Complete Nucleotide Sequences of Two VIM-1-Encoding Plasmids from Klebsiella pneumoniae and Leclercia adecarboxylata Isolates of Czech Origin

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ABSTRACT

Two multidrug resistance (MDR) plasmids, carrying the VIM-1-encoding integron In110, were characterized. Plasmid pLec-476cz (311,758 bp), from a Leclercia adecarboxylata isolate, consisted of an IncHI1 backbone, a MDR region, and two accessory elements. Plasmid pKpn-431cz (142,876 bp), from a sequence type 323 (ST323) Klebsiella pneumoniae isolate, comprised IncFIIY-derived and pKPN3-like sequences and a mosaic region. A 40,400-bp sequence of pKpn-431cz was identical to the MDR region of pLec-476cz, indicating the \textit{en bloc} acquisition of the VIM-1-encoding region from one plasmid by the other.

KEYWORDS
carbapenemases, metallo-β-lactamases, IncHI1, IncFIIY, integrative conjugative elements

VIM-producing \textit{Enterobacteriaceae} have been observed since 2001 in Greece (1). For at least a decade, VIM producers were the main carbapenemase-producing \textit{Enterobacteriaceae} (CPE) in Europe (2). In the Czech Republic, the first two cases of VIM-producing \textit{Enterobacteriaceae} were identified in 2011. The first case was a sequence type 323 (ST323) \textit{Klebsiella pneumoniae} (Kpn-431cz) isolate cultured in April 2011 from a bronchoalveolar lavage sample of a patient treated in a Czech hospital. The second case included a \textit{Leclercia adecarboxylata} (Lec-476cz) isolate recovered (3) during a survey study focused on compliance with hand hygiene among the staff of a different Czech hospital in May 2011. Interestingly, the two isolates carried the VIM-1 carbapenemase-encoding integron In110 (\textit{bla}\textsubscript{VIM-1}-\textit{aacA4-aadA1}) (4), localized on plasmids pKpn-431cz and pLec-476cz. In the present study, we characterized the complete nucleotide sequences of pKpn-431cz and pLec-476cz in order to examine the nature of the genetic elements involved in the acquisition and spread of In110 in the Czech Republic.

The \textit{bla}\textsubscript{VIM-1}-carrying plasmids were transferred from the clinical strains to rifampin-resistant \textit{Escherichia coli} A15 by conjugation in mixed broth cultures. Transconjugants were selected on MacConkey agar plates supplemented with rifampin (150 μg/ml) and ampicillin (50 μg/ml). Plasmid pKpn-431cz was transferred by conjugation at 37°C while pLec-476cz was capable of transferring at 30°C. Both \textit{bla}\textsubscript{VIM-1}-positive transconjugants exhibited similar resistance phenotypes (Table 1), showing resistance to piperacillin, piperacillin-tazobactam, and cephalosporins and decreased susceptibility to imipenem, while they remained susceptible to meropenem and ertapenem. Plasmid analysis revealed that the transconjugants harbored \textit{bla}\textsubscript{VIM-1}-positive plasmids of different sizes (~150 kb [pKpn-431cz] and ~290 kb [pLec-476cz]) (5). The two plasmids were non-


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TABLE 1  Antimicrobial susceptibility of L. adecarboxylata Lec-476, K. pneumoniae Kpn-431, and the E. coli A15 transconjugants producing the VIM-1 metallo-β-lactamase

