alignment shown in Fig. 3. Bootstrapping gave a high degree of resolution for internal nodes (greater than 50% majority consensus confidence) in all but...
hanced resistance to ceftazidime. However, such a mechanism in this species has not been reported.

In the course of cloning, a novel CTX-M type gene was characterized. The deduced enzyme, designated CTX-M-8, had 80 to 88% identity with previously described CTX-M enzymes. The conserved regions are known to have a critical role in the catalytic activity of active-site serine penicillin-

recognizing enzymes (23). In addition, CTX-M-8 harbors amino acid residues Ser-237 and Arg-276, which have been suspected of playing a part in the cephalosporin-hydrolyzing ac-
tivity of CTX-M enzymes (4, 14–16, 18, 27). The alignments of CTX-M enzymes showed the high level of polymorphism of the CTX-M family compared to those of the TEM and SHV fam-
ilies. The amino acid substitutions are generally conservative or semiconservative (43). Crystallographic data (18, 22) demon-
strate that the majority of the substitutions are localized far from the active site, in weakly conserved zones of class A β-lactamases. This explains the close similarities in the cata-
ytic activities of CTX-M enzymes.

The phylogenetic study of the CTX-M family showed four major types of CTX-M: the CTX-M-1 type, the CTX-M-2 type, Toho-1, and CTX-M-8. The evolutionary distances between the four types of CTX-M were large, suggesting that the four clusters of CTX-M branched off early from an unknown protein. Thus, these enzymes could be mutant derivatives from a distant common ancestor. Closely related enzymes of the CTX-M-1 type (M-1, M-3) and of the CTX-M-2 type (M-4, M-5, M-6, M-7) have been observed in a concentrated geographic area where they may have occurred as a result of point mutations, which suggests the existence of many unknown inter-
mediate enzymes. In contrast, CTX-M-2 and Toho-1, clas-
sified on the same branch of the CTX-M-2 cluster, have been characterized in geographically distant areas (Japan and South America). The expansion of the CTX-M family may therefore be due to the spread and mutations of the CTX-M-encoding genes, but independent genetic events cannot be excluded.

The catalytic properties of CTX-M-8 are close to those previ-
ously reported for CTX-M enzymes. Cephalothin is the best sub-
strate. Catalytic efficiency against cefotaxime is better than that against ceftazidime. CTX-M-8 is slightly more susceptible to the inhibitors than TEM penicillinases (10). Tazobactam is the best inhibitor, as previously described (5, 15, 16, 27).

The strains studied in this report were selected from 18 strains of the family Enterobacteriaceae chosen to characterize the different ESBLs present in Brazil. The majority of these strains (10 of 18) produced SHV type ESBLs. Two TEM type ESBLs were also identified. Four strains produced CTX-M type enzymes: E. cloacae Rio-1, C. amalonaticus Rio-2, and E. aerogenes Rio-3 (CTX-M-8) and P. mirabilis Rio-4 (CTX-
M-2). Another CTX-M-like enzyme produced by an E. coli strain and a broad-spectrum cephalosporin-hydrolyzing en-
zyme not related to the CTX-M, SHV, and TEM type enzymes is the best inhibitor, as previously described (5, 7, 11, 16, 27).

The starting point of this work was the observation of three clinical strains that exhibited a positive double-disk synergy test, resistance to broad-spectrum cephalosporins, and a β-lac-
tamase of pl 7.6. A distinctly higher level of resistance to cefotaxime than to ceftazidime was observed with the trans-
conjugants obtained. However, C. amalonaticus Rio-2, unlike transconjugant TrRio-2, exhibited an ESBL phenotype of the cefotaximase type. These differences of behavior between the wild-type strain Rio-2 and its transconjugant suggest an addi-
tional resistance mechanism directed mainly against ceftazi-
dime in C. amalonaticus Rio-2. A chromosomally mediated cephalosporinase, which has mainly cefotaximase activity, in C. amalonaticus has been previously reported (8), but no β-lac-
tamase of corresponding pl (5.5 and 6.05) was detected in our strain. Decreased permeability might also explain the en-

time. The distance along the vertical axis has no significance. *, previously designated CTX-M-5 (15).

one branch (49%). The dendrogram showed four major branches, whose members were closely related, separated by large evolutionary distances. Four types of CTX-M were ob-
tained: the CTX-M-1 type including CTX-M-1 and CTX-M-3; the CTX-M-2 type including CTX-M-2, Toho-1, CTX-M-5, CTX-M-4, CTX-M-6, and CTX-M-7; and Toho-2 and CTX-
M-8, both of which had only one member. Doubt over the length and also the position of the Toho-2 branch persists because of disagreements relating to its sequence (25).

**DISCUSSION**

The starting point of this work was the observation of three clinical strains that exhibited a positive double-disk synergy test, resistance to broad-spectrum cephalosporins, and a β-lactamase of pl 7.6. A distinctly higher level of resistance to cefotaxime than to ceftazidime was observed with the trans-
conjugants obtained. However, C. amalonaticus Rio-2, unlike transconjugant TrRio-2, exhibited an ESBL phenotype of the cefotaximase type. These differences of behavior between the wild-type strain Rio-2 and its transconjugant suggest an addi-
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tamase of corresponding pl (5.5 and 6.05) was detected in our strain. Decreased permeability might also explain the en-
ESBL-producing strains isolated in Brazil. Thus, like eastern Europe (11, 16, 17), South America could be an important source of CTX-M type β-lactamas. The spread of CTX-M enzymes and the paucity of TEM type mutants seem therefore to be a regional phenomenon in South America.

The first CTX-M-producing strains were sporadic isolates. Recently, outbreaks involving S. enterica serovar Typhimurium (11) and V. cholerae El Tor (Galas et al., 38th ICAAC, abstr. C-174) have been recently described. Nosocomial infections of the urinary tract induced by CTX-M-producing C. freundii have also been reported (17). In this study, E. aerogenes and E. cloaca, two species known to be responsible for nosocomial infections (3, 13) and isolated from patients hospitalized in separate intensive care units of different hospitals, produced CTX-M-8, suggesting that they are involved in nosocomial infections.

Since the first report of the MEN-1 (CTX-M-1) in the 1990s (4, 6), a great variety of CTX-M enzymes have been observed, all of which belong to a new group among the ESBL enzymes. Hence, CTX-M-8 constitutes a novel potential phylum of the CTX-Ms. The intensive use of broad-spectrum cephalosporins such as cefotaxime could account for the emergence and spread of the CTX-M plasmid-mediated enzymes among enteric pathogens. The analysis of a novel β-lactamase from patients infected with Salmonella typhimurium (11) and Vibrio cholerae C-174) have been recently described. Nosocomial infections of these organisms have also been reported (17). In this study, E. aerogenes and E. cloaca, two species known to be responsible for nosocomial infections (3, 13) and isolated from patients hospitalized in separate intensive care units of different hospitals, produced CTX-M-8, suggesting that they are involved in nosocomial infections.

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