Regional Dissemination of a Carbapenemase-encoding Plasmid: Plasmidome analysis Reveals Diverse Adaptations of Carbapenemase-Producing Enterobacteriaceae

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Abstract

Background. The global dissemination of carbapenem-resistant *Enterobacteriaceae* (CRE) threatens human health by limiting the range of usable antibiotics even against common bacterial infections. The spread of CRE is primarily due to the transmission of carbapenemase genes located on plasmids. However, few studies have comprehensively identified regionally spreading carbapenemase-encoding plasmids because of the difficulty to determine the complete sequence of a plasmid encoding carbapenemases. In a CRE surveillance study of 1,507 patients from 43 hospitals in northern Osaka, Japan, we previously found that 12% of the patients carried CRE and 95% of CRE isolates were IMP-6 producers. This result suggested a vast horizontal spread of a clonal plasmid carrying bla_{IMP-6} among *Enterobacteriaceae* in this region. In the current study, we aimed to describe the dynamics of this regional horizontal plasmid transmission.

Results. We systematically analysed the plasmids of 230 CRE isolates carrying bla_{IMP} obtained in our previous surveillance study by using whole genome sequencing and Southern blotting. We detected a major population (187 out of 230 bla_{IMP}-positive CRE isolates, 85.6%) that carried bla_{IMP-6} on the IncN plasmid pKPI-6, along with diverse minor subpopulations. Among the subpopulations, we identified a novel cluster carrying an IncF plasmid that leads to heteroresistance due to amplification of bla_{IMP-6}, resulting in covert transmission of bla_{IMP-6} or occasional chromosomal integration of bla_{IMP-6}. In addition, we detected one isolate that harboured bla_{IMP-1}, which is identical to bla_{IMP-6} except for a single point mutation, on pKPI-6 and thus had acquired a broader range of antimicrobial resistance.

Conclusions. Carbapenemase-encoding plasmid tracking revealed the clonal dissemination of pKPI-6 among chromosomally distinct isolates. Focusing on the mode of carbapenemase gene carriage is helpful for monitoring of horizontal spread of CRE isolates that is difficult to trace only by the comparisons of the whole genomes. A seemingly clonal horizontal dissemination of the predominant plasmid had embraced heterogenous subpopulations that contribute to diverse adaptations including covert transmission, stable chromosomal integration of bla_{IMP-6}, or broadened antimicrobial resistance patterns, ultimately leading to treatment failure.

Introduction

The rapid global dissemination of multidrug-resistant *Enterobacteriaceae* threatens healthcare systems worldwide [1]. Carbapenem-resistant *Enterobacteriaceae* (CRE) are of major concern because alternative treatment options are limited [2]. Carbapenem resistance is primarily conferred by carbapenemases, which are enzymes that hydrolyse carbapenem [3]. KPC, NDM, and OXA-48 are the most commonly detected carbapenemases [3]. Carbapenemase genes are generally plasmid-encoded and are frequently transmitted across species [4]. Genetic tracking of plasmids encoding carbapenemase genes has allowed monitoring the spread of CRE isolates. For example, structural similarities among plasmids from isolates obtained in a single hospital outbreak allowed elucidating links between patients carrying the isolates [5–7], and plasmid data accumulated globally revealed the worldwide spread of an epidemic plasmid

carrying *bla*$_{KPC}$.[8]. However, most regional surveillance studies compared the whole genomes of CRE isolates without analysing the clonality of the spreading carbapenemase-encoding plasmids, and few studies have comprehensively analysed regionally spreading carbapenemase-encoding plasmids in order to reveal the modes of horizontal plasmid transmission in a certain region [9].

We previously conducted a surveillance study of CRE in 1,507 patients from 43 hospitals in northern Osaka (population: 1,170,000, area: 307 km^2), Japan [10], and we reported that 12% of the patients carried CRE and 95% of CRE isolates harboured *bla*$_{IMP-6}$, the predominant carbapenemase in Japan. The predominance of this particular carbapenemase gene might have resulted from vigorous horizontal spreading of a specific plasmid carrying *bla*$_{IMP-6}$, pKPI-6 [11], in this region. The aim of the current study was to profile the mode of carriage of carbapenemase genes, primarily *bla*$_{IMP}$, to unveil their diversity within a defined geographical region.

Results

Dissemination of pKPI-6

All *bla*$_{IMP}$-positive CRE isolates of *Escherichia coli* (n = 135) and *Klebsiella pneumoniae* (n = 95) were classified into seven groups based on the results of S1-PFGE followed by Southern blot hybridization with probes for the *bla*$_{IMP}$ and *repA* genes (Figure 1). Ninety-nine of the 135 *E. coli* isolates (73%) and 88 of the 95 *K. pneumonia* isolates (93%) carried plasmids classified as Group pKPI-6 based on plasmid size and replicon type (Supplementary Figure S1). pKPI-6 was the predominant plasmid responsible for the transmission of *bla*$_{IMP-6}$ (187 out of 230 *bla*$_{IMP}$-positive CRE isolates, 85.6%). Next, we compared the similarity between pKPI-6 and 39 representative plasmids categorized as Group pKPI-6 based on WGS data. The overall sequence identity was 99 ± 0.28%, and the sequence coverage was 98 ± 4.0% (mean ± standard deviation) (Supplementary Figure S1). This analysis confirmed that pKPI-6 is the predominant plasmid carried by CRE isolates in the study area.

Genomic Analysis of Derivatives of the Predominant Plasmid, pKPI-6

During the characterization of the *bla*$_{IMP-6}$ plasmids mentioned above, nine *E. coli* isolates and three *K. pneumoniae* isolates possessed *bla*$_{IMP-6}$ plasmids categorized as Group IncN (Figure 1). Group IncN *bla*$_{IMP-6}$ plasmids were characterized by replicon type IncN and ranged from 35 to 264 kbp in size, which was different from the pKPI-6 plasmid of 50 kbp (Supplementary Figure S1). The complete sequences of these plasmids indicated that they had preserved the nearly complete locus of pKPI-6 and typically were multi-replicon plasmids that had integrated IncF-type plasmids framed by insertion sequences (Supplementary Figure S2 and Table S1). Additionally, two isolates (E208 and E328) of *K. pneumoniae* harboured plasmids categorized as Group Non-IncN KP (Figure 1B). These plasmids comprised a cassette carrying *bla*$_{IMP-6}$ without IncN-type *repA* of the pKPI-6 plasmid integrated into another plasmid (Supplementary Figure S3). Interestingly, *E. coli* isolate E119 and *K. pneumoniae* isolate E206

coharboured two distinct *bla*$_{IMP-6}$-encoding plasmids of different sizes and were categorized as Group double *bla*$_{IMP-6}$ (Figure 1 and Supplementary Figure S4). Barring occasional isolations of strains coharbouring different carbapenemase genes [12, 13], few studies have shown the coexistence of two identical carbapenemase genes on different plasmids within an isolate [14]. WGS revealed that isolate E119 carried pKPI-6 and an IncF-type plasmid (pEC743_1) that had a *bla*$_{IMP-6}$ cassette from pKPI-6 integrated (Supplementary Figure S5).