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Pip</th>
<th>Tzp</th>
<th>Ctx</th>
<th>Caz</th>
<th>Fep</th>
<th>Atm</th>
<th>Imp</th>
<th>Mem</th>
<th>Etp</th>
<th>Gen</th>
<th>Amk</th>
<th>Cml</th>
<th>Tet</th>
<th>Sxt</th>
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</thead>
<tbody>
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<td>L. adecarboxylata Lec-476cz</td>
<td>&gt;4</td>
<td>64</td>
<td>&gt;8</td>
<td>&gt;32</td>
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<td>1</td>
<td>8</td>
<td>4</td>
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<td>16</td>
<td>1</td>
<td>0.5</td>
<td>32</td>
<td>&gt;32</td>
<td>2</td>
</tr>
<tr>
<td>E. coli A15 pLec-476cz</td>
<td>64</td>
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<td>&gt;8</td>
<td>&gt;32</td>
<td>&gt;16</td>
<td>0.25</td>
<td>4</td>
<td>0.25</td>
<td>0.25</td>
<td>0.12</td>
<td>0.12</td>
<td>0.5</td>
<td>0.5</td>
<td>16</td>
<td>&gt;32</td>
</tr>
<tr>
<td>K. pneumoniae Kpn-431cz</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td>&gt;8</td>
<td>&gt;32</td>
<td>&gt;16</td>
<td>2</td>
<td>16</td>
<td>1</td>
<td>32</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>8</td>
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<tr>
<td>E. coli A15 pKpn-431cz</td>
<td>64</td>
<td>64</td>
<td>&gt;8</td>
<td>&gt;32</td>
<td>&gt;16</td>
<td>0.25</td>
<td>4</td>
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<td>0.12</td>
<td>0.12</td>
<td>0.12</td>
<td>0.5</td>
<td>16</td>
<td>&gt;32</td>
<td>0.5</td>
</tr>
<tr>
<td>E. coli A15 (recipient)</td>
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<td>≤0.06</td>
<td>≤0.25</td>
<td>≤0.12</td>
<td>≤0.25</td>
<td>≤0.12</td>
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<td>0.5</td>
<td>0.5</td>
<td>1</td>
<td>≤0.06</td>
<td></td>
</tr>
</tbody>
</table>

*Pip, piperacillin; Tzp, tazobactam; Ctx, cefotaxime; Caz, ceftazidime; Fep, cefepime; Atm, aztreonam; Imp, imipenem; Mem, meropenem; Etp, ertapenem; Gen, gentamicin; Amk, amikacin; Cml, chloramphenicol; Tet, tetracycline; Sxt, trimethoprim-sulfamethoxazole; Cip, ciprofloxacin.

The CP4-like prophage segment (nt 260350 to 296495) was a chimeric form of the CP4-6 and CP4-57 prophages. This segment included an IS-like integrated sequence, a putative relaxase-like protein (15) that usually recognizes a phage (14), followed by seven open reading frames (ORFs) that encoded a RadC DNA binding domain, a hypothetical protein, and an RdfS excisionase. This sequence was followed by a plasmidic segment, comprising the initiation replication (repHIA and repHIB genes), conjugal transfer (tra genes), and plasmid maintenance (parAB operon). Thermosensitivity is a well-known phenomenon for the conjugative apparatus of the IncHI plasmids (11), which is in agreement with the fact that pLec-476cz was capable of transferring at 30°C. Apart from the backbone, pLec-476cz carried a Tn4371-like integrative conjugative element (ICE) (12), a CP4-like prophage sequence (13), and a multidrug resistance (MDR) region.

The Tn4371-like ICE (nucleotide [nt] 76560 to 123914) was inserted into a gene encoding a hemolysin modulation protein. This region contained a putative int gene, encoding a tyrosine-based site-specific recombinase historically called a phage-like integrase (14), followed by seven open reading frames (ORFs) that encoded a RadC DNA repair protein, a ParB-like nuclease, and hypothetical proteins. The next region on the Tn4371-like ICE contained genes whose predicted products were related to the xenobiotic response element (XRE) transcriptional regulator, a lipoprotein with a DNA binding domain, a hypothetical protein, and an RdS excisionase. This sequence was followed by a plasmidic segment, comprising the initiation replication repA gene, the maintenance parAB operon, and a transfer region, including 3 tra genes (traF, traR, and traG) and 9 trb genes (trb8 through trbG, trbI, trbJ, and trbL) and virD2. VirD2 is a putative relaxase-like protein (15) that usually recognizes oriT, makes a single-strand DNA break in oriT, and covalently attaches to the 5'-end of the nicked DNA strand via a phosphotyrosyl linkage. In the region intervening between virD2 and traR, genes responsible for a putative AcrB inner membrane transporter, an ABC-type transport system, an RND family efflux pump, and a small multidrug export protein were found. Target site duplications of 9 bp (TTTTTTTGT) at the boundaries of the Tn4371-like ICE indicated integration by transposition. Of note is that a similar region (99%) has also been described in plasmid p8025 (10) and in the chromosomes of several Gram-negative rods, like Pseudomonas putida H8234 (GenBank accession no. CP005976), Enterobacter cloacae CAV1669 (CP011650), and Citrobacter freundii CFNIH1 (CP007557), further supporting its integrative nature.

