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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 IncFII$_Y$-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 *en bloc* acquisition of the VIM-1-encoding region from one plasmid by the other.

KEYWORDS carbapenemases, metallo-β-lactamases, IncHI1, IncFII$_Y$, integrative conjugative elements

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VIM-producing *Enterobacteriaceae* have been observed since 2001 in Greece (1). For at least a decade, VIM producers were the main carbapenemase-producing *Enterobacteriaceae* (CPE) in Europe (2). In the Czech Republic, the first two cases of VIM-producing *Enterobacteriaceae* were identified in 2011. The first case was a sequence type 323 (ST323) *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 *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 (*bla*$_{VIM-1}$-*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 *bla*$_{VIM-1}$-carrying plasmids were transferred from the clinical strains to rifampin-resistant *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 *bla*$_{VIM-1}$-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 *bla*$_{VIM-1}$-positive plasmids of different sizes (~150 kb [pKpn-431cz] and ~290 kb [pLec-476cz]) (5). The two plasmids were non-

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

Isolate	MIC (μg/ml) of[a]:														
	Pip	Tzp	Ctx	Caz	Fep	Atm	Imp	Mem	Etp	Gen	Amk	Cml	Tet	Sxt	Cip
L. adecarboxylata Lec-476cz	>64	64	>8	>32	>16	1	8	4	0.12	16	1	0.5	32	>32	2
E. coli A15 pLec-476cz	64	64	>8	>32	4	≤0.25	4	0.25	0.12	0.12	0.5	0.5	16	>32	≤0.06
K. pneumoniae Kpn-431cz	>64	>64	>8	>32	>16	16	16	8	2	16	1	32	>32	>32	8
E. coli A15 pKpn-431cz	64	64	>8	>32	8	≤0.25	4	0.5	0.25	0.12	0.5	4	16	>32	0.5
E. coli A15 (recipient)	≤0.5	1	≤0.06	≤0.25	≤0.12	≤0.25	≤0.12	≤0.12	≤0.12	0.12	0.5	≤1	0.5	1	≤0.06

[a]Pip, piperacillin; Tzp, piperacillin-tazobactam (inhibitor fixed at 4 μg/ml); 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.

typeable using the PCR-based replicon typing (PBRT) method (6). However, pKpn431cz was positive for the FII_Y replicon (7), and pLec-476cz was positive for the IncHI1 allele (8). Plasmids pKpn-431cz and pLec-476cz were extracted from *E. coli* transconjugants. Plasmids were sequenced using the Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA). Assembling the reads, filling sequence gaps, and analysis and annotation of the plasmid sequences were performed as described previously (9).

For pLec-476cz, assembly resulted in 24 contigs (range, 139 to 76,245 bp; N_{50} = 52,908 bp) with the length-weighted average coverage of 26× (range, 7× to 156×). After filling the gaps, the complete sequence of pLec-476cz resulted in a 311,758-bp molecule with a sequence closely related to the recently described IncHI1 plasmids p8025 (94% coverage; 99% identity) from the Czech Republic (10) and pNDM-CIT (80% coverage; 99% identity) from India (8) (Fig. 1). The pLec-476cz backbone was composed of regions responsible for replication (*repHIA* and *repHIB* genes), conjugative 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 Tn*4371*-like integrative conjugative element (ICE) (12), a CP4-like prophage sequence (13), and a multidrug resistance (MDR) region.

The Tn*4371*-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 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 Tn*4371*-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 RdfS 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 (*trbB* 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 (TTTTTTGTT) at the boundaries of the Tn*4371*-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-nega tive rods, like *Pseudomonas putida* H8234 (GenBank accession no. CP005976), *Enterob acter cloacae* CAV1669 (CP011650), and *Citrobacter freundii* CFNIH1 (CP007557), further supposing 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*1*-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

