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Complete Nucleotide Sequence of the IncN Plasmid Encoding IMP-6 and CTX-M-2 from Emerging Carbapenem-Resistant *Enterobacteriaceae* in Japan

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We have determined the DNA sequence of *Klebsiella pneumoniae* multidrug resistance plasmid pKPI-6, which is a self-transmissible IncN-type plasmid. pKPI-6 harboring bla_{IMP-6} and $bla_{CTX-M-2}$ confers a stealth-type carbapenem resistance phenotype on members of the family *Enterobacteriaceae* that is not detectable with imipenem. pKPI-6 is already epidemic in Japan, favoring the dissemination of IMP-6 and CTX-M-2 in members of the family *Enterobacteriaceae*.

Although still uncommon in Japan, carbapenem-resistant members of the family *Enterobacteriaceae* (CRE) have recently emerged and rapidly disseminated in the world (1). Stealth-type phenotypes, because of inability of automated systems to detect them, sometimes gave misleading results that caused a failure to stop the spread of CRE, e.g., *Klebsiella pneumoniae* carbapenemase-producing bacteria in Europe and the United States (2).

In 2011, five isolates of extended-spectrum β-lactamase (ESBL)-positive *K. pneumoniae* showed a paradoxical resistance phenotype, being highly resistant to almost all of the β-lactam antibiotics except imipenem (3). We therefore designated these isolates ISMRK (imipenem-susceptible but meropenem-resistant *Klebsiella*) (3). ISMRK bacteria carry the metallo-β-lactamase (MBL) gene bla_{IMP-6} in a cassette of a class 1 integron, In*722*, and the ESBL gene $bla_{CTX-M-2}$; both are on a ca. 47-kb self-transmissible plasmid designated pKPI-6. The combination of IMP-6 (3, 4) and CTX-M-2 gives ISMRK isolates a stealth phenotype not detectable with imipenem. We also alarmingly reported possible false diagnosis of ISMRK by clinical laboratories because not only imipenem but meropenem resistance of ISMRK cannot be screened for with the Vitek and RAISUS rapid automated susceptibility test systems (5). This is a more serious threat, leading to inappropriate antimicrobial therapy and a failure to stop the spread of ISMRK.

ISMRK strain a26 (3), isolated from a urine sample at the Hiroshima University Hospital, was used as the source of the pKPI-6 plasmid sequenced.

Whole-genome sequencing of pKPI-6 showed that the plasmid has a circularly closed 47,236-bp DNA sequence with an average G+C content of 50.99%, which is lower than that of the *K. pneumoniae* chromosome, which is about 57.7%, the highest G+C content of any species in the family *Enterobacteriaceae* (6). The numbering of pKPI-6 commences at the first nucleotide of the ATG start codon of *repA* (see Table S1 in the supplemental material). Annotation of the finished sequence data shows that pKPI-6 contains 53 putative genes, 4 of which encode proteins with no homology to known genes. Among the other 49 genes with known functions, the following 5 confer resistance to antibiotics: *aacA4′* (a derivative of *aacA4*) (7), *aadA2*, *tetR-tetA*, bla_{IMP-6}, and $bla_{CTX-M-2}$. pKPI-6 possessed a typical incompatibility group N (IncN) plasmid backbone (Fig. 1; see Table S1 in the supplemental material). IncN plasmids are important vehicles of clinically relevant resistance genes, including MBL and ESBL genes (8). There are three insertions of acquired extra DNAs containing resistance genes, an integron region including bla_{IMP-6}, a segment of Tn*1721* including *tetA* and *tetR*, and an IS*Ecp1* region including $bla_{CTX-M-2}$ (Fig. 1). Sequence analysis showed that the backbone gene organization of pKPI-6 is highly homologous to that of multidrug resistance-encoding IncN plasmids pKP96 (9) (accession no. EU195449; coverage, 100%; identity, 99%) and pN3 (accession no. FR850039; coverage, 97%; identity, 99%) (Fig. 1). Multilocus sequence typing (MLST) (8) shows that pKPI-6,