The CP4-like prophage segment (nt 260350 to 296495) was a chimeric form of the CP4-6 and CP4-57 prophages. This segment included an IS-like surrounded sequence encoding an AcrR transcriptional regulator, an RND/MDR efflux transporter, and an outer membrane lipoprotein component. Furthermore, the CP4-like prophage segment...
encoded a toxin-antitoxin system, composed of the toxin (YpjF-YfjZ) of CP4-6 and the antitoxin (YkfI-YafW) of CP4-57, the IntA integrase, and the transcriptional regulator AlpA. A similar region (99% identity), inserted in exactly the same position, has also been described in plasmid pNDM-CIT (8).

The MDR region (nt 132292 to 173347) of pLec-476cz included the VIM-1-encoding integron In110 (4). In pLec-476cz, the 5′ conserved sequence (5′-CS) of In110 was disrupted by IS26, while the 3′-CS was intact (Fig. 2). The 5′-CS-associated IS26 comprised part of the Tn6020 composite transposon, which includes the aphA1 resistance gene. A second integron that is similar to In-194-B from pNL194, whose variable region comprised the dfrA1 and aadA1 cassettes (conferring resistance to trimethoprim and aminoglycosides, respectively) (16), was located upstream from the second IS26 of Tn6020. In-194-B lacked the entire 3′-CS due to an insertion of IS1 at the recombination site of the aadA1 cassette. IS1 was found at the boundary of the plasmid backbone.
Downstream from the 3'-CS of In110, a Tn1696 fragment (ΔTn1696-1), consisting of IS6100, the resI site, the mercury resistance operon (mer), and the 38-bp inverted repeat (IRmer) of the transposon, was identified. Next to ΔTn1696-1, a 21,780-bp sequence (nt 151568 to 173347), sharing extensive similarity with a contiguous sequence carried by p8025 (100% coverage; 99% identity) (10), was found. This sequence consisted of a Tn1721 fragment (ΔTn1721) including a tetracycline resistance operon (tet), pecM and tnpA, a gene encoding an EcoRII methylase, a parB-like gene, an arsenic resistance operon (ars), and a tnpA. The ars-associated tnpA was found at the boundary of the plasmid backbone.

For the second plasmid, pKpn-431cz, initial assembly resulted in 16 contigs (range, 250 to 57,604 bp; N50 = 34,778 bp). After filling the gaps, the complete sequence of pKpn-431cz was 142,876 bp in size, with the length-weighted average coverage of 379× (range, 100× to 1,132×). The plasmid included a contiguous segment of 50,175 bp (nt 1 to 50175) sharing extensive similarity with the backbone of the recently described IncFIIY-type plasmids (17–20) (Fig. 3), encoding NDM-like carbapenemases. This segment was composed of regions responsible for replication (repA gene), conjugative transfer (tra and trb genes), and plasmid maintenance (ardA gene and psiAB operon). pKpn-431cz lacked the repB gene and parAB operon that are characteristic for the IncFIIY-type plasmids (17). However, in the 19,699-bp segment adjoining the boundary of the presumably IncFIIY-derived part (nt 123178 to 142876), sequences resembling the FIBKPN replicon (repFIB gene), the maintenance sopAB and stbDE operons, and the umuD gene of pKPN3-like plasmids were identified (21).

The remaining 73,002-bp sequence (nt 50176 to 123177) of pKpn-431cz comprised a mosaic structure. This mosaic structure contained a 40,400-bp segment (nt 82778 to 123177) encoding VIM-1, which exhibited high similarity to the MDR region of pLec-476cz (100% coverage; 99% identity) (Fig. 2). Similarities between the two plasmids extended from IS1 to the ars-associated tnpA. Unlike pLec-476cz, the IS1 element lacked 681 bp of its 5′ end (ΔIS1). The ars-associated tnpA was found at the boundary of the pKPN3-like plasmid backbone, downstream of repFIB.