Characterization of IncF Plasmids Encoding *bla*$_{IMP-6}$

In addition to the *K. pneumoniae* isolates carrying Group Non-IncN KP plasmids, *E. coli* isolates carrying plasmids without IncN replicon were found in a single hospital (hospital D; Figure 1A). WGS of these isolates revealed that they harboured nearly identical *bla*$_{IMP-6}$-encoding plasmids with an IncFIA-type replicon (categorized as Group IncF) (Supplementary Figure S6A and Table S1). These plasmids were generated by integration of a cassette carrying *bla*$_{IMP-6}$ on pKPI-6 into another IncF plasmid at IS*26*. This IncF plasmid (pEC302/04; Supplementary Figure S6B) has been reported to transmit antimicrobial resistance since 1965 [15].

The minimum inhibitory concentrations (MICs) of meropenem for the *E. coli* isolates carrying Group IncF plasmids were low when compared with those of *E. coli* isolates harbouring other *bla*$_{IMP-6}$-encoding plasmids, such as pKPI-6 (Supplementary Figure S7). Mutations or deletions in the porin (OmpF) gene in *E. coli* have been reported to enhance resistance to β-lactams [16]. However, all *E. coli* isolates carrying Group IncF plasmids had a premature termination codon within *ompF*, whereas the other isolates carried wild-type *ompF* (Supplementary TableS2 and S3). MICs of meropenem were low for these Group IncF plasmid-carrying isolates, despite them being OmpF-deficient. To investigate carbapenem resistance in the same genetic background, plasmids from representative isolates in each *bla*$_{IMP-6}$ carriage group were transformed into the *E. coli* TOP10 strain and MICs for the transformants were determined. Transformant T305 carrying pE305_IMP6$_{single}$ of Group IncF from *E. coli* isolate E305 was more susceptible to meropenem than transformants carrying *bla*$_{IMP-6}$-harbouring plasmids of groups (Supplementary Table S4). *bla*$_{IMP-6}$ transcription in the pE305_IMP6$_{single}$ transformant was significantly lower than that in the pKPI-6 transformant (Supplementary Figure S8A), although the plasmid copy numbers in the bacterial cells were comparable (Supplementary Figure S8B). These results indicated that the lower MICs of meropenem in *E. coli* isolates carrying Group IncF plasmids were due to reduced transcription of *bla*$_{IMP-6}$.

Heteroresistance to Carbapenems: Enhanced Resistance Through Gene Amplification

E. coli isolates E305 and E318 were found to carry Group IncF plasmids, and WGS revealed that their chromosomes were nearly identical (query: E318, identity 100%, coverage 100%; query: E305, identity 100%, coverage 98% in BLASTN). Isolate E318 harboured genes encoding extended-spectrum β-lactamases (ESBLs), such as *bla*$_{CTX-M-14}$ and *bla*$_{TEM-1B}$, on a plasmid other than pE318_IMP6, whereas isolate E305 did not have these genes (Supplementary TableS5). IMP-6 confers resistance to

cephalosporins and meropenem, but it hydrolyses penicillins very poorly [17]. Therefore, isolate E318 exhibited broader antimicrobial resistance than isolate E305. In contrast, the MIC of meropenem for E305 was higher than that for E318.

WGS of E305 and E318 revealed the complete sequence of pE318_IMP6; however, it failed to determine the complete sequence of pE305_IMP6. Therefore, to analyse the structure of pE305_IMP6, we used a combination of WGS, Southern blotting, and qPCR analysis. The length and depth of each contig of pE305_IMP6 deduced from WGS are shown in the *de-novo* assembly graphs generated using the Bandage software [18] in Figure 2A. The total length of pE305_IMP6 deduced from WGS data was approximately 149 kbp. However, according to Southern blotting results, pE318_IMP6 and pE305_IMP6 were ~145 kbp and ~200 kbp in size, respectively (Figure 2B). Based on the depth of each contig, the copy number of each contig was predicted as follows: Contig3, 1 copy; Contig2 and Contig5, 6 copies; Contig1 and Contig6, 3 copies; Contig4, 5 copies (Figure 2A). Therefore, pE305_IMP6 was predicted to have a ~19-kbp repeat region consisting of triplication of Contig1 and Contig6, sextuplication of Contig2 and Contig5, and quintuplication of Contig4 (Figure 2C). Except for the repeat region, pE305_IMP6 and pE318_IMP6 exhibited high sequence similarity (identity; 99.27%, coverage; 100%) (Figure 2D). The $bla_{\text{IMP-6}}$ gene was located on Contig6 and was predicted to be triplicated. qPCR analysis corroborated that pE305_IMP6 carried three copies of $bla_{\text{IMP-6}}$, whereas pE318_IMP6 harboured a single copy (appendix p10). $bla_{\text{IMP-6}}$ transcription was significantly higher in isolate E305 than in isolate E318 (Figure 2E), even though the $bla_{\text{IMP-6}}$-carrier plasmid copy numbers in the cells of these isolates were not significantly different (Supplementary Figure S9B). Triplication of $bla_{\text{IMP-6}}$ in tandem resulted in a higher transcription level in E305, resulting in a higher level of resistance to meropenem.

