Complete Nucleotide Sequence of the pCTX-M3 Plasmid and Its Involvement in Spread of the Extended-Spectrum β-Lactamase Gene blaCTX-M-3

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Here we report the nucleotide sequence of pCTX-M3, a highly conjugative plasmid that is responsible for the extensive spread of the gene coding for the CTX-M-3 extended-spectrum β-lactamase in clinical populations of the family Enterobacteriaceae in Poland. The plasmid belongs to the IncI1/M incompatibility group, is 89,468 bp in size, and carries 103 putative genes. Besides blaCTX-M-3, it also bears the blaTEM-1, aacC2, and armA genes, as well as integrons aadA2, dfrA12, and sul1, which altogether confer resistance to the majority of β-lactams and aminoglycosides and to trimethoprim-sulfamethoxazole. The conjugal transfer genes are organized in two blocks, tra and trb, separated by a spacer sequence where almost all antibiotic resistance genes and multiple mobile genetic elements are located. Only blaCTX-M-3, accompanied by an ISEcp1 element, is placed separately, in a DNA fragment previously identified as a fragment of the Kluyvera ascorbata chromosome. On the basis of sequence analysis, we speculate that pCTX-M3 might have arisen from plasmid pEL60 from plant pathogen Erwinia amylovora by acquiring mobile elements with resistance genes. This suggests that plasmids of environmental bacterial strains could be the source of those plasmids now observed in bacteria pathogenic for humans.

Bacterial plasmids constitute an important part of the so-called horizontal gene pool, the pool of genes that can be acquired by various bacterial strains by means of horizontal gene transfer (56). The ability of plasmids to spread by conjugation greatly enhances their impact on the genetic plasticity, metabolic potential, and environmental adaptability of bacteria, which in the case of plasmids carrying virulence or antimicrobial resistance genes is a source of serious clinical and epidemiological problems (see references 35, 56, and 61 and references therein).

Among the genes that are often located in conjugative plasmids are those coding for extended-spectrum β-lactamases (ESBLs), which in large part are responsible for the resistance of the members of the family Enterobacteriaceae to newer β-lactams (11, 24, 35). The epidemiology of ESBLs has recently been dominated by the extremely rapid and worldwide spread of organisms producing enzymes of the CTX-M family, both in nosocomial environments and in the community (10, 36, 45). To date, over 60 CTX-M-type β-lactamases have been identified, showing the intensive evolution of this group (http://www.lahey.org/studies/webt.stm). blaCTX-M genes are derivatives of the chromosomal β-lactamase genes of the genus Kluyvera (17, 29, 43, 46, 49). In transmissible plasmids found in clinical isolates, they usually reside in fragments of Kluyvera sp. chromosomes in association with either ISEcp1-like insertion sequences (30, 34, 37, 47) or CR1 elements inserted in sul1-type class 1 integrons (3, 19, 41, 50, 58). The role of ISEcp1-like sequences in the mobilization of Kluyvera genes and details of this transposition process have been demonstrated recently (35, 47, 48); moreover, these elements are also frequently responsible for the high-level expression of blaCTX-M genes (10, 13, 47).

In Poland, the first gene of the blaCTX-M type, blaCTX-M-3, was identified in 1996 in clinical isolates of Citrobacter freundii and Escherichia coli from one of the hospitals in Warsaw. It was associated with a large plasmid that could be easily transferred to E. coli via conjugation and that conferred resistance to penicillins, cephalosporins, and aztreonam, as well as aminoglycosides, gentamicin, and tobramycin (25). Later, the rapid spread of CTX-M-3-producing organisms was observed in the same center (44) and all over the country (7, 8), and it has mainly been attributed to the horizontal transmission of plasmids. Of the several types of plasmids with blaCTX-M-3 genes identified by restriction fingerprinting, one family of similar molecules in particular has been widely disseminated into multiple locales and multiple species, including E. coli, Klebsiella pneumoniae, Klebsiella oxytoca, Enterobacter cloacae, C. freundii, Morganella morganii, Serratia marcescens, and Salmonella enterica (7, 8). The most prevalent variant of that family was the plasmid first observed in 1996 in C. freundii isolates, in which CTX-M-3 had been originally identified (25).

