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Plasmid Content of a Clinically Relevant *Klebsiella pneumoniae* Clone from the Czech Republic Producing CTX-M-15 and QnrB1

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The entire plasmid content of a multidrug-resistant, CTX-M-15-producing *Klebsiella pneumoniae* ST416 clone was investigated. Two FII_K plasmids, pKDO1 (127 kb) and pKPN-CZ (207 kb), were identified and found to carry a formidable set of genes conferring resistance to toxic compounds, metals, and antimicrobial drugs and exhibiting novel features putatively associated with adaptation and fitness of the bacterium in the human host.

Extended-spectrum-β-lactamase (ESBL)-producing *Klebsiella pneumoniae* strains are opportunistic pathogens responsible for nosocomial infections primarily in intensive care units, and represent a high risk, especially for immunocompromised patients treated in these wards (1, 2). CTX-M-15 is the dominant type of cephalosporinase in *Enterobacteriaceae* in hospitalized patients as well as in the broader community (3) and is mobilized by plasmids of several incompatibility (Inc) groups, predominantly IncI1 and IncF (4, 5).

Plasmids of the IncF group represent one of the most frequent plasmid types, playing a major role in the dissemination of antimicrobial resistance in *Enterobacteriaceae*. They contribute to the fitness of their bacterial hosts by providing virulence and antimicrobial resistance genes and show rapid evolution (6).

Recently, an outbreak caused by ESBL-producing *Enterobacteriaceae*, mainly *K. pneumoniae*, was observed in a pediatric oncological clinic in the Czech Republic (7). Thirty-five *K. pneumoniae* strains belonging to seven sequence types (STs), carrying related CTX-15-positive $IncFII_K$ plasmids, were previously identified and characterized (7) (Table 1). In this study, the entire plasmid content of the most frequently isolated *K. pneumoniae* clone, ST416, was investigated to ascertain the structure and features of the CTX-M-15-carrying plasmid and to identify other plasmids contributing to the successful dissemination of this clone.

The complete DNA sequence of plasmids was obtained by applying the 454 genome sequencer FLX procedure on a library constructed from total plasmid DNA purified from an ST416 *K. pneumoniae* strain, by using an Invitrogen PureLink HiPure plasmid filter midiprep kit (Invitrogen, Milan, Italy), according to the manufacturer's procedure (Roche Diagnostics, Monza, Italy). Sixty-two contigs ranging from 33,451 to 252 bp, with at least 30-fold coverage, were obtained using GS-FLX gsAssembler software and compared to GenBank data using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). After the read status output and BLAST results were obtained, contigs were assembled by PCR-based gap closure and sequencing.

DNA sequence analysis showed the presence of two plasmids: pKDO1 (JX424423) and pKPN-CZ. pKDO1 is 127,509 bp and has high similarity to several *K. pneumoniae* plasmids, such as pKF3-94 (GenBank accession no. FJ876826), pKPHS2 (CP003224), and pKpQIL (GU595196; 52 to 61% coverage, 84 to 100% nucleotide identity) (Fig. 1). It belongs to the incompatibility group FII_K, as it carries the FII_{K7} replicon allele (assigned by replicon sequence typing [8]). pKDO1 also carries another replicon, encoding a Rep-3 family initiator replication protein showing 99.6% amino acid identity with replication proteins of pK245 (NC_010886) and pKF3-94, identified in *K. pneumoniae* strains from Taiwan and China, respectively. pKPN-CZ is 207,819 bp, carries replicon FII_{K8} and an additional FIB_{KPN} replicon specific for pKPN3-like *Klebsiella* plasmids (marked by violet boxes in Fig. 1), and is highly related (62 to 72% coverage, 81 to 100% nucleotide identity) to the $IncFII_K$ $IncFIB_{KPN}$ plasmids pKPN-IT (JN233704), from the KPC-producing *K. pneumoniae* ST258 clone (9), and pKPN3 (CP000648) (Fig. 1).

