ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 2005, p. 1567–1571 Vol. 49, No. 4
0066-4804/05/$08.00+0 doi:10.1128/AAC.49.4.1567–1571.2005

CTX-M-10 Linked to a Phage-Related Element Is Widely Disseminated among *Enterobacteriaceae* in a Spanish Hospital

Antonio Oliver,[1]* Teresa M. Coque,[2] Diana Alonso,[1] Aránzazu Valverde,[2] Fernando Baquero,[2] and Rafael Cantón[2]

Servicio de Microbiología, Hospital Universitario Son Dureta, Palma de Mallorca,[1] and Servicio de Microbiología, Hospital Universitario Ramón y Cajal, Madrid,[2] Spain

Received 17 August 2004/Returned for modification 22 November 2004/Accepted 17 December 2004

CTX-M-10 has been widely disseminated among multiple clones of several species of *Enterobacteriaceae*, harboring seemingly different plasmids, for over a decade in Ramón y Cajal University Hospital, Madrid, Spain. Cloning and sequencing of a 12.2-kb DNA fragment from plasmid pRYCE21 from *Klebsiella pneumoniae* strain KP4aC revealed a novel phage-related element immediately upstream of $bla_{CTX-M-10}$ conserved among different CTX-M-10-producing strains. This is the first report showing an extended-spectrum-β-lactamase gene linked to a phage-related element.

In 1989 a new group of extended-spectrum β-lactamases (ESBL), not related to TEM or SHV enzymes, was described in Germany and was designated CTX-M-1 due to its preferential hydrolysis of cefotaxime (3). Simultaneously, another CTX-M enzyme, later called CTX-M-2, was found to be widely disseminated among *Salmonella* strains in Argentina (4). Nevertheless, the first-published CTX-M β-lactamase was actually FEC-1, described in Japan in 1986 (18), which was later found to be almost identical to CTX-M-3, characterized in Poland in 1996 (12), when both nucleotide sequences were available (5). In the last few years, there has been an explosive dissemination of CTX-M enzymes, and today they are probably the most widespread ESBL group (5).

Five different major groups of plasmid-mediated CTX-M β-lactamases have been recognized so far, all of which share high degrees of homology with the chromosomal β-lactamases of several species of the genus *Kluyvera* (5). Chromosomal β-lactamases of *Kluyvera ascorbata* and *Kluyvera georgiana* are almost identical to the plasmid-mediated enzymes from the CTX-M-2 and CTX-M-8 groups, respectively, whereas that of *Kluyvera cryocrescens* is closely related to those of the CTX-M-1 group, although it does not seem to be its direct ancestor (10, 15, 24). The surprisingly rapidly increasing recognition of clinical isolates containing different CTX-M β-lactamases worldwide has led to a growing interest in the investigation of the genetic elements responsible for their explosive emergence and spread. IS*Ecp1*-like insertion sequences have been frequently found upstream of several bla_{CTX-M} genes from different groups and from different geographical origins, and it is believed that they might play a role in CTX-M β-lactamase mobility and expression (8, 11, 16, 25, 29). Genes encoding CTX-M-9 and CTX-M-2 have also been found as part of class 1 integrons containing open reading frame 513 (ORF513) (1, 28).

CTX-M-10 was initially described in 2001 to occur in an *Escherichia coli* strain isolated in 1997 at Ramón y Cajal University Hospital in Madrid, Spain (20). Long-term molecular epidemiology studies of ESBL-producing *Enterobacteriaceae* in this institution revealed that this enzyme, present since at least 1990, was widely disseminated among strains of *E. coli*, *Klebsiella pneumoniae*, and different species of the genus *Enterobacter* (7, 9; T. M. Coque, M. C. Varela, A. Oliver, M. I. Morosini, F. Baquero, and R. Cantón, Abstr. 41st Intersci. Conf. Antimicrob. Agents Chemother., abstract C2-298, p. 118, 2001). Since $bla_{CTX-M-10}$ had been detected for over a decade in multiple clones of these species and was found to be harbored by seemingly different transferable plasmids by restriction fragment length polymorphism analysis (7, 9), we decided to elucidate whether there was a common genetic environment that could explain the efficient dissemination of this gene among different plasmids and/or bacterial organisms in our institution.

