

Sequences of β -Lactamase Genes Encoding CTX-M-1 (MEN-1) and CTX-M-2 and Relationship of Their Amino Acid Sequences with Those of Other β -Lactamases

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Amino acid sequences determined either by protein sequencing or by DNA sequencing are identical for cefotaximases CTX-M-1 and MEN-1, whereas CTX-M-2 is 84% identical to CTX-M-1/MEN-1. Both β -lactamases are distantly related to other plasmidic class A enzymes (homology to TEM-1 is 38.1% for CTX-M-1/MEN-1 and 36.5% for CTX-M-2); the closest relationship was with the chromosomal β -lactamase of *Klebsiella oxytoca* E23004 (homologies of 74.5% for CTX-M-1/MEN-1 and 77.9% for CTX-M-2). The cefotaximases CTX-M-1/MEN-1 and CTX-M-2 represent two members of a new subgroup of plasmidic class A β -lactamases.

Extended-spectrum (ES) β -lactamases have been classified by the relationships of their activities against ceftazidime and cefotaxime as ceftazidimases (MICs for ceftazidime higher than those for cefotaxime; e.g., TEM-5, TEM-6, TEM-7, and TEM-10) or cefotaximases (MICs for cefotaxime equal to MICs for ceftazidime; e.g., TEM-3 and TEM-4) (10). Recently, plasmidic ES β -lactamases which are much more active against cefotaxime than against ceftazidime were described, namely, the cefotaximases CTX-M-1 (5), MEN-1 (6), and CTX-M-2 (4). The determination of the amino acid sequence of the MEN-1 β -lactamase (3) and comparison of it with the amino acid sequences of known β -lactamases indicate that MEN-1 is not closely related to the known plasmidic ES β -lactamases but shows homology to chromosomal β -lactamases of *Klebsiella oxytoca* E23004 (2) and *K. oxytoca* D488 (13). The cefotaximase CTX-M-1, described in 1990 (5), has a phenotype very similar to that of MEN-1. The phenotype of CTX-M-2 is clearly different from those of MEN-1 and CTX-M-1 by its activity against cephalosporins (MICs mostly four to eight times higher) and its isoelectric point (7.9 for CTX-M-2 versus 8.9 for CTX-M-1). We therefore attempted to clarify the genetic relationship between CTX-M-1, MEN-1, and CTX-M-2 by cloning and sequencing their genes and analyzing the results for similarity.

Bacterial strains. Resistant wild-type strains were *Escherichia coli* GRI, isolated from exudate from the ear of a 4-month-old child suffering from otitis media in Weingarten, Germany (5); *E. coli* MEN-1, isolated by C. Tancrede, Institut Gustave Roussy, Villejuif, France, from a patient from Italy (6); and *Salmonella typhimurium* CAS-5, isolated from the feces of a 16-month-old child suffering from enteritis in Argentina (4). The recipient strain was *E. coli* C600, which is resistant to nalidixic acid.

Vectors. pSelect and pBluescript were obtained from Stratagene (Heidelberg, Federal Republic of Germany [FRG]).

Antibiotics. The following antibiotics were obtained from their manufacturers: cefotaxime (Hoechst, Frankfurt, FRG), ceftazidime (Cascan, Wiesbaden, FRG), and clavulanic acid (SmithKline Beecham, Munich, FRG).

Plasmid DNA preparation. Plasmid DNA was released from

the cells by alkaline lysis (7). The DNA was purified with an anion-exchange column according to the recommendations of the manufacturer (Qiagen, Hilden, FRG).