The plasmid pKDO1 contains a multidrug resistance region (MRR) of 39,761 bp carrying an interesting set of antibiotic resistance genes (red line in Fig. 1). The MRR is flanked on one side by IS*26* followed by *qnrB1* and ΔIS*3000* and on the other by additional common resistance genes with the end defined by an integron terminal repeat (IRt) of an In4-type class 1 integron. In particular, the MRR contains the ESBL $bla_{CTX\text{-}M\text{-}15}$ gene in a region showing high similarity to the MRR of plasmids pEK516 (EU935738) and pC15-1a (NC_005327), previously identified in *Escherichia coli* strains belonging to pandemic lineage ST131 (Fig. 1) (10, 11). It also carries the plasmid-mediated quinolone resistance *qnrB1* gene, for the first time associated with an $IncFII_K$ plasmid, flanked by an IS*26* element on the left and ΔIS*3000* truncated by Tn*5403* on the right. The same *qnrB1*-carrying element structure (99% nucleotide identity) was identified in *K. pneumoniae* plasmid pUC38-7 (EF682134). In addition, pKDO1 harbors the EcoRII restriction/antirestriction system flanked by the In4-type class 1 integron with the *dfrA14* gene cassette on the left, which showed 0 to 5 nucleotide differences from a region on some IncN plasmids and the IncF plasmid pK245 (Fig. 1), suggesting the

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TABLE 1 Multidrug-resistant *Klebsiella pneumoniae* strains harboring $IncFII_K$ replicons[a]

MLST	Strain	Plasmid size(s) (kb)[b]	PCR result for[c]:				
			repA	*fecA*	*clpK*	*yliB*	*mrk*
ST416	16	200, 120	+	+	+	+	+
	3	190, 120	+	+	+	+	+
	7	200, 120	+	+	+	+	+
	9	210, 120	+	+	+	+	+
	22	200, 120	+	+	+	+	+
	55	200, 120	+	+	+	+	+
	13	220, 120	+	−	+	+	−
	23	220, 140	+	−	+	+	−
	49	120	−	−	−	−	−
ST323	20	180, 120, 45	+	+	+	−	−
	27	180, 120, 45	+	+	+	−	−
	25	180, 100	+	+	+	−	−
	19	280, 220, 100	+	+	+	−	−
	11	255, 220, 100	+	+	+	−	−
	5	255, 220, 100	+	+	+	−	−
	12	120, 70	−	−	−	−	−
ST627	15	235	+	+	+	−	−
	30	235	+	+	+	−	−
	33	235	+	+	+	−	−
	39	235, 100	+	+	+	−	−
	43	235	+	+	+	−	−
	44	235	+	+	+	−	−
ST321	41	205, 120	+	+	+	−	−
	8	217, 120, 70	+	+	+	−	−
	2	203, 92, 63	+	+	+	−	−
	4	203, 115, 63	+	+	+	−	−
ST626	46	160, 120	−	+	+	−	−
	52	160, 120	−	+	+	−	−
	29	150, 120	−	+	+	−	−
ST280	56	220, 120, 60	+	+	+	−	−
	18	220, 100, 55	+	+	+	−	−
	42	220, 120, 55	+	+	+	−	−
ST628	6	183, 68	+	−	+	−	−
	17	180, 120	+	−	+	−	−

[a] All the strains except those belonging to ST627 and strain no. 6 carry $bla_{CTX\text{-}M\text{-}15}$. *K. pneumonae* strain 16 was selected for complete plasmid sequencing. All the strains are positive for the FII_K replicon.

[b] The plasmid sizes were determined by S1 pulsed-field gel electrophoresis in a previous study (7).

[c] Results of PCR-based screening for putative virulence determinants found in pKPN-CZ in a collection of *K. pneumoniae* strains from pediatric oncological patients. *repA* is used for detection of the FIB_{KPN} replicon, *fecA* for iron(III) dicitrate transport system, *clpK* for the marker of thermoresistance, *yliB* for the peptide/nickel ABC transporting system of *Pantoea* origin, and *mrk* for the junction between the type 3 fimbria cluster *mrkABCDF* and the pKPN-CZ plasmid scaffold.

exchange of the EcoRII restriction/antirestriction module among unrelated plasmids. In pKDO1, this region is flanked by two IS*26* elements that were likely involved in the acquisition of this module.

