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,1 Fernando Baquero,2 and Rafael Cantón2

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

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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\textsubscript{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\text{Ecp1}-like insertion sequences have been frequently found upstream of several\text{bla\textsubscript{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\text{bla\textsubscript{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\text{bla\textsubscript{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 pBG18-* (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 CaCl2 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.
lected in MacConkey agar plates containing 50 μg of kanamycin per ml with and without 2 μg of cefotaxime per ml. Transformants were selected 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 blaCTX-M-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 blaCTX-M-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 E. coli (4321).

**Table 1. Primers used for PCR amplification of different regions of the 12.2-kb DNA fragment containing blaCTX-M-10 from plasmid pRYCE21.**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’→3’)</th>
<th>Positions</th>
<th>PCR amplification</th>
<th>PCR product size (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RYCE21-F1</td>
<td>CAGGAGCCGCGATTATCAC</td>
<td>335–351</td>
<td>RYCE21-F1–RYCE21-R1</td>
<td>1.5</td>
</tr>
<tr>
<td>RYCE21-R1</td>
<td>GGCTGGGATGGCCGTAAC</td>
<td>1785–1767</td>
<td>RYCE21-F1–RYCE21-R1</td>
<td>1.5</td>
</tr>
<tr>
<td>RYCE21-F2</td>
<td>GACATTTCATCGAAGGCC</td>
<td>2039–2058</td>
<td>RYCE21-F2–RYCE21-R2</td>
<td>2.0</td>
</tr>
<tr>
<td>RYCE21-R2</td>
<td>GCCGAGGGATTAAATCAGG</td>
<td>4032–4014</td>
<td>RYCE21-F2–RYCE21-R2</td>
<td>2.0</td>
</tr>
<tr>
<td>RYCE21-F3</td>
<td>CCCATGAGCCGCTTACG</td>
<td>3880–3897</td>
<td>RYCE21-F2–RYCE21-R3</td>
<td>2.1</td>
</tr>
<tr>
<td>RYCE21-R3</td>
<td>GAGCCGACAAATGTAGTCG</td>
<td>8922–8940</td>
<td>RYCE21-F3–RYCE21-R3</td>
<td>2.1</td>
</tr>
<tr>
<td>RYCE21-R4</td>
<td>ATCGACAAGGTCATGCTGATG</td>
<td>6425–6405</td>
<td>RYCE21-R4–RYCE21-R5</td>
<td>1.0</td>
</tr>
<tr>
<td>RYCE21-R5</td>
<td>GAGCCGACAAATGTAGTCG</td>
<td>6828–6808</td>
<td>RYCE21-R4–RYCE21-R5</td>
<td>1.0</td>
</tr>
<tr>
<td>RYCE21-R6</td>
<td>CTCGCTTACTAATTCCCAGC</td>
<td>8030–8011</td>
<td>RYCE21-R5–RYCE21-R6</td>
<td>2.2</td>
</tr>
<tr>
<td>RYCE21-R7</td>
<td>CCATGCTGTTTTCCGTAGTAC</td>
<td>6828–6808</td>
<td>RYCE21-R5–RYCE21-R6</td>
<td>2.2</td>
</tr>
<tr>
<td>RYCE21-R8</td>
<td>ATCGACAAGGTCATGCTGATG</td>
<td>5815–5833</td>
<td>RYCE21-R6–RYCE21-R7</td>
<td>2.7</td>
</tr>
<tr>
<td>RYCE21-R9</td>
<td>CAGGAGCCGCGATTATCAC</td>
<td>5885–5867</td>
<td>RYCE21-R7–RYCE21-R8</td>
<td>2.6</td>
</tr>
</tbody>
</table>

blaTX-M-10 corresponded to four 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 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 blaCTX-M-10 and an unknown 483-bp ORF designated ORF7) was 90% identical to the Klyuera cryocrescens chromosomal β-lactamase region. Interestingly, the Klyuera cryocrescens-homologous region apparently contained two inversion sequences: first, a 700-bp fragment, 97% identical at the nucleotide level to Tn5708 (GenBank accession number AJ010745) containing the left inverted repeat and a 366-bp ORF (ORF8) with unknown function, and second, a complete copy of IS4321. 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 IS5.

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 blaCTX-M-10 including the phase-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
fragment corresponding to the Tn1000-like transposase region was not amplified. The downstream region of bla_{CTX-M-10} was found to be more varied. All strains shared the 0.6-kb *Kluyvera cryocrescens*-homologous region downstream of bla_{CTX-M-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 Tn5708 fragment (positive PCR amplification with RYCE21-F4 and RYCE21-R5), but none of them were interrupted by IS4321 (negative PCR amplification with RYCE21-F4 and RYCE21-R6). The remaining strains had neither the Tn5708 fragment nor IS4321 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 Tn5708 fragment was observed within the studied collection.

CTX-M-10 belongs to the CTX-M-1 group of CTX-M/oxacillin-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 \( \beta \)-lactamases are compared. bla_{CTX-M-10} differs from bla_{CTX-M-3} in 21 nucleotides (2.4% of the coding sequence). This high degree of polymorphism suggests that both plasmid-mediated \( \beta \)-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-M-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...
to the findings from our institution, IS\textsuperscript{Ecp1} preceded \texttt{bla\_CTX-M-0} in the French strain, as did many other CTX-M genes, suggesting that the \texttt{bla\_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 \textit{Enterobacteriaceae} is a consequence of frequent modifications of a single \texttt{bla\_CTX-M-10}-bearing plasmid remains to be elucidated. Nevertheless, the results of this work suggest that the transfer of \texttt{bla\_CTX-M-10} from the chromosome of \textit{Kluyvera} spp. to a transmissible 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 \textit{	extgamma\textbeta\textomega\textalpha\textlambda\textomicron\textnu\textomicron\textlambda\textomicron\textomicron}-lactamase gene transfer mediated by a bacteriophage was reported in 1972 (30).

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

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\textbf{REFERENCES}


\begin{table}[h]
\centering
\begin{tabular}{|c|c|}
\hline
\textbf{Gene} & \textbf{Sequence Accession Number} \\
\hline
\texttt{bla\_CTX-M-0} & AY598759 \\
\hline
\end{tabular}
\caption{Nucleotide sequence accession number.}
\end{table}