Subculture of the clonal isolate E305 in broth medium revealed a mixture of subpopulations of bacteria carrying a plasmid with multiple $bla_{\text{IMP-6}}$ copies (which represented the majority) and bacteria carrying a plasmid with a single $bla_{\text{IMP-6}}$ copy. In Southern blotting for $bla_{\text{IMP-6}}$, a faint band at ~145 kbp was observed in addition to the major band at ~200 kbp (Figure 2B). It was also found that T305 (transformant of pE305_IMP6 $_{\text{single}}$ extracted from E305) carried a ~145-kbp plasmid without $bla_{\text{IMP-6}}$ amplification due to *recA* deficiency in the recipient *E. coli* TOP10 strain (Supplementary Figure S10) [19]. qPCR analysis confirmed that T305 carried one $bla_{\text{IMP-6}}$ copy on its plasmid (Supplementary Figure S9C). These results indicated the existence of a subpopulation carrying a plasmid with one $bla_{\text{IMP-6}}$ copy within *E. coli* isolate E305, whereas the majority of the population carried a plasmid harbouring three copies of $bla_{\text{IMP-6}}$.

Comparison of CRE Isolates Carrying pKPI-6 with Those Carrying Other Groups of Plasmids Harbouring $bla_{\text{IMP-6}}$

$bla_{\text{CTX-M-2}}$, which is an ESBL gene located distant from $bla_{\text{IMP-6}}$ on pKPI-6, compensated for the narrow range of hydrolysis of β-lactams by IMP-6 [11, 17]. However, toweverhhhhese two β-lactamase genes were not always transferred together from pKPI-6 to another plasmid. Plasmids categorized as Group

Non-IncN KP and Group IncF did not carry ESBL genes (Supplementary Table S6) and rarely conferred resistance to penicillins, in contrast to pKPI-6, which confers broad resistance to β-lactams (Figure 1). We next measured the conjugation efficiency of representative plasmids in each group (Supplementary Table S7). pKPI-6 plasmids and Group IncN plasmids, which had the entire pKPI-6 plasmid incorporated, showed a higher conjugation efficiency than Group Non-IncN KP/IncF plasmids. These characteristics may have facilitated the vast horizontal dissemination of pKPI-6 in the study area.

Compared with the chromosomal diversity among *E. coli* isolates bearing pKPI-6, *K. pneumoniae* isolates carrying pKPI-6 exhibited higher clonality as indicated by *Xba*I-PFGE analysis (Figure 1). This may be explained by the presence of the *kikA* gene on pKPI-6, the product of which reportedly promotes cell death of *K.pneumoniae* following conjugation [20]. The conjugation efficiency of pKPI-6 into *K. pneumoniae* ATCC13883 was considerably lower than that into *E. coli* TUM3456 (3.3×10^{-4} and 3.7×10^{-1}, respectively). Maybe only "*kikA*-resistant" *K. pneumoniae* are able to acquire pKPI-6, leading to clonal similarity among the *K. pneumoniae* isolates bearing pKPI-6.

Chromosomal Integration of bla_{IMP-6}

Unlike most CRE isolates, which carried the predominant pKPI-6 or other bla_{IMP-6}-encoding plasmids, three out of 135 *E. coli* isolates (E138, E300, and E302) harboured bla_{IMP-6} on their chromosomes as indicated by S1-PFGE followed by Southern blotting with bla_{IMP-6} probes (Figure 1A and Figure 3A). I-*Ceu*I-PFGE followed by Southern blotting with probes for the bla_{IMP-6} and 16S rRNA genes confirmed chromosomally located bla_{IMP-6} (Figure 3B). WGS revealed that the chromosome of isolate E138 had a cassette harbouring bla_{IMP-6} integrated, framed by a set of IS*15* (Figure 3C). The chromosomes of E300 and E302 had IncFIA plasmids carrying bla_{IMP-6} integrated (Figure 3D,E). While these plasmids were essentially identical to pE301_IMP6 (*E. coli*, Group IncF), these isolates were phylogenetically distinct on the *Xba*I-PFGE phylogenetic tree (Figure 1).

Emergence of pKPI-6-like Plasmid Harbouring bla_{IMP-1}

One *K. pneumoniae* isolate, E105, harboured bla_{IMP-1}, which is a single-nucleotide variant of bla_{IMP-6}, within a clonal cluster of pKPI-6 carriers (Figure 1B). Due to this mutation, E105 was resistant to imipenem, whereas most isolates carrying bla_{IMP-6} were susceptible to this antibiotic. WGS revealed that plasmids pKPI-6, pE013_IMP6 (plasmid group pKPI-6), and pE105_IMP1 were 99.8% identical, with a coverage of 100% (query: pE013_IMP6) (Figure 4). The only difference was the presence of a 714-bp region bracketed by a set of homologous regions in pE013_IMP6.

Discussion

IMP-producing *Enterobacteriaceae* have been reported sporadically on a global basis [2]. IMP-4-producing *Enterobacteriaceae* are endemic to Australia [21], and IMP-1-, 4-, and 8-producers have been occasionally detected in China [22]. Our study revealed the exclusive dissemination of IMP-6 producers (95% of CRE

isolates) in northern Osaka, Japan, consistent with findings in previous studies [11, 23, 24]. By tracking plasmids carrying *bla*$_{IMP-6}$, we clarified the relationships between *bla*$_{IMP}$-harbouring isolates that seemed diverse based on *Xba*I-PFGE analysis or comparison of short-read WGS results.

The current study revealed predominant dissemination of pKPI-6 in the study area, which may have resulted in the emergence of heterogeneous subpopulations. Group IncF plasmids possessed similar genomic structures, consisting of the globally disseminated IncF plasmid and a *bla*$_{IMP-6}$ cassette cointegrated on the pKPI-6 genome, without accompaniment of *bla*$_{CTX-M-2}$ (Figure S6). Our analysis revealed that *bla*$_{IMP-6}$ transcription was lower from Group IncF plasmid (pE305_IMP6 $_{single}$) than from pKPI-6 in *E. coli* cells of the same genetic background (Supplementary Figure S8). Low carbapenemase gene transcription is considered as one of the reasons for reduced resistance to meropenem [25]. Therefore, CRE isolates carrying Group IncF plasmids might have a reduced fitness cost for the carriage of *bla*$_{IMP-6}$, leading to further environmental dissemination of *bla*$_{IMP-6}$ [26].