Cloning and sequencing of the *bla*_{CTX-M-2} gene. Cloning was performed by standard procedures (15). All enzymes were purchased from Boehringer (Mannheim, FRG). The resistance plasmids carrying the *bla*_{CTX-M-1} (pMVP-3) and the *bla*_{CTX-M-2} (pMVP-4) genes were prepared from the *E. coli* C600 transconjugant strains. Digestion of the plasmids, ligation into vector pSelect or pBluescript, and transformation of *E. coli* DH5 α resulted in cefotaxime-resistant transformants. Further analysis of the recombinant *E. coli* strains revealed a 3.5-kb *Pst*I fragment containing the *bla*_{CTX-M-1} gene cloned into vector pSelect (pMVP-3-1) and a 2.2-kb *Hind*III fragment containing the *bla*_{CTX-M-2} gene in vector pBluescript (pMVP-4-1). Sequencing was performed with consecutive primers for both strands and an automatic sequencer (Applied Biosystems, Weiterstadt, FRG). Within the inserts of the recombinant plasmid, only the region containing the *bla*_{CTX} genes was sequenced. Since a relatively close relationship of CTX-M-1 and CTX-M-2 to MEN-1 was expected, the sequence of the first pair of primers was chosen on the basis of the amino acid sequence of MEN-1 (3) and the DNA sequence of its closest relative, the chromosomal *bla* gene of *K. oxytoca* E23004 (2). By using these two primers, a PCR was performed with a relatively low annealing temperature (55°C) (1 min). The resulting PCR product of 605 bp was sequenced and used for selection of the following primers.

Sequence analysis. Sequencing was stopped as soon as the information about the complete open reading frames of the two *bla* genes had been obtained. Related β -lactamases were identified by comparison with the EMBL and SwissProt databases (Fasta). Multiple alignment was performed with Clustal V (8, 9).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the EMBL database under accession numbers X92506 for *bla*_{CTX-M-1} and X92507 for *bla*_{CTX-M-2}.

Sequence analysis. The nucleotide sequences of both the *bla*_{CTX-M-1} and *bla*_{CTX-M-2} genes had not been previously determined. Our sequence data indicate an open reading frame of 876 bp, corresponding to 291 amino acids, for both CTX-M-1 (Fig. 1) and CTX-M-2 (Fig. 2). Comparison of the nucle-

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TTTTA ATG ATG ACT CAG AGC ATT CGC CGC TCA ATG TTA ACG GTG ATG GCG ACG CTA CCC CTG CTA TTT AGC
      M  M  T  Q  S  I  R  R  S  M  L  T  V  M  A  T  L  P  L  L  F  S

AGC GCA ACG CTG CAT GCG CAG GCG AAC AGC GTG CAA CAG CAG CTG GAA GCC CTG GAG AAA AGT TCG GGA GGT
S  A  T  L  H  A ↓ Q  A  N  S  V  Q  Q  Q  L  E  A  L  E  K  S  S  G  G
      20                                30                                40

CGG CTT GGC GTT GCG CTG ATT AAC ACC GCC GAT AAT TCG CAG ATT CTC TAC CGT GCC GAT GAA CGT TTT GCG
R  L  G  V  A  L  I  N  T  A  D  N  S  Q  I  L  Y  R  A  D  E  R  F  A
      50                                60

ATG TGC AGT ACC AGT AAG GTG ATG GCG GCC GCG GCG GTG CTT AAA CAG AGC GAG AGC GAT AAG CAC CTG CTA
M  C  S  T  S  K  V  M  A  A  A  A  V  L  K  Q  S  E  S  D  K  H  L  L
      70                                80                                90

AAT CAG CGC GTT GAA ATC AAG AAG AGC GAC CTG GTT AAC TAC AAT CCC ATT GCG GAG AAA CAC GTT AAC GGC
N  Q  R  V  E  I  K  K  S  D  L  V  N  Y  N  P  I  A  E  K  H  V  N  G
      100                                110

ACG ATG ACG CTG GCT GAG CTT GGC GCA GCG GCG CTG CAG TAT AGC GAC AAT ACT GCC ATG AAT AAG CTG ATT
T  M  T  L  A  E  L  G  A  A  A  L  Q  Y  S  D  N  T  A  M  N  K  L  I
      120                                130

GCC CAT CTG GGT GGT CCC GAT AAA GTG ACG GCG TTT GCT CGC TCG TTG GGT GAT GAG ACC TTC CGT CTG GAC
A  H  L  G  G  P  D  K  V  T  A  F  A  R  S  L  G  D  E  T  F  R  L  D
      140                                150                                160

AGA ACC GAG CCC ACG CTC AAT ACC GCC ATT CCA GGC GAC CCG CGT GAT ACC ACC ACG CCG CTC GCG ATG GCG
R  T  E  P  T  L  N  T  A  I  P  G  D  P  R  D  T  T  T  P  L  A  M  A
      170                                180