The *K. pneumoniae* clinical strain KP4aC containing $bla_{CTX-M-10}$ in a 60-kb plasmid (pRYCE21) (9), recovered from a urine sample in 1997, was used to characterize the genetic environment of this gene. Plasmid pRYCE21 was transferred to a rifampin-resistant mutant of the *E. coli* laboratory strain BM21 (BM21R) by conjugation with the filter mating method. Transconjugants were selected on MacConkey agar plates containing rifampin (100 μg/ml) plus cefotaxime (2 μg/ml). Plasmid pRYCE21 was isolated from a BM21R transconjugant with the Plasmid Midi Kit (QIAGEN, Hilden, Germany). Analysis of pRYCE21 DNA digested with BamHI or EcoRI showed the presence of three BamHI fragments (360 bp, 8.5 kb, and >40 kb) and 10 EcoRI fragments (ranging from 500 bp to 20 kb). DNA from pRYCE21 digested with either EcoRI or BamHI was ligated to pBGS18$^-$ (31), digested with the same enzymes, by using T4 DNA ligase at 16°C overnight. Recombinant plasmids were transformed into the *E. coli* strain XL1-Blue made competent with $CaCl_2$ and were further se-

* Corresponding author. Mailing address: Servicio de Microbiología, Hospital Son Dureta, C. Andrea Doria No. 55, 07014 Palma de Mallorca, Spain. Phone: 34 971 175 185. Fax: 34 971 175 185. E-mail: aoliver@hsd.es.

TABLE 1. Primers used for PCR amplification of different regions of the 12.2-kb DNA fragment containing $bla_{CTX\text{-}M\text{-}10}$ from plasmid pRYCE21

Primer	Sequence (5′→3′)	Positions	PCR amplification	PCR product size (kb)
RYCE21-F1	CAGGACGCGGTATCACC	335–351		
RYCE21-R1	GGCTGGGATGTCGCGTAAC	1785–1767	RYCE21-F1–RYCE21-R1	1.5
RYCE21-F2	GACATTTCCATCGAAGAGCC	2039–2058		
RYCE21-R2	GCCGAGCGGATTAATCAGG	4032–4014	RYCE21-F2–RYCE21-R2	2.0
RYCE21-F3	CCCATGAGCCCGCTTACG	3880–3897		
RYCE21-R3	GAGCCACAAAGTGTAGCGC	4944–4936	RYCE21-F3–RYCE21-R3	2.1
CTX-M-F8	CCGCGCTACACTTTGTGGC	4924–4942		
CTX-M-R3	TTACAAACCGTCGGTGACG	5885–5867	CTX-M-F8–CTXM-R3	1.0
RYCE21-F4	CCCAACCTAAGGCAGAAAG	5815–5833		
RYCE21-R4	ATCGACAAGGTCATGCTGATG	6425–6405	RYCE21-F4–RYCE21-R4	0.6
RYCE21-R5	CCATGCTGTTTTCCGTAGTAC	6828–6808	RYCE21-F4–RYCE21-R5	1.0
RYCE21-R6	CTCGCTTACTAATTCCCAGC	8030–8011	RYCE21-F4–RYCE21-R6	2.2
RYCE21-R7	GGGTTCTGTCACCCTGAC	8551–8534	RYCE21-F4–RYCE21-R7	2.7
RYCE21-F5	GCTGAATGTGGACTATGTC	8922–8940		
RYCE21-R8	GCACGATCATTCCTGATAC	11523–11505	RYCE21-F5–RYCE21-R8	2.6