Unlike for other plasmids in Group IncF, the complete sequence of pE305_IMP6 could not be obtained by long-read or short-read sequencing because of a signature 19-kbp repeat sequence unit. Based on combined WGS, Southern blotting, and qPCR data, we proposed a hypothetical structure of pE305_IMP-6 (Fig. 2C). Our results indicated that, despite its clonal origin, CRE isolate E305 comprised two different populations: a major population carrying pE305_IMP-6 with multiple *bla*$_{IMP-6}$ copies and a minor population carrying pE305_IMP-6$_{single}$ with a single *bla*$_{IMP-6}$ copy (Supplementary Figure S10). Moreover, the amplification of *bla*$_{IMP-6}$ on the IncF plasmid enhanced the transcription of *bla*$_{IMP-6}$ (Fig. 2E), resulting in increased resistance to meropenem (Supplementary Table S5). These results are consistent with previous studies reporting higher resistance to carbapenem through amplification of *bla*$_{OXA-58}$ [27] and *bla*$_{NDM-1}$ [19].

All *E. coli* isolates carrying Group IncF plasmids were found to possess *ompF* with a premature termination codon (Supplementary Table S3). When an isolate producing wild-type OmpF carries this plasmid with a single copy of *bla*$_{IMP-6}$, it is difficult to detect due to weaker resistance to meropenem. However, when an isolate with a porin mutation acquires a Group IncF plasmid with multiple *bla*$_{IMP-6}$ copies, it may abruptly exhibit strong resistance to meropenem without any direct trace of horizontal transfer. These types of plasmids may act as “hidden transmitters” of *bla*$_{IMP-6}$.

Moreover, we demonstrated chromosomal integration of Group IncF plasmids in some *E. coli* isolates. Carbapenemase genes have been reported to be transmitted primarily through plasmid conjugation [4], and chromosomal integration has been reported in a limited number of strains [28]. In our study, three out of 135 *E. coli* isolates (2.2%) exhibited chromosomal integration of *bla*$_{IMP-6}$, which presumably occurred during the vast horizontal spread of pKPI-6. Compared with *bla*$_{IMP-6}$ on plasmids, chromosomal *bla*$_{IMP-6}$ was not readily transmissible to another patient. However, these isolates may stably possess *bla*$_{IMP-6}$ within a patient and not lose carbapenem resistance through the elimination of plasmids harbouring *bla*$_{IMP-6}$.

In the early 1990s, some metallo-β-lactamases were reported in Japan [29, 30], followed by the identification of IMP-1 [31]. Since then, these β-lactamases have been frequently identified in Japan [32]. The single amino-acid variant, IMP-6, was identified in 2001 [17]. IMP-1 producers have disseminated mainly in eastern Japan, including Tokyo [23, 24, 33], whereas IMP-6 producers have been almost exclusively found in western Japan, including Osaka [7, 10, 11, 24]. Consistent herewith, in this study, only one *K. pneumoniae* isolate carrying bla_{IMP-1}, E105, was isolated in hospital A, where CRE carrying pKPI-6 were dominant. The patient carrying CRE isolate E105 was hospitalized for 512 days with other inpatients carrying CRE with pKPI-6, and the isolate showed ~ 83% similarity with a cluster of *K. pneumoniae* isolates carrying pKPI-6 in the *Xba*I-PFGE phylogeny (Fig. 1B). In addition, WGS of the plasmids revealed that a 714-bp region bracketed by 32-bp homologous regions was the only difference between pE105_IMP1 and pE013_IMP6 (Fig. 4A). This very small fragment appeared to have been removed by homologous recombination in pE105_IMP1 (Fig. 4B). Our results suggest that bla_{IMP-6} had disseminated via the transmission of pKPI-6, and spontaneous mutation may have generated the bla_{IMP-1}-encoding plasmid providing broader antimicrobial resistance, resulting in increased fitness in the clinical setting.

Conclusions

This multi-institutional surveillance study uncovered the clonal dissemination of a plasmid encoding a specific carbapenemase IMP-6 and demonstrated that a seemingly clonal horizontal dissemination of CRE isolates had embraced heterogeneous minor subpopulations, which exhibited broadened antimicrobial resistance, stable carriage of bla_{IMP-6} through chromosomal integration, or heteroresistance related to covert bla_{IMP} transmission. Such diverse gene adaptations might also be common among CRE isolates carrying other carbapenemase genes. By focusing on the modes of carbapenemase gene carriage, this study revealed the clonal dissemination of a carbapenemase-encoding plasmid, along with the presence of diverse subpopulations that would ensure and facilitate the dissemination of carbapenemase genes in various environments, resulting in serious complications in clinical settings.

Materials And Methods

CRE Isolates and PFGE Phylogenetic Analysis. We performed a CRE surveillance study of 1,507 patients hospitalized in 43 hospitals located in northern Osaka between December 2015 and January 2016 [10]. In the current study, we analysed 230 CRE isolates carrying bla_{IMP} obtained in the surveillance study, including 135 *E. coli* isolates and 95 *K. pneumoniae* isolates. All isolates were subjected to *Xba*I-digested PFGE for phylogenetic analysis [34]. Dendrograms were generated from PFGE patterns by the UPGMA method using BioNumerics software (version 6.6) (Applied Maths NV, Sint-Martens-Latem, Belgium).

Classification of bla_{IMP} **Carriage by PFGE and Southern Blotting.** The size and replicon type of bla_{IMP}-harboring plasmids were determined by S1-nuclease-digested PFGE followed by Southern hybridization (S1-nuclease was obtained from Takara Bio, Shiga, Japan). S1-PFGE and Southern blot hybridization for the bla_{IMP-6} and *repA* genes encoded on the IncN-type plasmid were performed as described in our

previous study [35]. The sizes of *bla*$_{IMP}$-encoding plasmids were determined using BioNumerics software (version 7.5) (Applied Maths NV). The modes of *bla*$_{IMP}$ carriage were classified into seven groups based on the sizes and replicon types of the plasmids carrying *bla*$_{IMP}$. The groups and their associated characteristics are as follows: Group pKPI-6, a pKPI-6-like *bla*$_{IMP-6}$-encoding plasmid (~ 50 kbp, encoding *repA* for IncN plasmid); Group IncN, a *bla*$_{IMP-6}$-encoding plasmid (not ~ 50 kbp, encoding *repA* for IncN plasmid); Group Non-IncN KP, a *bla*$_{IMP-6}$-encoding plasmid (without *repA* for IncN plasmid) harboured by *K. pneumoniae* isolates; Group IncF, a *bla*$_{IMP-6}$-encoding plasmid (without *repA* for IncN plasmid) harboured by *E. coli* isolates; Group Double *bla*$_{IMP-6}$, multiple plasmids with *bla*$_{IMP-6}$ harboured by a single isolate; Group Chromosome, chromosomal *bla*$_{IMP-6}$; Group Non-Typeable, a *bla*$_{IMP-6}$-encoding plasmid of unknown size; Group IMP1, a *bla*$_{IMP-1}$-carrier plasmid.