In Taiwan, where CTX-M-3 is the most common ESBL, it has been encoded by large, transmissible IncI1-like plasmids (37),...
while in Spain the dissemination of CTX-M-3 and other CTX-M-1 like β-lactamases is ascribed to the broad-host-range plasmids of the IncA/C2, IncL/M, and IncN groups (42).

In this paper we report the sequence of the pCTX-M-3 plasmid originating from C. freundii isolate 2526 from the Praski Hospital in Warsaw (25). The results of our study demonstrate the physical link between the blaCTX-M-3 gene, the IncL/M replicon, and an efficient conjugal transfer system. We also discuss the possible evolution of pCTX-M-3 from a putative ancestor pEL60 from a plant pathogen, Erwinia amylovora (22).

MATERIALS AND METHODS

Bacterial strains and plasmids. E. coli A15(pCTX-M3), a transconjugant of C. freundii 2526 (25), was the initial source of pCTX-M3 in this study. Clinical isolates K. pneumoniae 179, S. marcescens 12, and E. coli 279 were described previously (44); and the other clinical strains or transconjugants used in the study included E. coli 2112, E. coli 3624, E. coli 1145, E. coli 8330, E. coli 1775, K. pneumoniae 9172, and K. pneumoniae 2113, which were also described previously (7). E. coli DH5α (27) was used as a host strain in all cloning experiments. In the matings, E. coli DH5α(pCTX-M3) and Agrobacterium tumefaciens UBAPF(pCTX-M3) (39) were used as the donors and E. coli JE2571 Rfr (lev thi thi lacY thi pyr flr Rfr) (12) and DH5α(pACYC184) were used as the recipients, respectively. The shotgun library was prepared in vector pCR4BluntTOPO (Invitrogen, Carlsbad, CA). Plasmid pEM3, the pUC19 derivative with the bla gene replaced by cat of pACYC184 (the Avall-NarI fragment of pUC19 blunt ended by use of the Klenow fragment and ligated with FnuDII-digested pACYC184), was used as the vector for cloning of the IncL/M and IncI1 orf sequences. Plasmid pGZ221, a ColI-P9 derivative (14), was used as an IncI1 helper plasmid in orf mobilization experiments.

Plasmid conjugal transfer. One-milliliter volumes of cultures of the donor and recipient strains (108 CFU of each strain per ml) grown in Luria-Bertani (LB) broth (Biocorp, Warsaw, Poland) were mixed and incubated for 30 min at 37°C. Conjagation was stopped by vigorous vortexing for 30 s and placing the mating mixture on ice. Transconjugants were selected on LB agar (Biocorp) supplemented with cefotaxime (5 μg/ml; Polfa Tarchomin, Warsaw, Poland) and rifampin (50 μg/ml; Polfa Tarchomin). Solid mating was performed likewise, with an additional step of filtering of the mating mixture through a sterile HA 45-μm-pore-size filter (Millipore, Billerica, MA), which was then incubated on an LB agar plate. In the control experiments, the frequencies of spontaneous mutation of both the donor and the recipient to the phenotype of the transconjugants were measured.

DNA cloning and sequencing. Plasmid DNA was isolated by the alkaline lysis method, and cloning procedures were performed by standard protocols (53). The pCTX-M3 DNA for construction of a shotgun library was purified by CsCl-ethidium bromide gradient ultracentrifugation (53). The shotgun library was prepared in the pCR4BluntTOPO plasmid with the use of a TOPO shotgun subcloning kit (Invitrogen). The pCTX-M3 DNA for construction of a shotgun library was purified by CsCl-ethidium bromide gradient ultracentrifugation (53). The shotgun library was prepared in vector pCR4BluntTOPO (Invitrogen, Carlsbad, CA). Plasmid pEM3, the pUC19 derivative with the bla gene replaced by cat of pACYC184 (the Avall-NarI fragment of pUC19 blunt ended by use of the Klenow fragment and ligated with FnuDII-digested pACYC184), was used as the vector for cloning of the IncL/M and IncI1 orf sequences. Plasmid pGZ221, a ColI-P9 derivative (14), was used as an IncI1 helper plasmid in orf mobilization experiments.