lected in MacConkey agar plates containing 50 μg of kanamycin per ml with and without 2 μg of cefotaxime per ml. Transformants were checked by plasmid extraction and digestion with restriction enzymes to obtain a genetic library containing all 10 EcoRI fragments and the 360-bp and the 8.5-kb BamHI fragments described above (we failed to obtain a recombinant plasmid harboring the >40-kb BamHI fragment). Transformants selected on MacConkey agar plates containing kanamycin (50 μg/ml) plus cefotaxime (2 μg/ml) were used to select bacterial colonies containing the $bla_{CTX\text{-}M\text{-}10}$ gene, which was found to be present in 7.2-kb EcoRI and 8.5-kb BamHI DNA fragments. The different EcoRI or BamHI DNA fragments were overlapped by PCR using specific primers from the ends of the sequences. A 12.2-kb region surrounding the $bla_{CTX\text{-}M\text{-}10}$ gene was then fully sequenced. Comparisons of DNA and amino acid sequences were carried out with BLASTN and BLASTP programs, available at www.ncbi.nlm.nih.gov/BLAST. Multiple sequence alignments were done using the Clustal W 1.8 program (32) available at www.infobiogen.fr. Additionally, specific sets of primers (Table 1) were used to amplify this 12.2-kb region in two additional strains of *K. pneumoniae* (KP30C and KP36C) (9), three *E. coli* strains (EC16, EC22, and EC54) (T. M. Coque, M. C. Varela, A. Oliver, M. I. Morosini, F. Baquero, and R. Cantón, Abstr. 41st Intersci. Conf. Antimicrob. Agents Chemother., abstr. C2-298, p. 118, 2001), two *Enterobacter cloacae* strains (ECL4 and ECL5), and one *Enterobacter gergoviae* strain (7). Strains in each species were selected from different clones harboring apparently different CTX-M-10-producing plasmids, by restriction fragment length polymorphism analysis, as described in their respective published studies.

Figure 1 shows the 12.2-kb DNA fragment containing the $bla_{CTX\text{-}M\text{-}10}$ gene from pRYCE21. The 3.5-kb fragment upstream from $bla_{CTX\text{-}M\text{-}10}$ corresponded to four conserved phage-related ORFs, which were preceded by a Tn*1000*-like transposase. Three of these four ORFs (ORF2, ORF3, and ORF4) showed homology to conserved phage tail proteins, closest to those found in phage sequences from the genomes of *Chromobacterium violaceum* or *Neisseria meningitidis* (46 to 54% identity) (6, 21). Interestingly, a phage-related DNA invertase, 50 to 52% identical to different Pin, Cin, and Gin DNA invertases, preceded by a 1.5-kb invertible region was found 200 bp immediately upstream from the $bla_{CTX\text{-}M\text{-}10}$ gene (Fig. 1). Figure 2 shows results from multiple sequence alignment of the plasmid pRYCE21 DNA invertase with other related enzymes. The orientation of the invertible sequence modified the nature of one of the phage tail proteins, making it similar to that found in other phage DNA invertases.

Downstream of the DNA invertase, a 1.5-kb DNA fragment (including $bla_{CTX\text{-}M\text{-}10}$ and an unknown 483-bp ORF designated ORF7) was 90% identical to the *Kluyvera cryocrescens* chromosomal β-lactamase region. Interestingly, the *Kluyvera cryocrescens*-homologous region apparently contained two insertion sequences: first, a 700-bp fragment, 97% identical at the nucleotide level to Tn*5708* (GenBank accession number AJ010745) containing the left inverted repeat and a 366-bp ORF (ORF8) with unknown function, and second, a complete copy of IS*4321*. A 38-bp fragment homologous to the *K. cryocrescens* chromosomal region contiguous to the above-described 1.5-kb fragment was also found (Fig. 1). Finally, the 12.2-kb fragment was completed by a 651-bp ORF (ORF10) coding for a protein that is 65% identical to a conserved hypothetical protein from *E. coli*, a 747-bp ORF (ORF11) coding for a nucleoprotein- or polynucleotide-associated enzyme that is 56% identical to YaiL from *E. coli* K-12, and a complete copy of IS*5*.

Conservation of the 12.2-kb genetic element identified in pRYCE21 from *K. pneumoniae* strain KP4aC among different clinical strains is represented in Fig. 1. The 5 kb upstream of $bla_{CTX\text{-}M\text{-}10}$ including the phage-related region with the DNA invertase and its invertible region was found to be present in all the studied strains, with the exception of Kp36C, for which the