Isolates classified as chromosomal *bla*$_{IMP}$ carriers were further analysed to identify the location of *bla*$_{IMP}$. In brief, I-*Ceu*I endonuclease-digested PFGE followed by Southern blotting using probes for *bla*$_{IMP-6}$ and 16S rRNA genes was performed to confirm the location of the *bla*$_{IMP}$ gene in three *E. coli* isolates E138, E300, and E302, as previously described [28].

Antimicrobial Susceptibility Testing. Susceptibility to ampicillin, ampicillin/sulbactam, piperacillin/tazobactam, piperacillin, cefotaxime, cefepime, imipenem, and meropenem was determined by the broth microdilution method according to the Clinical and Laboratory Standards Institute document M100-S28 [36]. MICs of meropenem were determined using ETEST® (bioMérieux, Marcy l'Etoile, France), following the manufacturer's instructions.

Whole-Genome Sequencing and Genomic Analysis. Genomic DNA for long- and short-read sequencing was extracted using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany). Short-read sequencing was conducted on an Illumina HiSeq 3000 sequencer using the KAPA library preparation kit (Kapa Biosystems, Woburn, MA, USA) or on an Illumina MiSeq sequencer using the KAPA HyperPlus Library Preparation Kit (Kapa Biosystems). Long-read sequencing was conducted on a Nanopore GridION sequencer (Oxford Nanopore Technologies, Oxford, UK) using the SQK-LSK109 1D Ligation Sequencing Kit and the EXP-NBD103 Native Barcoding Kit. The reads were assembled and polished using Unicycler [37]. In cases where the complete plasmid sequences could not be constructed, sequences were assembled with CANU (version 1.8) [38] or flye [39] and improved using Pilon [40] or Racon [41]. The PlasmidFinder [42] and ResFinder [43] databases were used to identify antimicrobial resistance genes and plasmid replicon types, respectively. A detailed analysis of the insertion sequence was performed using ISfinder [44]. The sequences were annotated with RASTtk [45], and the genomic structures were compared with EasyFig [46]. Plasmids similar to those found in this study were identified using BLAST.

Transformation and Bacterial Transconjugation Assay. Plasmids were prepared from overnight cultures of *E. coli* isolates E033, E066, E174, and E305, and *K. pneumoniae* isolates E187, E188, E196, E208, and E328, using the Plasmid Miniprep Kit (Qiagen). Electro-competent TOP10 *E. coli* cells (Invitrogen, Waltham, MA, USA) were electroporated with the extracted plasmids using a Gene Pulser Xcell System

(Bio-Rad, Hercules, CA, USA). Following incubation in S.O.C. Medium (Invitrogen) for 2 h (6 h for isolate E305), transformants were selected on Luria–Bertani (LB) agar supplemented with 0.125 µg/mL meropenem (2 µg/mL cefotaxime for isolate E305).

Bacterial conjugation assays were performed using the transformants as donors and the sodium azide-resistant *E. coli* strain TUM3456 [47] as a recipient. After mixing overnight cultures of donors and recipients at a 1:10 volumetric ratio, the mixture (10 µL) was incubated on LB agar for 24 h at 37 °C. Transconjugants were selected on LB agar containing cefotaxime (2 µg/mL) and sodium azide (150 µg/mL). The conjugation frequency was calculated from the CFU as the number of transconjugants divided by the number of donors plus transconjugants.

Determination of the Plasmid Copy Number per Host Bacterial Cell. DNA of *E. coli* isolates E305 and E318, and *E. coli* transformants with plasmids pE188_IMP6 and pE305_IMP6 $_{single}$ (T188 and T305, respectively) was extracted using the DNA Mini Kit (Qiagen). Using qPCR, the copy numbers of the *repA2* gene on plasmids pE305_IMP6 and pE318_IMP6 and the bla_{IMP-6} gene on pE188_IMP6 were compared with the copy number of the *rrsA* gene encoding 16S ribosomal RNA on the chromosome. qPCRs were carried out using THUNDERBIRD SYBR qPCR Mix (TOYOBO Life Science, Osaka, Japan) on a LightCycler 96 System (Roche Life Science, Penzberg, Germany). Primers used for this assay are list in Table S8. qPCR analysis was performed using data from repeated experiments (n = 6), and the plasmid copy number per cell was calculated from Ct values using the comparative Ct method [48].

Determination of the Copy Number of bla_{IMP-6} **per Plasmid.** Plasmids of *E. coli* isolates E305 and E318 were extracted using the Plasmid Miniprep Kit (Qiagen). Using qPCR, the copy numbers of the bla_{IMP-6} gene were compared with those of the *repA2* gene on plasmids pE305_IMP6 and pE318_IMP6. qPCRs were carried out using THUNDERBIRD SYBR qPCR Mix on a LightCycler 96 System. Primers used for this assay are listed in Table S8. qPCR analysis was performed using data from repeated experiments (n = 5), and the bla_{IMP-6} copy number per plasmid was calculated from Ct values using the comparative Ct method.

Transcription of bla_{IMP-6}. *E. coli* isolates E305 and E318, and *E. coli* transformants T188 and T305 were incubated in LB broth until the optical density at 600 nm (OD_{600}) reached 0.3–0.4. Total RNA was extracted using the RNeasy Mini Kit (Qiagen). RNA was treated with ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO Life Science) to remove contaminating DNA and to reverse-transcribe the RNA into cDNA. For quality control, DNase-treated RNA that had not been reverse-transcribed was subjected to a DNA contamination test by qPCR. The *rrsA* gene encoding 16S ribosomal RNA served as an endogenous control for normalization. qPCRs were carried out using THUNDERBIRD SYBR qPCR Mix on a LightCycler 96 System. Primers used for this assay are listed in Table S8. qPCR analysis was performed using data from repeated experiments (n = 7), and transcript levels were calculated from Ct values using the comparative Ct method.

Declarations

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Availability of data and materials

The WGS data are available from the DDBJ (DNA Data Bank of Japan) database under the following accession numbers; AB616660, AP19402, AP19405, and AP022349 to AP022369. Raw data of isolate E305 are available at NCBI under accession numbers DRX184368 and DRX182679.