CAG ACC CTG AAA AAT CTG ACG CTG GGT AAA GCG CTG GCG GAA ACT CAG CGG GCA CAG TTG GTG ACG TGG CTT
Q  T  L  K  N  L  T  L  G  K  A  L  A  E  T  Q  R  A  Q  L  V  T  W  L
      190                                200                                210

AAG GGC AAT ACT ACC GGT AGC GCG AGC ATT CGG GCG GGT CTG CCG AAA TCA TGG GTA GTG GGC GAT AAA ACC
K  G  N  T  T  G  S  A  S  I  R  A  G  L  P  K  S  W  V  V  G  D  K  T
      220                                230

GGC AGC GGA GAT TAT GGC ACC ACC AAC GAT ATC GCG GTT ATC TGG CCG GAA AAC CAC GCA CCG CTG GTT CTG
G  S  G  D  Y  G  T  T  N  D  I  A  V  I  W  P  E  N  H  A  P  L  V  L
      240                                250                                260

GTG ACC TAC TTT ACC CAA CCG GAG CAG AAG GCG GAA AGC CGT CGG GAT ATT CTG GCT GCG GCG GCG AAA ATC
V  T  Y  F  T  Q  P  E  Q  K  A  E  S  R  R  D  I  L  A  A  A  A  K  I
      270                                280

GTA ACC CAC GGT TTC TGA TGCAATAAATGGAGCGAGGTATCGCTCCA
V  T  H  G  F
      290

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FIG. 2. Nucleotide sequence of the *bla*_{CTX-M-2} gene (pMVP-4-1). The deduced amino acid sequence of CTX-M-2 is shown below the nucleotide triplets. The putative cleavage site of the signal peptidase is marked by a vertical arrow. A terminator hairpin following the stop codon is marked by horizontal arrows.

cefotaximases were found in *K. oxytoca* D488 (77.2% homology to CTX-M-2 and 72.6% homology to CTX-M-1/MEN-1) and in *Citrobacter diversus* ULA27 (75.3% homology to CTX-M-2 and 73.4% homology to CTX-M-1/MEN-1).

As expected from the results for MEN-1 (3), CTX-M-2 also is not closely related to TEM or SHV β -lactamases (homology between TEM-1 and CTX-M-2, 36.5%; homology between SHV-1 and CTX-M-2, 37.5%) (Table 1).

The plasmidic β -lactamases CTX-M-1 and CTX-M-2 were initially characterized as cefotaximases because of their higher activities against cefotaxime than against ceftazidime as expressed by MICs (MIC for cefotaxime of *E. coli* GRI *bla*_{CTX-}

M-1 32 times greater than that for ceftazidime; MIC of *S. typhimurium* CAS-5 *bla*_{CTX-M-2} for cefotaxime 16 times greater than that for ceftazidime) and higher relative V_{\max} s (cephaloridine = 100) for cefotaxime in comparison with ceftazidime (12.5 versus 0.02 for CTX-M-1; 14 versus 0.04 for CTX-M-2) (4). This indicates a substrate profile different from those of TEM- or SHV-derived ES β -lactamases; most of them are more active against ceftazidime than against cefotaxime, while only a few (e.g., TEM-3 and TEM-4) show about equal activities against ceftazidime and cefotaxime (10). Other characteristics, e.g., a large reduction of activity by clavulanate (by factors of 32 or 64 for CTX-M-1 or CTX-M-2, respectively) or the

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                                     30          40
CTX-M2      MMTQSIRRSM LTVMATLPLLFSSATLHAQANS----VQQQLEALEKSSGG
CTX-M1/MEN-1 MVKKS L RQFTLMATATV TLL LGSVPLYAQTAD----VQQKLAELERQSGG
KOX_E23004  MLKSSWRKTALMAAAVPLLLASGSLWASADA----IQQKLADLEKRS GG
KOX_D488    -----STDA----IHQKLTDL EKRS GG
CDIV_ULA27  MFKKRGRQTVL-IAAVLAFFTASSPLLARTQGEPTQVQQKLAALEKQSGG
                                     * * ** ***