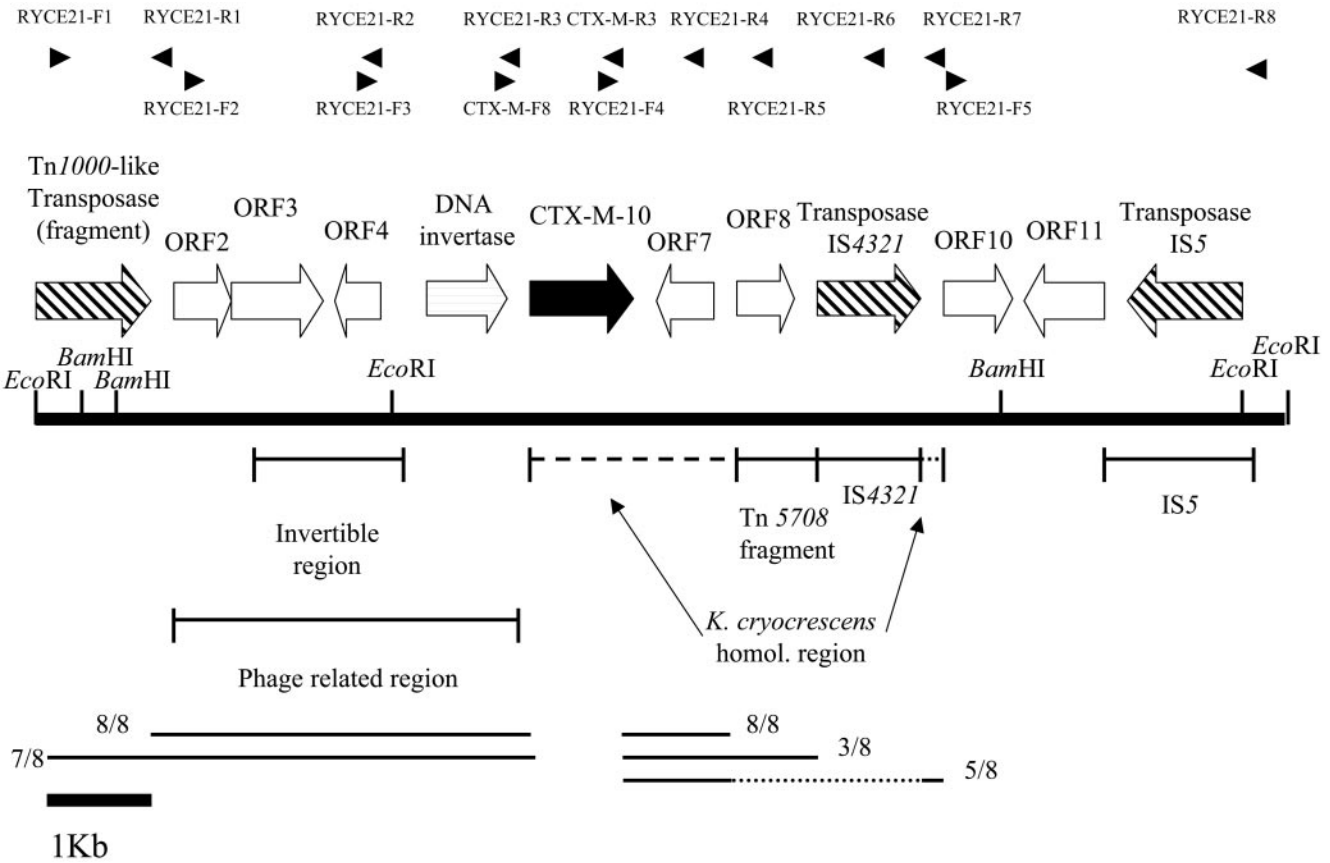

FIG. 1. Representation of the 12.2-kb region of pRYCE21 harboring the $bla_{CTX\text{-}M\text{-}10}$ gene. EcoRI and BamHI restriction sites used for cloning are represented with vertical lines. The locations of the primers used for the mapping of the 12.2-kb region by PCR amplification in different CTX-M-10-producing strains of *Enterobacteriaceae* are represented with black arrows. The conserved regions and the numbers of strains in which they are found are represented at the bottom of the figure. homol., homologous.