Authors' contributions

R.A. and Y.A. designed research; R.A. and R.K. performed research; R.A., Y.A., Y.S., D.T., Y.M., and D.M. analysed data; R.A., Y.A., N.Y., K.T., Y.F., and S.H wrote the paper. All authors edited and approved of the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interest

The authors declare that they have no competing interests.

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Figures

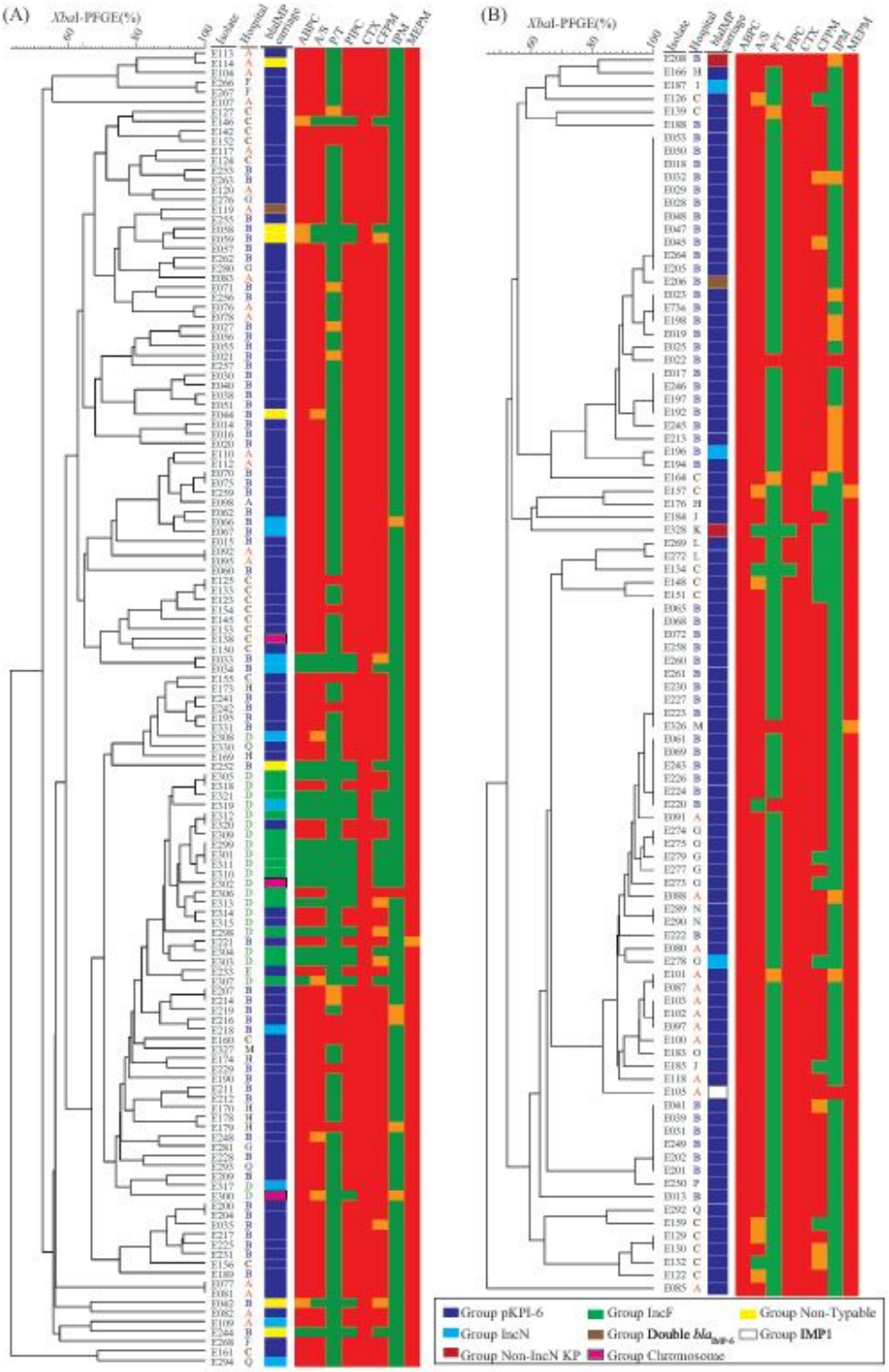

Figure 1

Phylogenetic trees based on XbaI-PFGE and classification of blaIMP carriage and antimicrobial resistance patterns. blaIMP carriage of (A) E. coli and (B) K. pneumoniae isolates was classified according to the size and replicon type of the blaIMP-carrier plasmids, determined by S1-PFGE and Southern blotting for blaIMP-6 and repA on the IncN plasmid. blaIMP carriage was classified designated as follows: blue, Group pKPI-6, pKPI-6-like plasmid (~50 kbp, encoding repA for IncN plasmid); light blue,

Group IncN, plasmid with repA for IncN, but not ~50 kbp; red, Group Non-IncN KP, plasmid without repA for IncN harboured by K. pneumoniae; green, Group IncF, plasmid without repA for IncN harboured by E. coli; brown, Group Double blaIMP-6, multiple plasmids with blaIMP-6 carried by a single isolate; enclosed pink, Group Chromosome, chromosomal blaIMP-6; yellow, Group Non-Typeable, failure to determine the size of plasmid carrying blaIMP-6; white, Group IMP1, blaIMP-1-carrier plasmid. Hospitals where the isolates were obtained are indicated as A to Q. Antimicrobial resistance measured by the broth microdilution method is indicated as follows: red, resistant; orange, intermediate; green, susceptible. Abbreviations: ABPC, ampicillin; A/S, ampicillin/sulbactam; P/T, piperacillin/tazobactam; PIPC, piperacillin; CTX, cefotaxime; CFPM, cefepime; IPM, imipenem; MEPM, meropenem