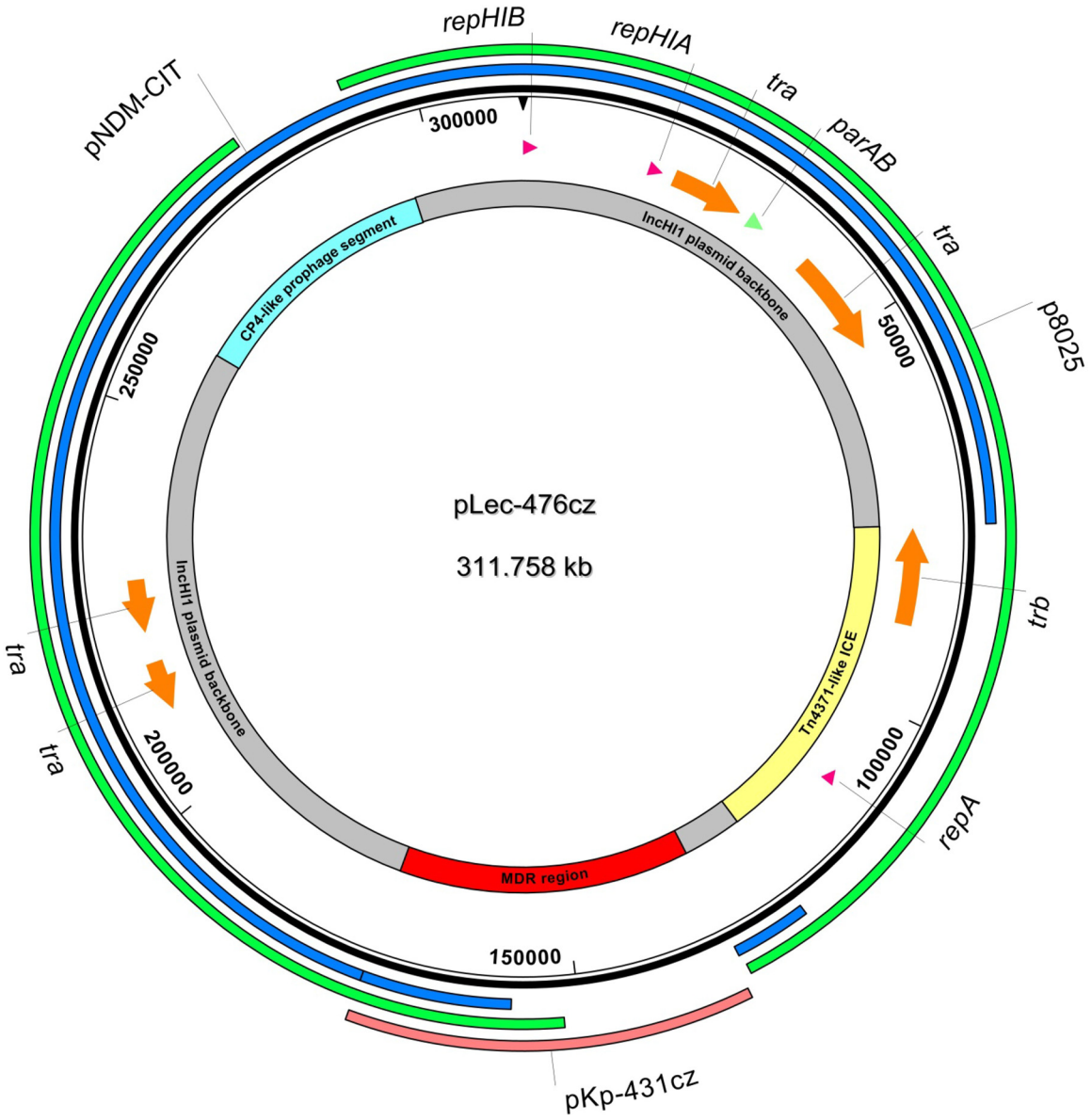

FIG 1 Overview of the IncHI1 plasmid pLec-476cz. The innermost circle shows the main regions of pLec-476cz. In the next circle, indicative genes and the direction of transcription are shown by arrows. Replicons of the plasmid are indicated as pink arrows. Genes responsible for plasmid transfer and maintenance are shown in orange and light green, respectively. Similarities with other plasmids are shown in the outer circle. Each color represents a unique plasmid.

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 IS*26*, while the 3′-CS was intact (Fig. 2). The 5′-CS-associated IS*26* comprised part of the Tn*6020* 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 IS*26* of Tn*6020*. In-194-B lacked the entire 3′-CS due to an insertion of IS*1* at the recombination site of the *aadA1* cassette. IS*1* was found at the boundary of the plasmid backbone.