Plasmid backbone. Only one replication region was identified in the sequence of pCTX-M3. This replication cassette (positions 54341 to 55979) was confirmed to be identical to that of the prototypical IncI/M replicon of pMU407.1 (4, 5). The segregational stability of the plasmid is ensured by an active partition system encoded by parAB genes (position 17203 to 18633) related to those of IncI1 plasmid ColIb-P9 (parA, 64% similarity; parB, 34% similarity). The presence of a single replicon in pCTX-M3, as well as the functionality of the parAB system, were confirmed in a separate analysis (39). Two other regions possibly involved in stable plasmid maintenance were identified; and these included (i) region pemIK (positions 757 to 1348), which is identical to that of IncFII plasmid R100 (pYVE8081 (GenBank accession no. AF074611; 76% similarity), pCTX-M3 also bears mucAB genes (positions 4698 to 6435), which are members of the umuDC-like family of UV resistance genes (28).

Conjugal transfer system. Genes coding for the pCTX-M-3 conjugal transfer system are organized in two blocks, tra (positions 31620 to 54603) and trb (positions 89418 to 83109). The system is related to that of IncI1 plasmid ColIb-P9 in terms of the amino acid sequences of specific proteins (30% to 60%) and of a conserved gene order (Fig. 2). Nevertheless, significant differences are observed between the two systems. First, the oriT region (oriT, nikAB) in ColIb-P9 is located downstream of the trb cluster, and they are both convergently tran-
scribed, while in pCTX-M3 this region is a part of the tra operon. Second, the trb operon in the IncI1 system is placed next to tra and is transcribed in the same direction, whereas in pCTX-M3, trb lies 28.5 kb apart from the tra genes and is transcribed in the opposite direction. Third, some genes present in one of the plasmids are missing from the other; e.g., pCTX-M3 lacks traEFG, the three initial genes of the tra operon, and traV, which was found to be essential for the conjugal transfer of ColIb-P9 (33). In contrast, ColIb-P9 lacks a counterpart of orf36, which in pCTX-M3 separates traL and
traM, and the traST genes of ColIb-P9 are replaced in pCTX-M3 by a single gene, orf37. Interestingly, no homology between orf37 and either traS or traT was detected. It is noteworthy that pil genes have not been found in pCTX-M3; thus, its host should not produce thin pili, which in IncI1 carriers enable liquid mating (31, 32). Therefore, it should be underlined that pCTX-M3 is able to transfer in liquid medium (7, 25, 44) at the same frequency as it does on solid support, reaching a value of 0.1 transconjugants/donor (the frequency of spontaneous mutations of donor and recipient strains to the value of 0.1 transconjugants/donor (the frequency of spontaneous mutations of donor and recipient strains to the

transfer system in an Agrobacterium

oriT

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As shown recently by Mierzejewska et al. (39), the broad host range of pCTX-M3 is not limited to the family Enterobacteriaceae but also includes members of the classes Alpha-, Beta-, and Gammaproteobacteria. We managed to transfer pCTX-M3 back from a soil bacterium, A. tumefaciens

is located (positions 56790 to 57990) next to the replicon. The

Beta-, and

Entero-

results indicate that the mobilization of pCTX-M3 by a single gene,

traST

and the

3792

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sible mobile elements (see below), which removed a part of its 27-kb insert, close to the

trub

region. The second part of Tn1

1

with the replicon. The second part of Tn1

1

was disrupted by other

genes have not been found in pCTX-M3; thus, dfrA12, orfF, and aadA2. An identical array of gene cassettes was found in an integron of plasmid pLEW517 from E. coli