Plasmid pKPN-CZ showed an increased number of putative virulence-encoding clusters compared with other similar plasmids previously identified in *K. pneumoniae* strains, such as a KPC-producing ST258 clone (Fig. 1). In particular, a peptide/nickel ABC transporting system, previously found in plasmids pPANA10 (HE617161) and PAGR_p (CP003086) and on the chromosome (CP001875) of the human- and plant-pathogenic species *Pantoea anatis*, was identified on pKPN-CZ. The involvement of peptide transporting systems in increasing colonization abilities and virulence of bacterial strains was described previously (12, 13). pKPN-CZ contains the *mrkABCDF* gene cluster, showing >99% nucleotide identity with the *mrk* cluster, encoding type 3 fimbria, identified in the chromosomes of *K. pneumoniae* strains HS11286 (CP003200) and MGH 78578 (CP000647) (14). The same cluster was previously identified on IncX plasmids from *E. coli*, profoundly enhancing the ability of *E. coli* to form biofilms and increasing its conjugation efficiency (15, 16). To the best of our knowledge, this is the first evidence of a type 3 fimbria cluster associated with *Klebsiella* plasmids. The thermoresistance cluster (positions 63,633 to 78,484) identified in the chromosome of the human *Cronobacter sakazakii* strain ATCC 29544 (92% coverage, 99% nucleotide identity; accession no. FR714908) was also iden-