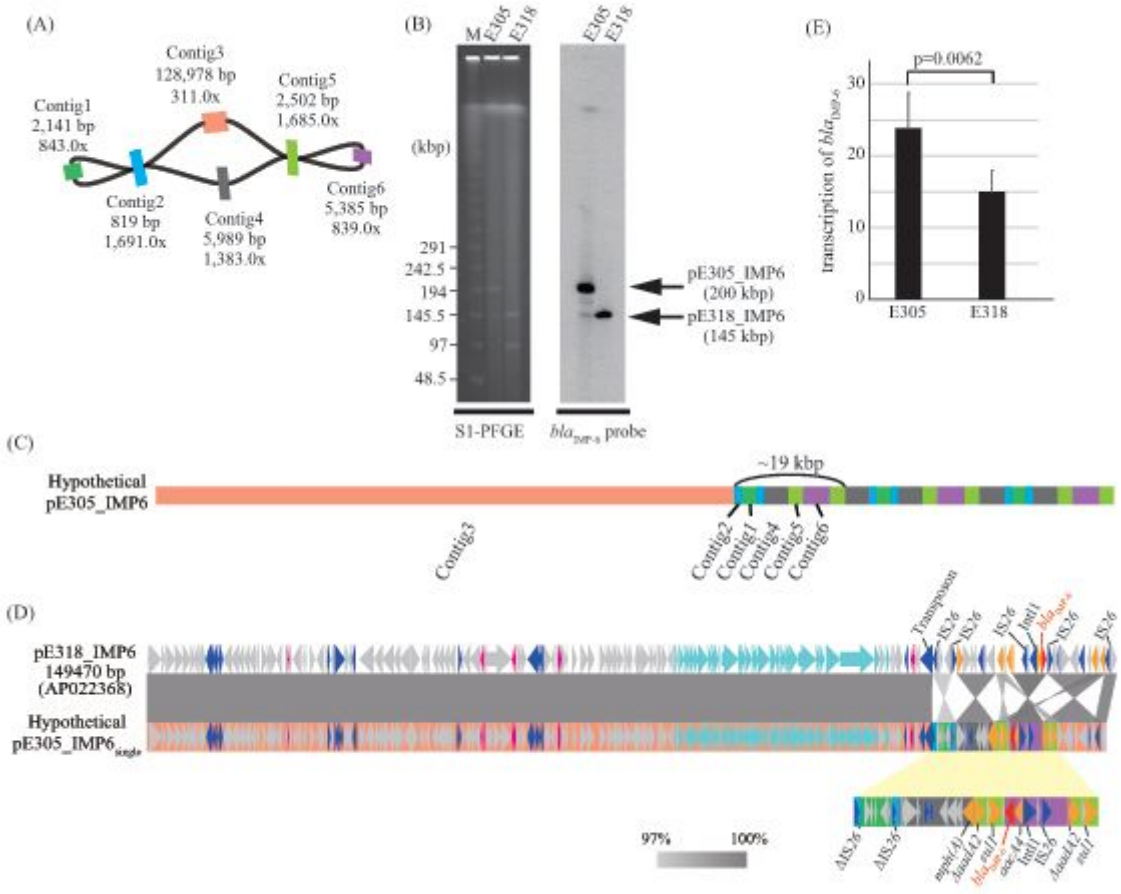

Figure 2

Genomic structure of Group IncF plasmid pE305_IMP6 and enhanced transcription of blaIMP-6. (A) Genomic structure of plasmid pE305_IMP6. De-novo assembly graph of plasmid pE305_IMP6 visualized by Bandage displays connections between contigs. The length and depth of each contig are shown. Contig2 connects Contig1 with Contig3 or Contig4, and Contig5 connects Contig6 with Contig3 or Contig4. (B) Sizes of plasmids pE305_IMP6 and pE318_IMP6. PFGE of S1-digested genomic DNA from E. coli isolates E305 and E318, followed by Southern blotting with a blaIMP-6 probe indicated the size of each plasmid. M, DNA size marker (lambda ladder; Bio-Rad). (C) Hypothetical structure of pE305_IMP6.

The colours correspond to the colours of contigs in (A). (D) Genomic comparison of pE318_IMP6 and hypothetical pE305_IMP6single. According to the overlap between contigs of pE305_IMP6, we assembled the hypothetical sequence shown and compared it with the sequence of plasmid pE318_IMP6. Except for the repeating, pE305_IMP6single and pE318_IMP6 were highly similar. Block arrows indicate confirmed or putative open reading frames (ORFs), and their orientations. Arrow size is proportional to the predicted ORF length. The colour code is as follows: red, carbapenem resistance gene; yellow, other antimicrobial resistance gene; light blue, conjugative transfer gene; blue, mobile element; purple, toxin-antitoxin. Putative, hypothetical, or unknown genes are represented as grey arrows. The grey-shaded area indicates regions with high identity between the two sequences. Accession numbers of the plasmids are indicated in brackets. The colours under arrows of pE305_IMP6single correspond to the colours of contigs in (A). (E) Transcript levels of blaIMP-6 in E. coli isolates E305 and E318. qPCR revealed significantly higher transcription of blaIMP-6 in isolate E305 than in isolate E318. The bar chart represents the mRNA transcript ratio of blaIMP-6 to the housekeeping gene rrsA, which was used as a reference gene. Bars indicate the mean ± standard deviation, calculated from sextuplet experiments. The p-value was calculated by the Mann–Whitney U test.

