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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. Tancrède, 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* DH5α 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 *Hin*dIII 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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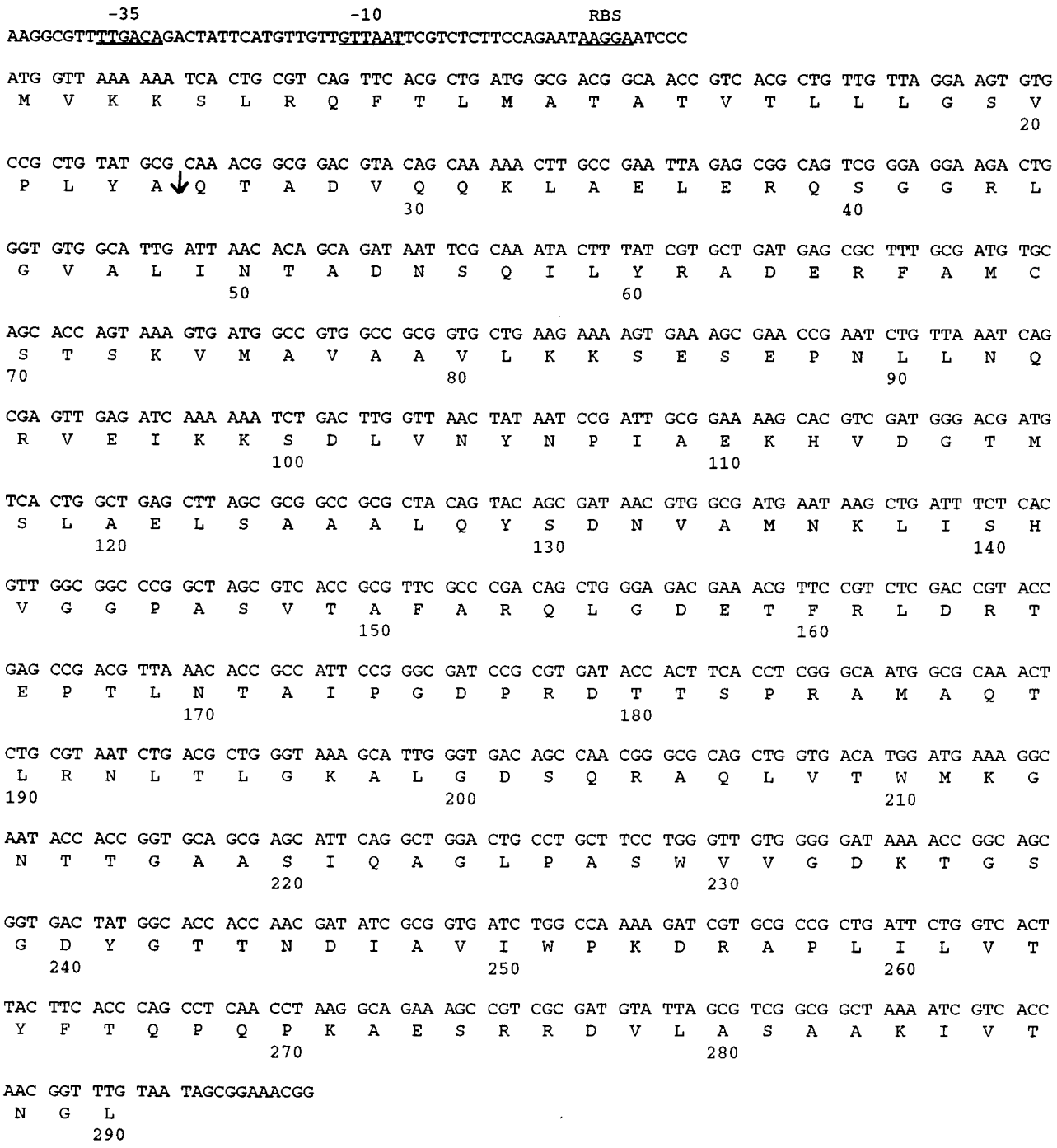

FIG. 1. Nucleotide sequence of the $bla_{CTX-M-1}$ gene (pMVP-3-1). The deduced amino acid sequence of CTX-M-1 is shown below the nucleotide triplets. The putative cleavage site of the signal peptidase is marked by an arrow. Possible promoter sequences (−35 and −10) and a ribosome binding site (RBS) upstream of the start codon are underlined.

otide and deduced amino acid sequences with known β-lactamase sequences (EMBL and SwissProt databases) revealed a relationship to β-lactamases of Ambler class A (Fig. 3). The four conserved motifs characteristic of class A β-lactamases (11), including an active-site serine at position 70, were detectable in both CTX-M-1 and CTX-M-2.