In the remaining part of the mosaic structure (nt 50176 to 82777), a 505-bp fragment of a Tn3-like transposon, consisting of the IRntp of the transposon and the 3′ end of tnpA, was found at the boundary of the IncFIIY plasmid backbone downstream of repA (Fig. 2). The tnpA gene was probably deleted due to the insertion of a Tn1696-like transposon.
The Tn1696-like sequence (nt 50950 to 73449) of pKpn-431cz included an integron similar to In37 from pHSH2, whose variable region comprised the aacA4, blaOXA-1, catB3, and arr-3 cassettes (22). The IRi of In37 was located between the resI and resII sites of the Tn1696 module in precisely the same position as In4 in Tn1696. Similar to In37 in the plasmid pHSH2 (22), the 3′-CS of the integron was duplicated. Between the two copies of the 3′-CS, an ISCR1 element, the qnrA1 resistance gene, and ampR were identified. The second copy of the 3′-CS bounded with a Tn1696 fragment, consisting of IS6100, the resI site, and the mer operon. The Tn1696-like transposon of pKpn-431cz was flanked by the IRtnp and IRmer of Tn1696, with IRtnp, disrupted by IS4321 while IRmer remained intact. However, direct repeats were not found at the boundaries of the Tn1696-like transposon structure, excluding its transposition into pKpn-431cz. Interestingly, resistance islands composed of a class 1 integron and multiple transposons included within a class II transposon structure have been previously identified in IncA/C, MDR plasmids (23, 24).

Adjacent to the Tn1696-like sequence, pKpn-431cz included a 3,002-bp segment (nt 73557 to 76558), exhibiting 99% identity with a contiguous sequence described in the IncN MDR plasmid pNL194 (16). This segment comprised three ORFs, a 320-bp fragment of a Tn5501-like transposon, ΔfipA, nuc, and the 3′ end of traG (ΔtraG). A Tn3-like transposon consisting of inverted repeats (IRs) of the transposon, tnpA, tnpR, and two ORFs was identified upstream of ΔtraG and next to ΔIS1. It is likely that insertion of the Tn3-like transposon deleted the remaining parts of ΔtraG and ΔIS1.
In the Czech Republic, the occurrence of VIM-producing *Enterobacteriaceae* is rare, with most of the isolates recovered from patients with a history of travelling abroad. However, the complete nucleotide sequences of two plasmids, carrying the VIM-1-encoding In110 integron from *Enterobacteriaceae* isolates of Czech origin, were presented in this study. In the *L. adecarboxylysta* isolate, In110 was localized on the IncHI1 plasmid pLec-476cz. Previous studies from the Czech Republic have reported the emergence of IncHI1 plasmids in *E. coli* and *Salmonella enterica* isolates of veterinary origin (10, 25). These findings may indicate that IncHI1 plasmids circulate in different species of *Enterobacteriaceae* in this specific geographical area. However, in the *K. pneumoniae* isolate, In110 was carried by the IncFII, plasmid pKpn-431cz. Interestingly, sequencing data showed that pLec-476cz and pKpn-431cz shared the same VIM-1-encoding mosaic region. *En bloc* acquisition of the VIM-1-encoding mosaic region by an IncFII-type plasmid from pLec-476cz is a plausible hypothesis regarding the formation of pKpn-431cz. The presence of a 21,780-bp sequence, previously described in the IncHI1 plasmid p8025, in pLec-476cz and pKpn-431cz further supports this notion. pKpn-431cz evolved more through acquisitions, deletions, and recombinations that may have resulted in the observed structure. These findings, which are in agreement with our previous results (26), punctuate the potential of large MDR segments through reshuffling of enterobacterial plasmids. Furthermore, they indicate that acquisition of carbapenemase-encoding genes by clinically insignificant species, like *L. adecarboxylysta*, is disquieting since such bacteria can act as hidden sources of important resistance determinants.

**Accession number(s).** The nucleotide sequences of the plasmids pLec-476cz and pKpn-431cz have been assigned GenBank accession numbers KY320277 and KY020154, respectively.

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We declare no conflicts of interest.

**REFERENCES**