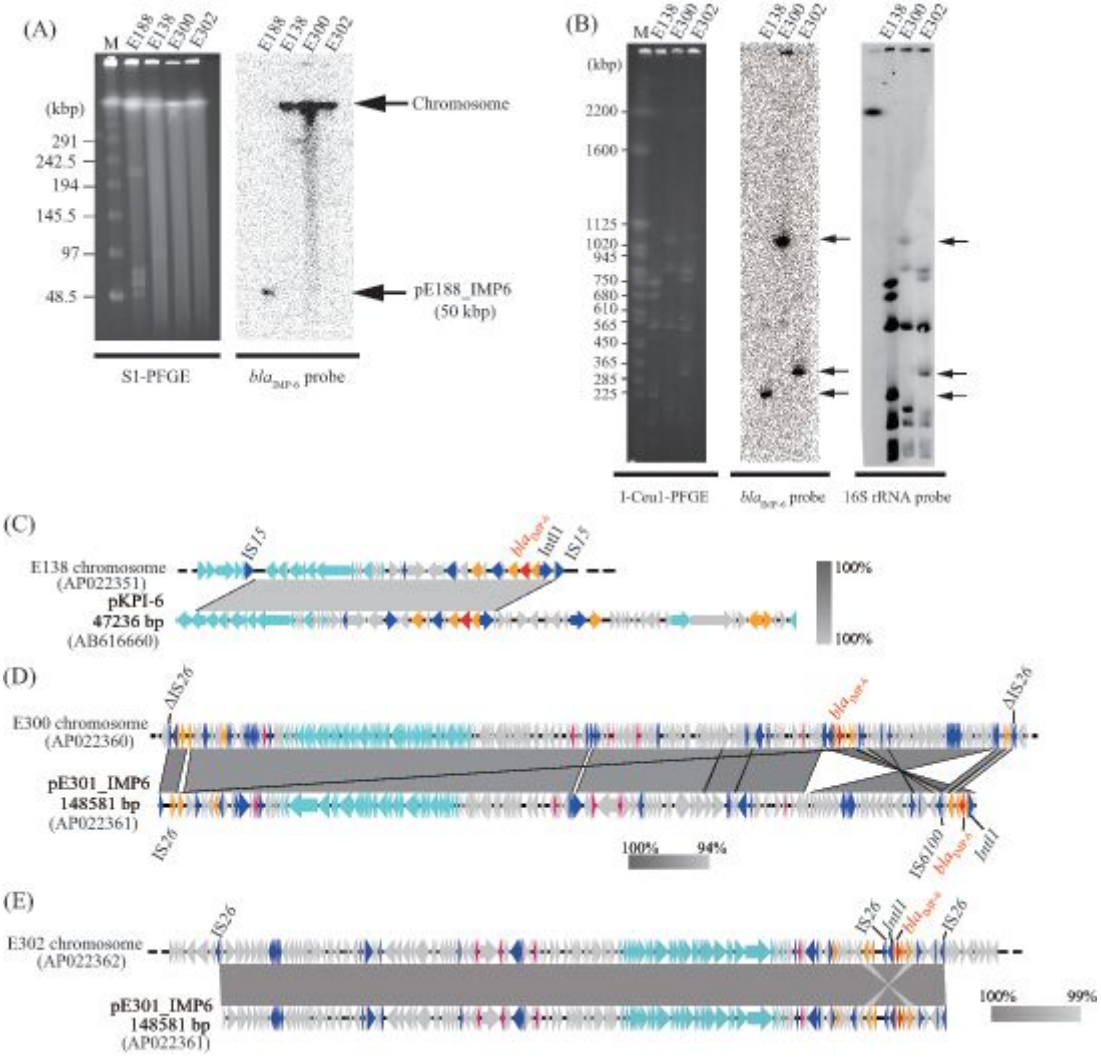

Figure 3

Chromosomal integration of blaIMP-6. (A) S1-PFGE followed by Southern blotting with a blaIMP-6 probe. Arrows indicate the segments carrying blaIMP-6 in E. coli isolates E138, E300, and E302 (Group Chromosome), and K. pneumoniae isolate E188 (Group pKPI-6). M, DNA size marker (lambda ladder; Bio-Rad). (B) I-Ceu1-PFGE followed by Southern blotting with blaIMP-6 and 16S rRNA probes. Arrows indicate the segments encoding blaIMP-6 or 16S rRNA proving that blaIMP-6 and 16S rRNA were located on the same segment. M, DNA size marker (Saccharomyces cerevisiae ladder; Bio-Rad). (C) Chromosomal integration of a region carrying blaIMP-6 in isolate E138. A 23-kbp region containing blaIMP-6 of plasmid pKPI-6 was integrated in the chromosome of isolate E138. This region was bracketed by a set of IS15. (D) Comparison of the chromosomal genomic structure of isolate E300 with plasmid pE301_IMP6. Isolate E300 carried chromosomal blaIMP-6, and the region bracketed by a set of mutated IS26 showed high similarity with plasmid pE301_IMP6 in Group IncF. (E) Chromosomal integration of plasmid pE301_IMP6 in isolate E302. Isolate E302 acquired chromosomal blaIMP-6 by incorporation of plasmid pE301_IMP6 bracketed by a set of IS26. The colour code is the same as that described in the legend of Figure 2.

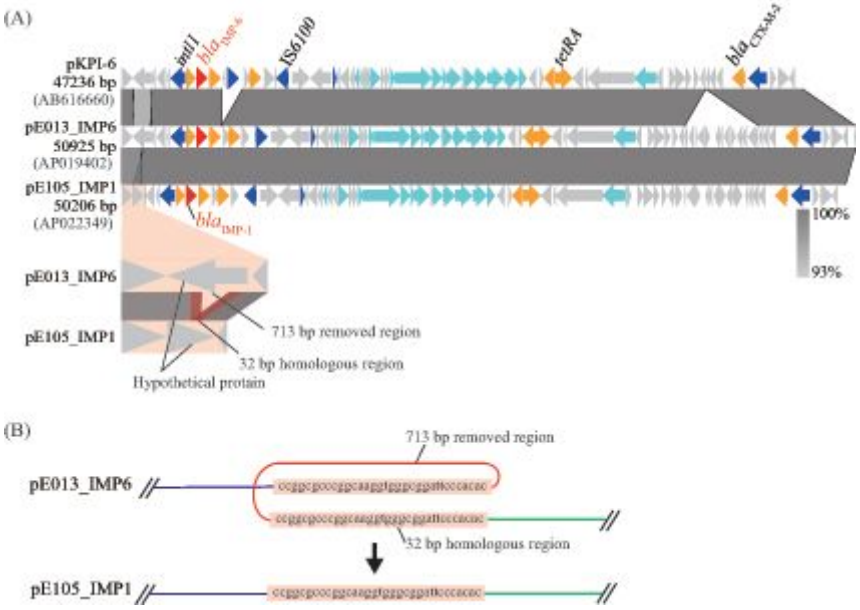

Figure 4

Plasmid pE105_IMP1 carrying blaIMP-1 was derived from plasmid pKPI-6 by homologous recombination. (A) Comparison of the pE105_IMP1 and pKPI-6 plasmids. The genomic structure of pE105_IMP1 (Group IMP1) was compared with plasmids pKPI-6 and pE013_IMP6 (Group pKPI-6) obtained from K. pneumoniae isolate E013. Differences between pE105_IMP1 and pE013_IMP6 are visually extended at the bottom. The colour code is the same as that described in the legend of Figure 2. (B) Schematic chart of homologous recombination. The 713-bp region of plasmid pE013_IMP6 was removed by homologous recombination at the 32-bp region.

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