The amino acid sequence of CTX-M-1 deduced from its nucleotide sequence was completely identical to the sequence described for MEN-1 as determined by direct amino acid sequencing of the protein (3). The total amino acid sequence of CTX-M-2 differed from that of CTX-M-1 or MEN-1 by 20.3%. For the mature protein, the difference was only 16%. The chromosomal β-lactamase of *K. oxytoca* E23004, which was described as the closest relative to MEN-1, is slightly more closely related to CTX-M-2 (77.9%) than to CTX-M-1/MEN-1 (74.5%) (Table 1). Other β-lactamases closely related to the

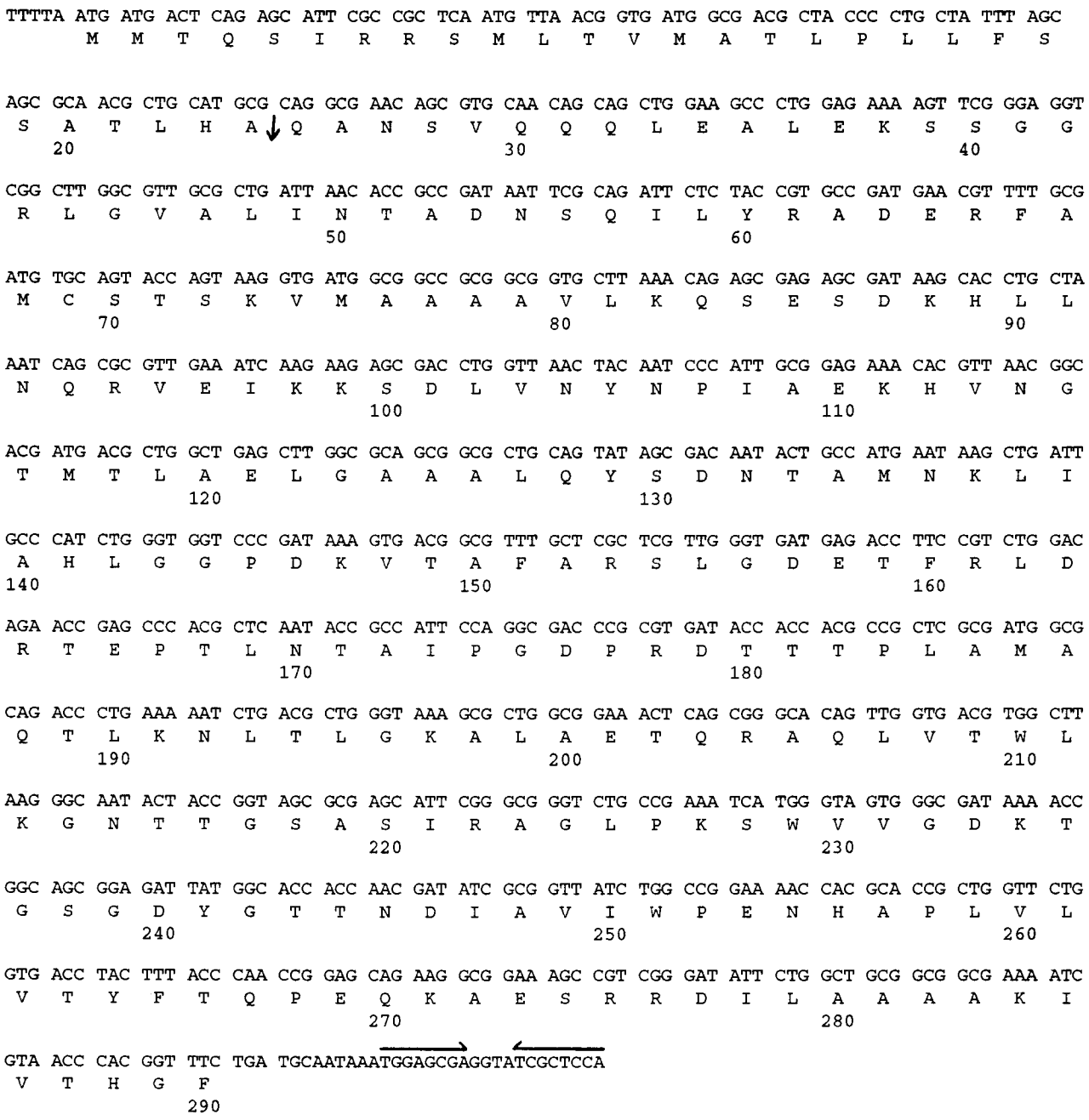

FIG. 2. Nucleotide sequence of the $bla_{CTX\text{-}M\text{-}2}$ gene (pMVP-4-1). The deduced amino acid sequence of CTX-M-2 is shown below the nucleotide triplets. The putative cleavage site of the signal peptidase is marked by a vertical arrow. A terminator hairpin following the stop codon is marked 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 $bla_{CTX\text{-}M\text{-}1}$ 32 times greater than that for ceftazidime; MIC of *S. typhimurium* CAS-5 $bla_{CTX\text{-}M\text{-}2}$ 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          MMTQSIRRSMLTVMATLPLLFSSATLHAQANS----VQQQLEALEKSSGG
CTX-M1/MEN-1    MVKKSLRQFTLMATATVTLLLGSVPLYAQTAD----VQQKLAELERQSGG
KOX_E23004      MLKSSWRKTALMAAAAVPLLLASGSLWASADA----IQQKLADLEKRSGG
KOX_D488        ----------------------------STDA----IHQKLTDLEKRSGG
CDIV_ULA27      MFKKRGRQTVL-IAAVLAFFTASSPLLARTQGEPTQVQQKLAALEKQSGG
                                                      * *  **  ***