(60) and in several others (16) (GenBank accession no. AF188331). The pCTX-M3 integron contains a complete 5’ conserved sequence (5’S-CS) with the terminal IR (IRi), whereas its 3’-CS is truncated 24 bp after the sull stop codon due to the insertion of a CR1 element with orfS13 (positions 71184 to 71218) (54). Downstream of CR1 there is a putative IS homologous to ISR391B (9) with truncated IRL (positions 71219 to 72092), the aminoglycoside resistance gene armA, an ISI330-like element (positions 73766 to 75094), and genes reported before to be associated with resistance to macrolides: mel (1) and mph1 and mph2 (40, 55). This region ends with the second copy of IS26 (positions 79595 to 80417). The whole segment flanked by two IS26 copies might be considered a putative large composite transposon, and a similar structure was found before in pMUR050, an IncN group plasmid (26). It differs from that in pCTX-M3 only by the lack of two integronic gene cassettes (dfrA12 and orfF) and by a peculiar structure of the integron’s 5’S-CS, where the integrase gene intI1 is truncated, duplicated, and surrounded by two IS26 copies. pMUR050 is of “animal” origin (it was isolated from an E. coli strain from a diarrheic pig), and it has been implicated in the conjugal dissemination of the armA gene (26).

The mosaic structure of the 27-kb replicon-trb region clearly suggests that it arose from multiple insertions (Fig. 3). As both DRs created by the Tn1 insertion are present at boundaries of the region, the first event had to be the Tn1 transposition. The subsequent integration events disrupted Tn1 by removing 156 initial codons of tnps and the whole res gene but leaving bla\(_{TEM-1}\), intact. These events caused the acquisition of several mobile elements, some of which were acquired together with other resistance genes, including those present in the class 1 integron. It is impossible to determine if all these elements were acquired by pCTX-M3 in a series of separate events or as bigger modules that were preformed earlier. Since no DRs were found on the flanks of the IS26 elements, the composite transposon-like structure emerged in pCTX-M3 rather not by transposition but by another recombination mechanism.

The IS\(E\)cp1- and bla\(_{CTX-M3}\)-containing region. IS\(E\)cp1 (positions 1457 to 3112) and bla\(_{CTX-M3}\) (positions 3161 to 4151) are the only mobile element and resistance gene, respectively, that are located outside the 27-kb replicon-trb region of pCTX-M3. The presence of IS\(E\)cp1 at a distance 128 bp upstream from the bla\(_{CTX-M3}\) and the bla\(_{CTX-M3-15}\) genes in pCTX-M3 and a similar plasmid, respectively, has been reported earlier (6). The pCTX-M3 fragment placed directly downstream from IS\(E\)cp1 was identified by Rodriguez et al. (49) to be a chromosomal fragment from a K. ascorbata strain. Apart from the

positions 58454 to 60070) is located behind bla\(_{TEM-1}\), with complete inverted repeats (IRs) and DRs. Its tnp reveals 97% similarity to that in plasmid pADP-1 (38). This IS is followed by orf39 to orf41 and the aacC2 gene, and the entire segment from Tn1 to aacC2 is almost identical to a fragment of recently described plasmid pU302L of S. enterica serovar Typhimurium (15).

The first of two IS26 copies present in pCTX-M3 (positions 63800 to 64622) and a class 1 integron (positions 64640 to 69064) are located downstream of the aacC2 gene. This integron bears three gene cassettes in its variable region, namely, dfrA12, orfF, and aadA2. An identical array of gene cassettes was found in an integron of plasmid pLEW517 from E. coli

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128-bp spacer and the entire coding region of bla
CTX-M-3, it contains 343 bp of a *Kluyvera* orf477 that, together with 204 bp of the pCTX-M3 sequence, constitutes an orf1 of unknown function. The 3' end of the insert is terminated by an 18-bp sequence which is a part of orf477 that most probably mimicked the right IR of IS*Ecp1*. The duplication of a pentanucleotide (TGCAG), the IS*Ecp1* target sequence (positions 1452 to 1456), was found next to this boundary (positions 4508 to 4512) and most probably was generated in the one-ended transposition event that mobilized *bla*CTX-M-3 from the *K. ascorbata* chromosome (48).