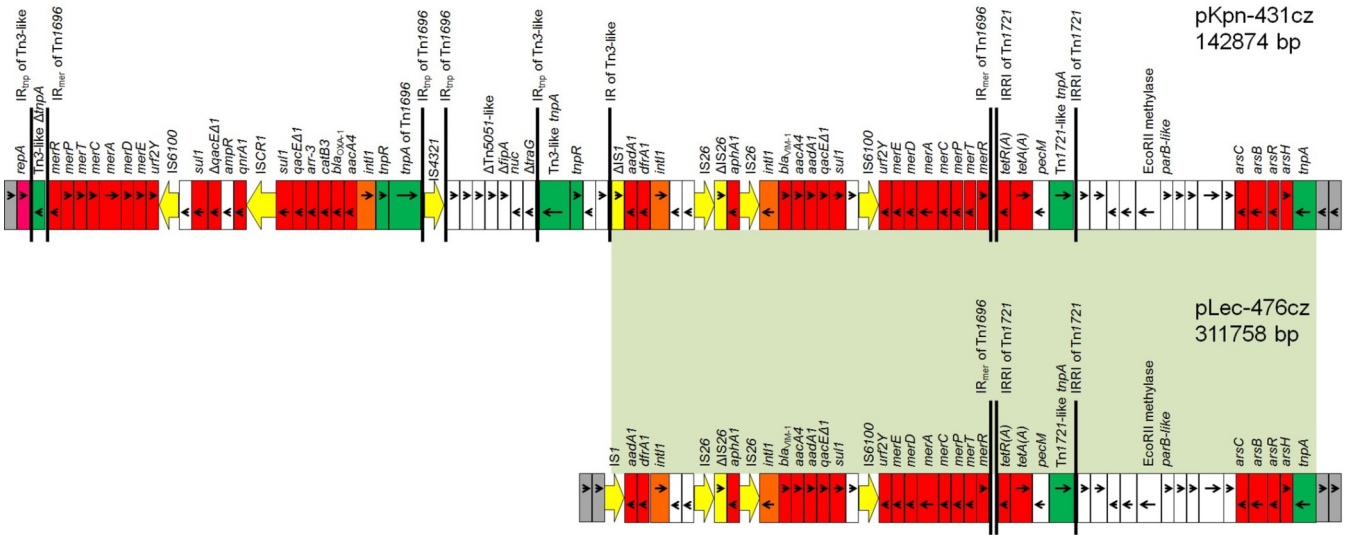

FIG 2 Detailed comparison of the MDR regions of the plasmids pLec-476cz and pKpn-431cz. Open reading frames (ORFs) are shown as rectangles (arrows within rectangles indicate the direction of transcription). Intact insertion sequences (ISs) are represented by arrows, while truncated IS elements appear as rectangles. Resistance genes, IS elements, and transposases are shown in red, yellow, and green, respectively. Orange rectangles indicate integrases. Gray rectangles indicate plasmid scaffold regions; the replicons of the plasmids are indicated as pink rectangles. The remaining genes are shown in white. The green shading shows the presence of the same VIM-1-encoding mosaic region on both pLec-476cz and pKpn-431cz.

Downstream from the 3′-CS of In110, a Tn*1696* fragment (ΔTn*1696*-1), consisting of IS*6100*, the *resI* site, the mercury resistance operon (*mer*), and the 38-bp inverted repeat (IR_{mer}) of the transposon, was identified. Next to ΔTn*1696*-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 Tn*1721* fragment (ΔTn*1721*) 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; N_{50} = 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 $IncFII_Y$-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 $IncFII_Y$-type plasmids (17). However, in the 19,699-bp segment adjoining the boundary of the presumably $IncFII_Y$-derived part (nt 123178 to 142876), sequences resembling the FIB_{KPN} 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 IS*1* to the *ars*-associated *tnpA*. Unlike pLec-476cz, the IS*1* element lacked 681 bp of its 5′ end (ΔIS*1*). 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 Tn*3*-like transposon, consisting of the IR_{tnp} of the transposon and the 3′ end of *tnpA*, was found at the boundary of the $IncFII_Y$ plasmid backbone downstream of *repA* (Fig. 2). The *tnpA* gene was probably deleted due to the insertion of a Tn*1696*-like