                                          Element 1
                     50        60        70        80        90
CTX-M2          RLGVALINTADNSQILYRADERFAMCSTSKVMAAAAVLKQSESDKHLLNQ
CTX-M1/MEN-1    RLGVALINTADNSQILYRADERFAMCSTSKVMAVAAVLKKSESEPNLLNQ
KOX_E23004      RLGVALINTADDSQTLYRGDERFAMCSTGKVMAAAAVLKQSESNPEVVNK
KOX_D488        RLGVALINTADNSQILYRGDERFAMCSTSKVMAAAAVLKQSESNKEVVNK
CDIV_ULA27      RLGVALINTADRSQILYRGDERFAMCSTSKTMVAAAVLKQSETQHDILQQ
                ********** ** *** ********* * *  ***** **

                                                  Element 2
                    100       110       120       130       140
CTX-M2          RVEIKKSDLVNYNPIAEKHVNGTMTLAELGAAALQYSDNTAMNKLIAHLG
CTX-M1/MEN-1    RVEIKKSDLVNYNPIAEKHVDGTMSLAELSAAALQYSDNVAMNKLISHVG
KOX_E23004      RLEIKKSDLVVWSPITEKHLQSGMTLAELSAAALQYSDNTAMNKMISYLG
KOX_D488        RLEINAADLVVWSPITEKHLQSGMTLAELSAATLQYSDNTAMNLIIGYLG
CDIV_ULA27      KMVIKKADLTNWNPVTEKYVDKEMTLAELSAATLQYSDNTAMNKLLEHLG
                   *   **    *  **     * **** ** ****** ***      *

                                     Element 3
                    150       160       170       180       190
CTX-M2          GPDKVTAFARSLGDETFRLDRTEPTLNTAIPGDPRDTTTPLAMAQTLKNL
CTX-M1/MEN-1    GPASVTAFARQLGDETFRLDRTEPTLNTAIPGDPRDTTSPRAMAQTLRNL
KOX_E23004      GPEKVTAFAQSIGDVTFRLDRTEPALNSAIPGDKRDTTTPLAMAESLRKL
KOX_D488        GPEKVTAFARSIGDATFRLDRTEPTLNTAIPGDERDTSTPLAMAESLRKL
CDIV_ULA27      GTSNVTAFARSIGDTTFRLDRKEPELNTAIPGDERDTTCPLAMAKSLHKL
                *   *****   ** ****** ** ** ***** ***  * ***  *  *

                                                 Element 4
                    200       210       220       230       240
CTX-M2          TLGKALAETQRAQLVTWLKGNTTGSASIRAGLPKSWVVGDKTGSGDYGTT
CTX-M1/MEN-1    TLGKALGDSQRAQLVTWMKGNTTGAASIQAGLPASWVVGDKTGSGDYGTT
KOX_E23004      TLGNALGEQQRAQLVTWLKGNTTGGQSIRAGLPASWAVGDKTGAGDYGTT
KOX_D488        TLGNALGEQQRAQLVTWLKGNTTGGQSIRVGLPESWVVGDKTGAGDYGTT
CDIV_ULA27      TLGDALAGAQRAQLVEWLKGNTTGGQSIRAGLPEGWVVGDKTGAGDYGTT
                *** **   ****** * ******  **  ***  * ****** ******

                   250       260       270       280       290
CTX-M2          NDIAVIWPENHAPLVLVTYFTQPEQKAESRRDILAAAAKIVTHGF
CTX-M1/MEN-1    NDIAVIWPKDRAPLILVTYFTQPQPKAESRRDVLASAAKIVTNGL
KOX_E23004      NDIAVIWPENHAPLVLVTYFTQPQQDAKSRKEVLAAAAKIVTEGL
KOX_D488        NDIAVIWPENHAPLVLVTYFTQPQQDAKNRKEVLAAAAKIVTEGL
CDIV_ULA27      NDIAVIWPEDRAPLILVTYFTQPQQDAKGRKDILAAAAKIVTEGL
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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 $bla_{\text{CTX-M-1}}$ gene confirmed (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	*K. oxytoca* E23004 β-lactamase	*K. oxytoca* D488 β-lactamase	*C. diversus* 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
K. oxytoca E23004				90.9	75.7	40.0	38.3
K. oxytoca D488					76.8	39.6	37.5
C. diversus 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 $bla_{\text{CTX-M-2}}$ gene in comparison with that of the $bla_{\text{CTX-M-1}}$ 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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