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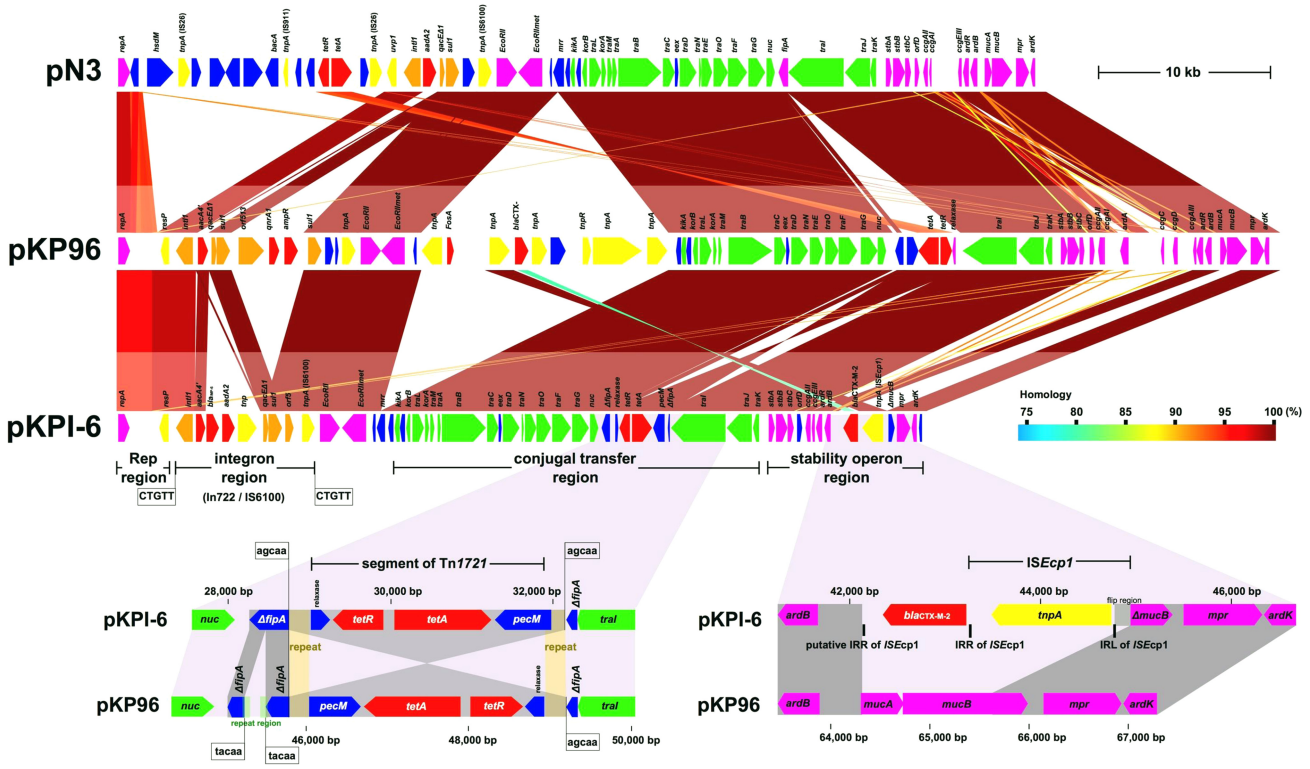

FIG 1 Structural comparison of pKPI-6, pKP96, and pN3. The nucleotide sequence of pKPI-6 plasmid DNA purified from a transconjugant was determined by the random shotgun sequencing method as described previously (15). Collected sequences were assembled by using Phrap software (version 1.080730) (16). Gaps were closed by direct sequencing of PCR products amplified with oligonucleotide primers designed to anneal each end to the neighboring contigs. Sequence reads were processed by using MetaGeneAnnotator (17), the InSilico molecular cloning software package, genomics edition (InSilico Biology Inc., Yokohama, Japan), the program BLASTP (18), and GenomeMatcher software (19). Color shadings between plasmids indicate homologous regions. Sequences of the terminal direct repeats of the acquired region are shown. ORFs are represented by pentagons, and annotated genes are colored on the basis of predicted gene function as follows: antimicrobial resistance genes, red; conjugation genes, green; transposons, yellow; integrons, orange; plasmid maintenance genes, violet; hypothetical genes, blue. Two regions, segments of Tn*1721* and $bla_{CTX\text{-}M\text{-}2}$-IS*Ecp1*, are visually extended at the bottom to show the difference between pKPI-6 and pKP96. Boxed nucleotide sequences represent direct duplications. Comparative analysis and generation of the image shown were performed with GenomeMatcher software (19). IRL, left inverted repeat; IRR, right inverted repeat.