                                     Element 1
                                     50          60          70          80          90
CTX-M2      RLGVALINTADNSQILYRADERFAMCSTSKVMAAAAVLKQSESDKHL LNQ
CTX-M1/MEN-1 RLGVALINTADNSQILYRADERFAMCSTSKVMAVAAVLKKSESEPNLLN Q
KOX_E23004  RLGVALINTADDSQTLYRGDERFAMCSTGKVMAAAAVLKQSESNPEVVNK
KOX_D488    RLGVALINTADNSQILYRGDERFAMCSTSKVMAAAAVLKQSESNKEVVNK
CDIV_ULA27  RLGVALINTADRSQILYRGDERFAMCSTSKTMVA AAVLKQSETQHDILQQ
***** ** *** ***** * * ***** **

                                     Element 2
                                     100         110         120         130         140
CTX-M2      RVEIKKSDLVNYPNIAEKHVNGTMTLAELGAAALQYSDNTAMNKLI AHLG
CTX-M1/MEN-1 RVEIKKSDLVNYPNIAEKHVDGTMSLAELSAALQYSDNVAMNKLI SHVG
KOX_E23004  RLEIKKSDLVVWSPITEKHLQSGMTLAELSAALQYSDNTAMNKMISYLG
KOX_D488    RLEINAADLVVWSPITEKHLQSGMTLAELSAATLQYSDNTAMNLI IGYLG
CDIV_ULA27  KMV IKKADLTNWNPVTEKYVDKEMTLAELSAATLQYSDNTAMNKLLEHLG
               * ** * ** * ***** ** ***** ** *

                                     Element 3
                                     150         160         170         180         190
CTX-M2      GPDKVTAFARSLGDETFR LDRTEPTLNTAIPGDPRDTTTP LAMAQTLKNL
CTX-M1/MEN-1 GPASVTAFARQLGDETFR LDRTEPTLNTAIPGDPRDTTSP RAMAQTLRNL
KOX_E23004  GPEKVTAFQAQSIGDVTFR LDRTEPALNSAIPGDKRDTTTP LAMAESLRKL
KOX_D488    GPEKVTAFARSIGDATFR LDRTEPTLNTAIPGDERDTSTP LAMAESLRKL
CDIV_ULA27  GTSNVTAFARSIGDTFR LDRKEPELNTAIPGDERDTTC PLAMAKSLHKL
               * ***** ** ***** ** ** ***** ** * ** * *

                                     Element 4
                                     200         210         220         230         240
CTX-M2      TLGKALAE TQRAQLVTWLKGN TTGSASIRAGLPKSWVVGDKTGSGDYGTT
CTX-M1/MEN-1 TLGKALGDSQRAQLVTW MKGN TTGAASI QAGLPASWVVGDKTGSGDYGTT
KOX_E23004  TLGNALGEQQRAQLVTWLKGN TTGGQSI RAGLPASWAVGDKTGAGDYGTT
KOX_D488    TLGNALGEQQRAQLVTWLKGN TTGGQSI RVGLPESWVVGDKTGAGDYGTT
CDIV_ULA27  TLGDALAGA QRAQLVEWLKGN TTGGQSI RAGLPEGWVVGDKTGAGDYGTT
               *** ** ***** * ***** ** *** * ***** *****

                                     250         260         270         280         290
CTX-M2      NDIAVIWPENHAPLVLV TYFTQPEQKAESRRDILAAA KIVTHGF
CTX-M1/MEN-1 NDIAVIWPKDRAPLILV TYFTQPQPKAESRRDVLASA AKIVTNGL
KOX_E23004  NDIAVIWPENHAPLVLV TYFTQPQDAKSRKEVLAAA AKIVTEGL
KOX_D488    NDIAVIWPENHAPLVLV TYFTQPQDAKNRKEVLAAA AKIVTEGL
CDIV_ULA27  NDIAVIWPEDRAPLILV TYFTQPQDAKGRKDI LAAA AKIVTEGL
               ***** ** ***** * * ** ***** *

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FIG. 3. Multiple sequence alignment of the amino acid sequences of CTX-M-2, CTX-M-1/MEN-1, and related chromosomal β -lactamases of *K. oxytoca* (KOX) E23004 (2), *K. oxytoca* D488 (13), and *C. diversus* (CDIV) ULA27 (12). Amino acids identical in all five sequences are marked by asterisks. Numbering is according to Ambler (1). Elements 1 to 4 (marked by boldface) are conserved residues of Ambler class A β -lactamases which surround the active site (11).

isoelectric points of the CTX-M enzymes (8.9 for CTX-M-1 and 7.9 for CTX-M-2), appear compatible with a possible relationship to SHV-type ES β -lactamases.