**Cooccurrence of the bla**CTX-M-3** gene with the IncL/M replicon and the conjugal transfer system.** Multiple plasmids purified from Polish CTX-M-3 producers were compared before by PstI restriction fingerprinting and were split into several types, with the highly predominant type A (pCTX-M3 family). Several pCTX-M3-like plasmid variants and the plasmid of type B were nonconjugative (7, 8, 44). We decided to check whether these differences could be due to differences in the conjugal transfer genes and whether the *bla*CTX-M-3 gene has always been linked to the IncL/M replicon and located in the same context as it is in pCTX-M3. The repA gene was chosen as an IncL/M marker, and the *traU* gene was chosen as a marker of the transfer system. *repA* is essential for IncL/M plasmid replication (7, 8, 44), whereas *traU* codes for one of the most conserved and essential proteins of the transfer system (25a, 51). The results of PCR tests carried out with several pCTX-M3-like variants, as well as plasmids of types B, C, and D, are shown in Table 1. All plasmids of types pCTX-M3, C, and D contained the *repA* and *traU* genes, whereas the plasmid of type B had neither of these. PCR mapping of the *bla*CTX-M-3 locus revealed that all of the types of plasmids carried the same fragment of the *K. ascorbata* chromosome described above. The results demonstrated clearly the major role of conjugative

![Diagram](https://example.com/diagram.png)

**FIG. 3.** Organization of the 27-kb region of the replicon and the *trb* genes. The partial pCTX-M3 sequence is drawn schematically out of scale. Arrows, ORFs; black lines, the sites of integration of particular sequence segments; black frames, hypothetical fragments of Tn1 and the integron that are not observed in pCTX-M3, probably due to integration events; vertical bars, IRs of the respective mobile elements and IRi of the integron; gray blocks, the regions of the plasmid backbone.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Plasmid type/variant by PstI fingerprinting</th>
<th>Mating</th>
<th><em>traU</em></th>
<th><em>repA</em></th>
<th><em>bla</em>CTX-M-3 locus</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. freundii 2526</td>
<td>A1–pCTX-M3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>K. pneumoniae 179</td>
<td>A4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S. marcescens 12</td>
<td>A5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E. coli 2112</td>
<td>A11</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>K. pneumoniae 2113</td>
<td>A12</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E. coli 3624</td>
<td>A18</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E. coli 11145</td>
<td>A33</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>K. pneumoniae 9172</td>
<td>A34</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>E. coli 279</td>
<td>B</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E. coli 8350</td>
<td>C</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E. coli 1775</td>
<td>D</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*a* Clinical isolates and their plasmid fingerprints were described previously (6, 24, 45); in cases marked with an asterisk, plasmids were purified from transconjugants for this analysis.

*b* + and −, positive and negative mating results, respectively.

*c* +, +, intense band of a PCR product; +, weak band; −, no band present.

*d* Defined as IS*Ecp1*, *bla*CTX-M-3 (128 bp from IS*Ecp1*), and orf477 (373 bp) (80).
InCl/M plasmids in the spread of the bla\textsubscript{CTX-M-3} gene in populations of the family Enterobacteriaceae in Poland and suggested the possible transfer of the bla\textsubscript{CTX-M-3}-containing element to other plasmids.

**Conclusions.** We have sequenced plasmid pCTX-M3, which seems to be in large part responsible for the rapid dissemination ofCTX-M-3-producing microorganisms in Poland (7, 8, 25, 44). The bla\textsubscript{CTX-M-3} gene observed in the country most probably emerged by a single IS\textsubscript{Ecp1}-mediated mobilization from the K. ascorbata genome (49), and our results suggest that it could have been transmitted to other plasmids of the same or different replicon types. The pCTX-M3 plasmid bears the InCl/M type of replicon, which enables replication in a broad range of hosts (39) and which codes for the conjugal transfer system that is most similar to that of IncI1 plasmids. Despite the lack of the pil genes, pCTX-M3 is able to transfer with a high efficiency both in liquid and on solid media, and the transfer system is also functional in A. tumefaciens. Moreover, the oriT sequence of pCTX-M3 may serve as a transfer origin both for its cognate InCl/M system and for the heterologous IncI1 system. The backbones of pCTX-M3 and pEPE60, a plasmid from bacterial species pathogenic for plants, reveal extended identity, with differences clustered in two regions packed with mobile genetic elements and antibiotic resistance genes. Our results allow us to speculate that an ancestral plasmid similar to pEPE60 might have originated from an environmental bacterium and that the plant bacterial community may be the source of plasmids utilized by species pathogenic for human in their rapid adaptation to quickly changing clinical environments.