pKP96, and pN3 belong to sequence type 5 (ST5). The class 1 integron of pKPI-6, designated In*722* (10) (Fig. 2), and IS*6100* are located at the same position on pKP96, suggesting that the class 1 integron was inserted by IS*6100*, creating a duplication of a 5-bp direct repeat (CTGTT) (Fig. 1). The fourth gene cassette of In*722* contains a gene encoding a potential transposase related to the IS*91* family of transposases (11). This transposase was identified as IS*Kpn22* by ISfinder (http://www-is.biotoul.fr//).

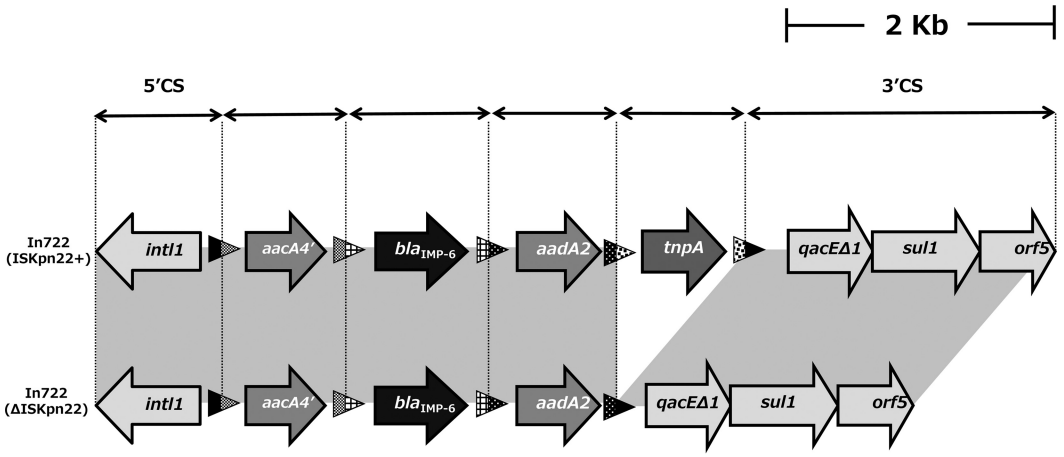

FIG 2 Schematic representation of the genetic content of the $bla_{IMP\text{-}6}$ integron (In*722*) lacking IS*Kpn22*. The *attI1* and *attC* elements are shown as triangles. 5′CS and 3′CS represent the 5′ and 3′ conserved segments, respectively.