fragment corresponding to the Tn*1000*-like transposase region was not amplified. The downstream region of $bla_{CTX\text{-}M\text{-}10}$ was found to be more varied. All strains shared the 0.6-kb *Kluyvera cryocrescens*-homologous region downstream of $bla_{CTX\text{-}M\text{-}10}$ (positive PCR amplification of RYCE21-F4 and RYCE21-R4) (Table 1; Fig. 1). On the other hand, only three additional strains (*E. coli* strains EC22 and EC54 and *E. cloacae* strain ECL4) had the Tn*5708* fragment (positive PCR amplification with RYCE21-F4 and RYCE21-R5), but none of them were interrupted by IS*4321* (negative PCR amplification with RYCE21-F4 and RYCE21-R6). The remaining strains had neither the Tn*5708* fragment nor IS*4321* inserted in the *Kluyvera cryocrescens*-homologous region. For these strains, a specific 0.7-kb band was obtained after RYCE21-F4–RYCE21-R7 amplification (Table 1; Fig. 1), as expected, when no additional sequences were inserted in the *Kluyvera cryocrescens*-homologous region. Sequencing of the 0.7-kb PCR products from two of the isolates confirmed this assumption. No temporal or spatial relationship of strains lacking or gaining the Tn*5708* fragment was observed within the studied collection.

CTX-M-10 belongs to the CTX-M-1 group of CTX-M β-lactamases, differing from CTX-M-3 (12) in only two amino acids (Ala27Val and Arg38Gln). Despite this high homology, an important degree of polymorphism is observed when the nucleotide sequences of the genes coding for these closely related β-lactamases are compared. $bla_{CTX\text{-}M\text{-}10}$ differs from $bla_{CTX\text{-}M\text{-}3}$ in 21 nucleotides (2.4% of the coding sequence). This high degree of polymorphism suggests that both plasmid-mediated β-lactamase genes may be derived from an independent chromosome mobilization process occurring in different strains from the same species of the genus *Kluyvera*. CTX-M-10 has been successfully disseminated in our hospital for over a decade, despite the fact that $bla_{CTX\text{-}M\text{-}10}$-containing plasmids, unlike integron-borne enzymes, do not harbor additional antibiotic resistance determinants. Nevertheless, the absence of CTX-M-10-producing strains reported in other studies until very recently suggested that the high dissemination in our institution was of only local dimensions. In a recent Spanish nationwide study of the prevalence of ESBL in a collection of 170 and 70 ESBL-producing *E. coli* and *K. pneumoniae* isolates collected in 2000, respectively, only one *K. pneumoniae* isolate and three *E. coli* isolates with CTX-M-10 were recovered, three of which (two *E. coli* isolates and one *K. pneumoniae* isolate) were found in two institutions not far from our hospital and the fourth of which (*E. coli*) was from northern Spain (13). In France, a single *E. coli* isolate was recently recognized to produce the CTX-M-10 enzyme (17). In contrast

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Pin             ------------MLIGYVRVSTNDQNTDLQRNALNCAGCELIFEDKISGTKSERPGLKKL
PinH            MASFLLLSGRSTMLIGYVRVSTNDQNTDLQRNALNCAGCELIFEDKISGTKSERPGLKKL
Cin P7          ------------MLIGYVRVSTNEQNTALQRNALESAGCELIFEDKASGKQTERPGLKKV
Cin P1          ------------MLIGYVRVSTNEQNTALQRNALESAGCELIFEDKASGKKAERPGLKKV
Gin Mu          ------------MLIGYVRVSTNDQNTDLQRNALVCAGCEQIFEDKLSGTRTDRPGLKRA
DNA INV.        ------------MIIGYARVSSNHQDTELQMQALRAAGCKLIFEEKASGRKTNRPILKKV
                            *:***.***:*.*:* ** :** .***: ***:* ** :::** **:

Pin             LRTLSAGDTLVVWKLDRLGRSMRHLVVLVEELRERGINFRSLTDSIDTSTPMGRFFFHVM
PinH            LRTLSAGDTLVVWKLDRLGRSMRHLVILVEELRERGVNFRSLTDAIDTSTPMGRFFFHVM
Cin P7          LRMLSRGDTLVVWKLDRLGRSMRHLVVLVEELRDRGINFRSLTDSIDTSTPMGRFFFHVM
Cin P1          LRMLSRGDTLVVWKLDRLGRSMRHLVVLVEELRDRGINFRSLTDSIDTSTPMGRFFFHVM
Gin Mu          LKRLQKGDTLVVWKLDRLGRSMKHLISLVGELRERGINFRSLTDSIDTSSAMGRFFFHVM
DNA INV.        VEMLEPGDELVIWKLDRIGRNVLHALLTFQSLAERNVNIRSITDGVDLSTASGRYNFRNI
                :. *. ** **:*****:**.: * :  . .* :*.:*:**:**.:* *:. **: *: :