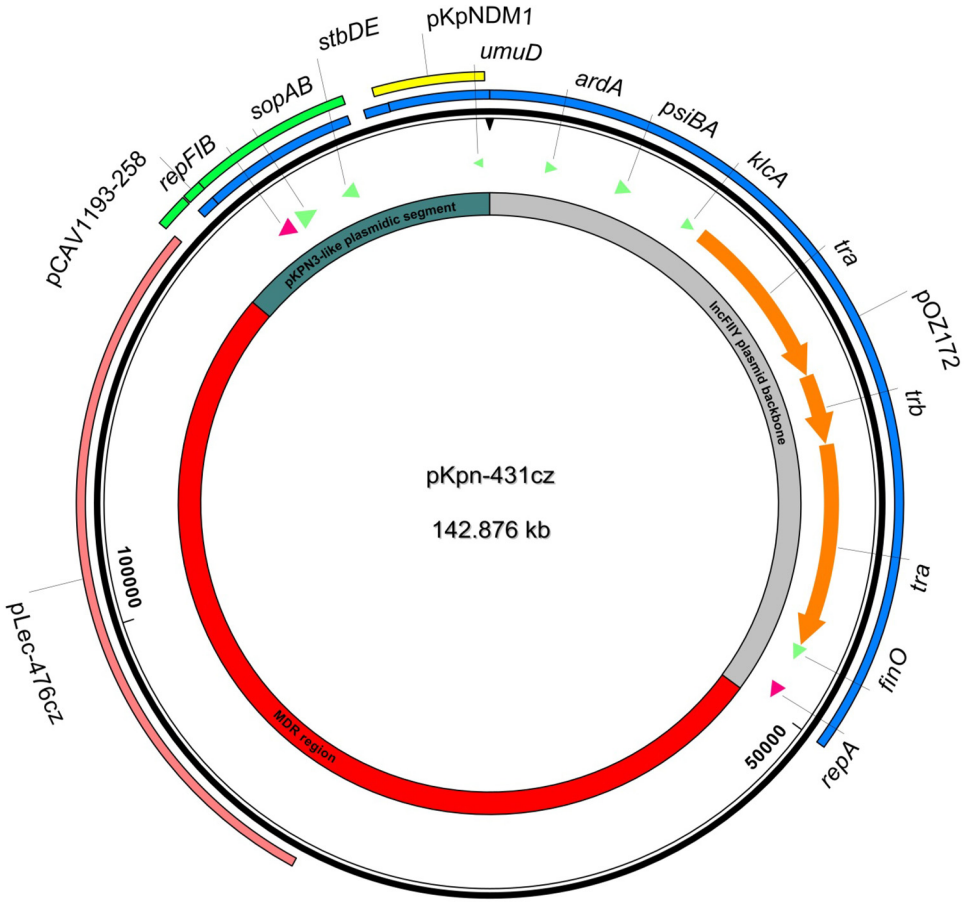

FIG 3 Overview of the IncFII$_Y$ plasmid pKpn-431cz. The innermost circle shows the main regions of pKpn-431cz. In the next circle, indicative genes and the direction of transcription are shown by arrows. Replicons of the plasmid are indicated as pink arrows. Genes responsible for plasmid transfer and maintenance are shown in orange and light green, respectively. Similarities with other plasmids are shown in the outer circle. Each color represents a unique plasmid.

transposon structure. The Tn*1696*-like sequence (nt 50950 to 73449) of pKpn-431cz included an integron similar to In37 from pHSH2, whose variable region comprised the *aacA4*, *bla*$_{OXA-1}$, *catB3*, and *arr-3* cassettes (22). The IRi of In37 was located between the *resI* and *resII* sites of the Tn*1696* module in precisely the same position as In4 in Tn*1696*. 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 IS*CR1* element, the *qnrA1* resistance gene, and *ampR* were identified. The second copy of the 3′-CS bounded with a Tn*1696* fragment, consisting of IS*6100*, the *resI* site, and the *mer* operon. The Tn*1696*-like transposon of pKpn-431cz was flanked by the IR$_{tnp}$ and IR$_{mer}$ of Tn*1696*, with IR$_{tnp}$ disrupted by IS*4321* while IR$_{mer}$ remained intact. However, direct repeats were not found at the boundaries of the Tn*1696*-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$_2$ MDR plasmids (23, 24).

Adjacent to the Tn*1696*-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 Tn*5501*-like transposon, Δ*fipA*, *nuc*, and the 3′ end of *traG* (Δ*traG*). A Tn*3*-like transposon consisting of inverted repeats (IRs) of the transposon, *tnpA*, *tnpR*, and two ORFs was identified upstream of Δ*traG* and next to ΔIS*1*. It is likely that insertion of the Tn*3*-like transposon deleted the remaining parts of Δ*traG* and ΔIS*1*.

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. adecarboxylata* 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 $IncFII_Y$ 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 $IncFII_Y$-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 spreading 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. adecarboxylata*, 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.

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