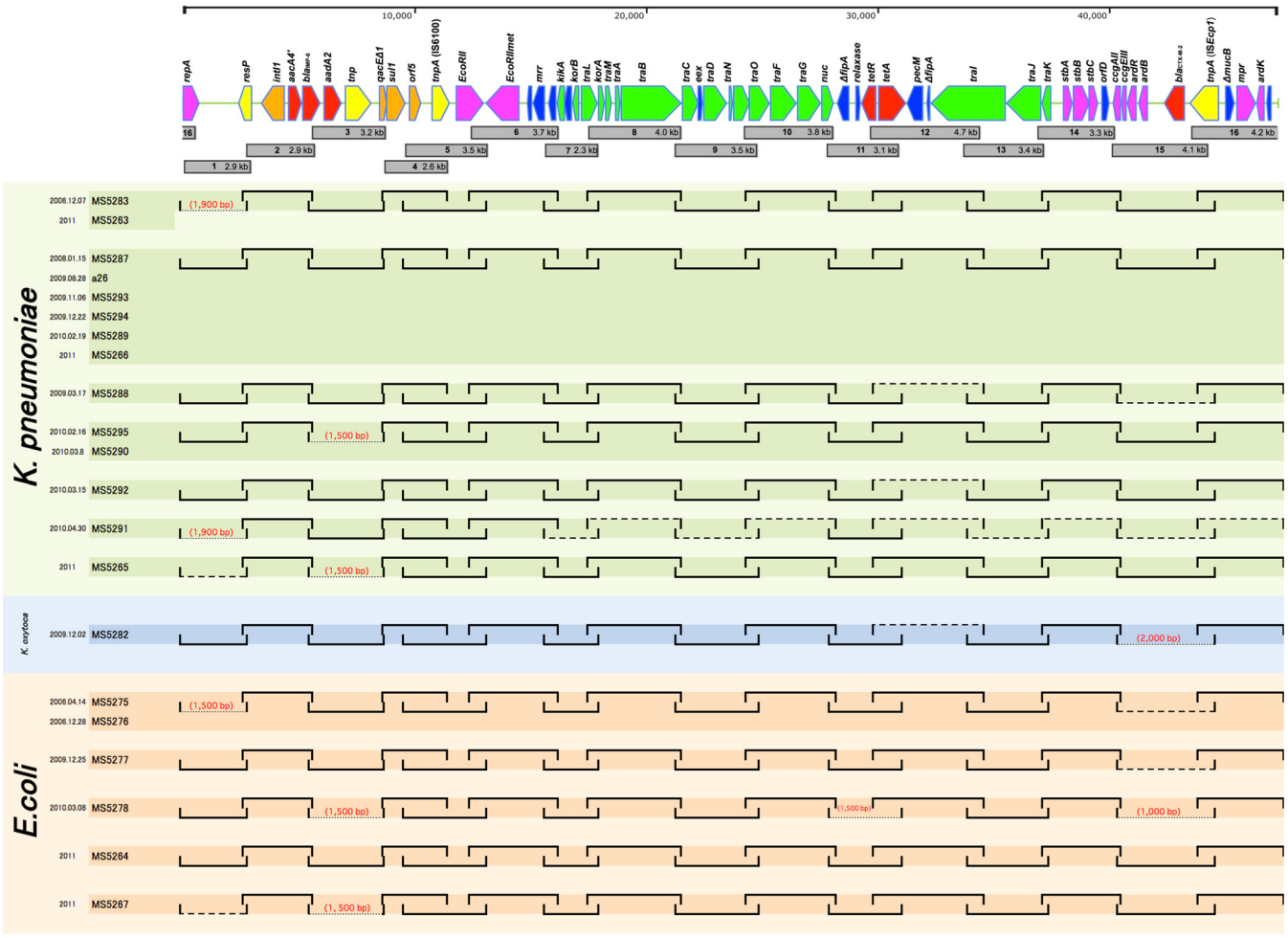

FIG 3 Summary of the PCR scanning analysis of IMP-6-positive plasmids from 13 *K. pneumoniae*, 1 *K. oxytoca*, and 6 *E. coli* clinical strains from the Kinki region. The plasmid structures in clinical isolates were examined by PCR scanning with 16 sets of primers (numbered 1 to 16; see Table S2 in the supplemental material) designed to cover all of plasmid pKPI-6. PCR amplification for the scan of pKPI-6 was performed with Quick *Taq* HS DyeMix (Toyobo, Tokyo, Japan) with 30 cycles of denaturing at 96°C for 20 s, annealing at 50°C for 30 s, and polymerization at 68°C for 5 min. By comparing the length of each amplified fragment to pKPI-6, the regional heterogeneity was determined. The gene arrangement of pKPI-6 is presented at the top. PCR products covering the entire plasmid sequence are depicted as numbered gray squares. The numbers represent the primer sets used for PCR scanning (see Table S2 in the supplemental material). Solid lines represent the regions amplified. Where the length of the amplified fragment is different from that of pKPI-6, the estimated size of the fragment is shown in parentheses. If no PCR product was obtained when a prolonged extension time was used, no estimated size is shown.

The gene array of the conjugal transfer system of pKPI-6 and pKP96 was conserved, except for the orientation of a segment of Tn*1721* including the *tetA* and *tetR* genes (GenBank accession no. X61367) that was inserted between *nuc* and *traI* (Fig. 1). Comparison of the pN3 DNA sequence to those of pKPI-6 and pKP96 shows that the 3′ segment of Tn*1721* juxtaposed with two 245-bp short stretches of DNA in the opposite orientation is inserted into *fipA* by homologous recombination at TTGCT, resulting in direct repeat AGCAA at both ends (Fig. 1, lower left). Inversion of the segment between pKPI-6 and pKP96 may be due to the inverted repeat of the 245-bp short stretches. Another resistance gene, $bla_{\text{CTX-M-2}}$, was mobilized with an IS*Ecp1* mobile element that appears to interrupt the *mucA* and *mucB* genes in the backbone structure of the IncN plasmid. There were 14-bp inverted repeats of IS*Ecp1* present at both ends where a 5′-flanking short stretch containing the left inverted repeat showed inversion in IS*Ecp1* (gray in Fig. 1).