Pin             GALAEMERELIVERTKAGLETARAQGRIGGRRPKLTPEQWAQAGRLIAAGTPRQKVAIIY
PinH            GALAEMERELIVERTKAGLEAARAQGRIGGRRPKLTPEQWAQAGRLIAAGIPRQKVAIIY
Cin P7          GALAEMERELIVERTRAGLDAARAEGRIGGRRPKYQEETWQQMRRLLENGIPRKQVAIIY
Cin P1          GALAEMERELIVERTRAGLDAARAEGRIGGRRPKYQEETWQQMRRLLEKGIPRKQVAIIY
Gin Mu          GALAEMERELIIERTMAGLAAARNKGRIGGRPPKLTKAEWEQAGRLLAQGIPRKQVALIY
DNA INV.        LSAAQYESDLNSERTLAGLAVARAKGRIGGRKPKFTDEHWDAFEREIKSGCSHRDIALKY
                 : *: * :*  *** *** .** :****** **     *    * :  * .::.:*: *

Pin             DVGVSTLYKRFPAGDK---------
PinH            DVGVSTLYKKFPAGDK---------
Cin P7          DVAVSTLYKKFPASSFQS-------
Cin P1          DVAVSTLYKKFPASSFQS-------
Gin Mu          DVALSTLYKKHPAKRAHIENDDRIN
DNA INV.        GVGLSTLYKRYPAQQEHQ-------
                .*.:*****:.**
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FIG. 2. Clustal W multiple sequence alignments of DNA invertase (DNA INV) from plasmid pRYCE21 with related phage DNA invertases: Pin DNA invertase from lambdoid prophage (23), PinH DNA invertase from prophage CP-933H (27), Cin from *Enterobacteriaceae* phages P1 and P7 (14, 26), and Gin from *Enterobacteriaceae* phage Mu (22).

to the findings from our institution, IS*Ecp1* preceded $bla_{\text{CTX-M-10}}$ in the French strain, as did many other CTX-M genes, suggesting that the $bla_{\text{CTX-M-10}}$ gene has been captured in France by a genetic environment different from that of our strains. This is an excellent example of the influence of local genetic patterns on the local dissemination of resistance genes (2).

Whether the phage-like structure found is self-transferable (functional) into different plasmids or whether the high plasmid diversity in CTX-M-10-producing strains of *Enterobacteriaceae* is a consequence of frequent modifications of a single $bla_{\text{CTX-M-10}}$-bearing plasmid remains to be elucidated. Nevertheless, the results of this work suggest that the transfer of $bla_{\text{CTX-M-10}}$ from the chromosome of *Kluyvera* spp. to a transferable plasmid may have been mediated by transduction by a bacteriophage, highlighting the potential role of phages in the dissemination of resistance determinants. The first description of a β-lactamase gene transfer mediated by a bacteriophage was reported in 1972 (30). More-recent works show that bacteriophages found in sewage frequently harbor β-lactamase genes of the OXA and PSE type (19). Our study of the genetic environment of the gene coding for the CTX-M-10 β-lactamase illustrates the importance of the environmental reservoir of genetic elements that, as phages, may serve as tools for natural genetic engineering processes that eventually lead to the evolution and spread of antibiotic resistance.

Nucleotide sequence accession number. The GenBank accession number for the 12.2-kb DNA fragment from pRYCE21 is AY598759.

This work was partially supported by the Red Española de Investigación en Patología Infecciosa (REIPI) from the Ministerio de Sanidad of Spain (C03-014), the Fondo de Investigaciones Sanitarias of the Ministerio de Sanidad of Spain (PI02043), the Ministerio de Ciencia y Tecnología of Spain (SAF 2003-09285), and the European Commission (LSHM-CT-2003-503335).

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