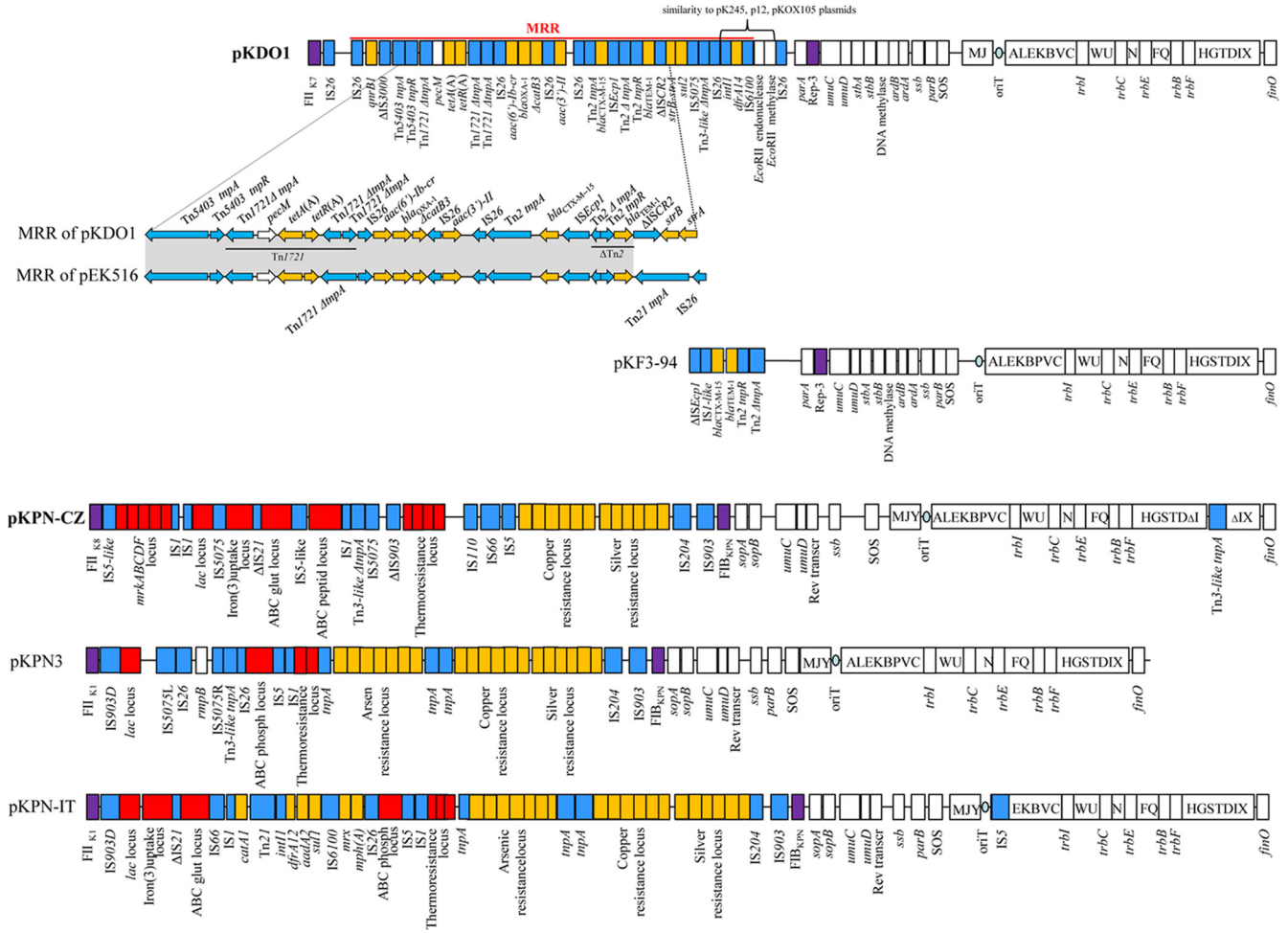

FIG 1 Major structural features of pKDO1 and pKPN-CZ. Plasmids sequenced in this study were compared as follows: pKDO1 was compared with pKF-94 (FJ876826); a part of the MRR of pKDO1 was compared with the similar region on pEK516 (EU935738), a plasmid identified in *E. coli* clone ST131; and pKPN-CZ was compared with pKPN-IT (JN233704) found in *K. pneumoniae* ST258 clones and with pKPN3 (CP000648). White boxes indicate plasmid scaffold regions that are in common among the plasmids. The *tra* locus is indicated by white boxes with capital letters indicating the respective *tra* genes (i.e., W represents *traW* and so on). Resistance genes are indicated by orange boxes. Transposon-related genes (*tnpA* and *tnpR*), the class 1 integrase gene, and insertion sequences are indicated by blue boxes. Other genes are indicated by colored boxes as follows: violet, replicase genes; red, the putative virulence clusters acquired by pKPN-IT, pKPN-CZ and pKPN3 including the *lac* operon, and loci corresponding to the iron(III) uptake system, phosphate/phosphonate ABC transport system (ABC phosph locus), nickel/peptide ABC transport system of *Pantoea* origin (ABC peptid locus), glutathione ABC transport system (ABC glut locus), type 3 fimbriae (*mrkABCDF*), and thermoresistance. The *lac* operon, the iron(III) uptake system, and ABC transport systems represent clusters formed by several genes but they are indicated by a unique block. Gray shading indicates >99% identity in the MRRs of pKDO1 and pEK516. Labels common to both MRRs appear only in the pKDO1 diagram, and only differences in pEK516 are labeled. The black lines show the extent of Tn*1721* and ΔTn*2* of MRR of pKDO1 and pEK516. The red line indicates the position of MRR in pKDO1. The region common to pKDO1, the IncN plasmids pKOX105 (FJ223605) and p12 (HM126016), and the IncF plasmid pK245 (DQ449578) is formed by an In4-type class 1 integron with the *dfrA14* gene cassette and EcoRII restriction/antirestriction system and is flanked by two IS*26* elements. The diagrams are not to scale.

tified in pKPN-CZ. Part of this cluster, including the gene *clpK*, that was demonstrated to increase bacterial thermal tolerance was previously found in other large IncFII$_K$ *Klebsiella* plasmids (17). We assume that transport systems, thermoresistance clusters, and fimbria clusters found in pKPN-CZ may be involved in virulence properties of the strains carrying them as well as in increased survival within the hospital environment, overall contributing to the successful dissemination and maintenance of these plasmids in *K. pneumoniae* during the hospital outbreak.

A PCR assay targeting five pKPN-CZ features, including the putative virulence determinants, was designed and tested on the 35 *K. pneumoniae* strains from the oncological clinic (Table 1; also, see Table S1 in the supplemental material). The FIB$_{KPN}$ replicon, the *clpK* gene involved in thermotolerance, and the iron(III) uptake system were largely prevalent in the strains of our collection, while the novel *Pantoea*-like peptide ABC transport system and the type 3 fimbria cluster were found only in *K. pneumoniae* ST416 clones harboring large plasmids (190 to 210 kb) (Table 1). Plasmid analysis and the PCR-based screening revealed high plasticity of the pKPN-CZ-like plasmids, likely due to plasmid rearrangements that occurred during the course of the outbreak. We propose this PCR-based approach as an efficient method for screening virulence determinants that can be identified in *K. pneumoniae* strains, presumably associated with pKPN-CZ-like plasmids, as observed in our collection.

Our study confirms that *K. pneumoniae* plasmids possess a

dynamic nature, the capacity for rapid evolution, and the ability to integrate new resistance and virulence determinants, overall increasing fitness and viability of the bacteria hosting them.

Nucleotide sequence accession numbers. The GenBank accession numbers for the sequences determined in this study are JX424423 (for pKDO1) and JX424424 (for pKPN-CZ).

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