A retrospective search for members of the family *Enterobacteriaceae* showing the ISMR phenotype in the Kinki region (the geographic center of Japan) identified 13 isolates of *K. pneumoniae*, 1 of *K. oxytoca*, and 6 of *Escherichia coli*; all 20 were positive for $bla_{\text{IMP-6}}$ and $bla_{\text{CTX-M-2}}$ (see Table S3 in the supplemental material). These strains were recognized primarily as ESBL producers but not as MBL producers because of their susceptibility to imipenem delineated by the automated susceptibility testing system.

Genomic profiling by pulsed-field gel electrophoresis performed as described elsewhere (12) and subsequent clustering analysis of *K. pneumoniae* carrying $bla_{\text{IMP-6}}$ and $bla_{\text{CTX-M-2}}$ identified a cluster with ≥80% similarity having eight isolates in the Kinki region and the representative *K. pneumoniae* isolate in Hiroshima, a26 (3). The MICs of a variety of antimicrobial agents clearly suggest that they have similar susceptibility profiles (shaded in Table S3 in the supplemental material). In addition, MLST (13) indicated that these isolates all belong to ST37.

We examined the genome organization of the bla_{IMP-6}- and $bla_{CTX-M-2}$-bearing plasmids from the 20 isolates of *K. pneumoniae*, *K. oxytoca*, and *E. coli* by the PCR scanning method (12). We generated 16 pairs of primers whose PCR products cover the entire 47.2-kb plasmid (Fig. 3; see Table S2 in the supplemental material). All of the primer pairs yielded PCR products of the expected sizes for seven isolates, including *K. pneumoniae* a26. In the other plasmids, some differences in fragment size were noted (one or two segments in each plasmid). Size variations were observed in limited regions, mainly regions 1, 3, and 15 shown in Fig. 3. The size of amplified region 1 was remarkably diverse, ranging from 1.5 to ~2.9 kb. This heterogeneity is due to the size variation in direct repeats called iteron sequences common in the replicative region of Gram-negative plasmids (14). Region 3 displayed two patterns, 3.2 and 1.5 kb. Sequencing analysis of the 1.5-kb DNA fragment showed that not only IS*Kpn22* but the entire gene cassette containing IS*Kpn22* was absent from the integron structure compared with the DNA sequence of In*722*; and this integron is designated In*722*ΔIS*Kpn22* (Fig. 2).

The In*722* and In*722*ΔIS*Kpn22* integrons both have a 6-bp insertion (CAGCAG) in the 5′ conserved segment that is not found in any other available class 1 integron sequence. This suggests that the two integrons are closely related and IS*Kpn22* is embedded within an integron gene cassette that can be excised through Intl-mediated recombination, which probably occurred in these strains, resulting in a shortened region 3. In*722*ΔIS*Kpn22* was found in three isolates of *K. pneumoniae* and two isolates of *E. coli* from the Kinki region (Fig. 3). In Hiroshima, we reported five ISMRK isolates from four different institutions, all of which carried In*722* but not In*722*ΔIS*Kpn22*. Some of the *K. pneumoniae* (two) and *E. coli* (three) isolates lack PCR products in region 15, which contains $bla_{CTX-M-2}$. PCR amplification with a 5′ primer designed for the $bla_{CTX-M-2}$ open reading frame (ORF) and a 3′ primer designed for *tnpA* yielded products of the expected size, suggesting that $bla_{CTX-M-2}$ is present next to *tnpA* in these plasmids.

The identification of pKPI-6 in clinical isolates of *K. oxytoca* and *E. coli* from the Kinki region strongly suggests that pKPI-6 has spread across species in at least two regions, but the unique phenotype suggests that it may have already spread further without being recognized in Japan.

Nucleotide sequence accession number. An annotated sequence of pKPI-6 has been submitted to the GenBank nucleotide sequence database under accession number AB616660.

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