Our nucleotide sequencing of the *bla*_{CTX-M-1} gene con-

firmed (100% identity) the amino acid sequence of MEN-1 obtained by direct amino acid sequencing of the protein. These data show that although CTX-M-1 represents an Ambler class A enzyme, it has an amino acid sequence very different from

TABLE 1. Homologies of amino acid sequences of various mature class A β -lactamases^a

β -Lactamase	% Homology with:						
	CTX-M-2	CTX-M-1/ MEN-1	<i>K. oxytoca</i> E23004 β -lactamase	<i>K. oxytoca</i> D488 β -lactamase	<i>C. diversus</i> ULA27 β -lactamase	TEM-1	SHV-1
CTX-M-2		84.0	77.9	77.2	75.3	36.5	37.5
CTX-M-1/MEN-1			74.5	72.6	73.4	38.1	38.3
<i>K. oxytoca</i> E23004				90.9	75.7	40.0	38.3
<i>K. oxytoca</i> D488					76.8	39.6	37.5
<i>C. diversus</i> ULA27						38.7	37.4
TEM-1							66.5

^a Determined by the neighbor-joining method (14).

those of TEM or SHV β -lactamases, as already pointed out by Barthélémy et al. (3). Thus, the question remained as to whether the ES β -lactamase initially characterized as CTX-M-2 by its phenotypic similarity to CTX-M-1 represents a second member of this new subgroup of plasmidic β -lactamases genotypically as well.

Our sequencing data for CTX-M-2 demonstrate that this enzyme has an amino acid sequence similar, but not identical, to that of CTX-M-1/MEN-1 (identity of 84% for the mature protein). At this time it cannot be excluded that the phenotypic differences between the two cefotaximases may be attributed to greater transcription of the *bla*_{CTX-M-2} gene in comparison with that of the *bla*_{CTX-M-1} gene because of differences in their promoter sequences. However, the relationship between these two β -lactamases and other enzymes of Ambler class A is distant enough for their classification as a new subgroup of plasmidic enzymes within this class. Other β -lactamases related to CTX-M-1/MEN-1 and CTX-M-2 are found in various members of the family *Enterobacteriaceae* (*K. oxytoca* E23004, *K. oxytoca* D488, and *C. diversus* ULA27); however, none of these enzymes was reported to be located on a plasmid.

Although CTX-M-1/MEN-1 and CTX-M-2 are closely related by their amino acid sequences, the difference of 16% is still too high to consider the possibility of direct descent from one to the other (or from the β -lactamase of *K. oxytoca* E23004, *K. oxytoca* D488, or *C. diversus* ULA27). Thus, there is no evident phylogenetic connection between the CTX-M cluster and other clusters of *bla* genes.

The CTX-M-1- and MEN-1-producing pathogens appeared at about the same time (1989 and 1990, respectively) at two different locations (Germany and Italy, respectively) in *E. coli* strains. Meanwhile, two other CTX-M-1-producing *E. coli* strains were isolated in Germany (Munich and Weingarten, both in 1994). In addition to the initial finding in an *S. typhimurium* strain from Argentina (1992), the CTX-M-2 β -lactamase was found in *Klebsiella pneumoniae* from Israel (1992), in *K. pneumoniae* from Paraguay (1994), and in *E. coli* and *Proteus mirabilis* from Argentina (1994) (unpublished data). These observations indicate the presence of the *bla*_{CTX} genes in various species of the family *Enterobacteriaceae* over a period of at least 5 years in geographically widely distant areas. Ongoing selective pressure due to therapy of bacterial infections by

β -lactams susceptible to the cefotaximases may be expected to favor the persistence and spread of the *bla*_{CTX} genes around the world.

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