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Co-carriage of *bla*$_{\text{KPC-33}}$ and *tmexCD2-toprJ2* on a Transferable Plasmid in an ST15-KL19 *Klebsiella pneumoniae* Isolate

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Abstract

Ceftazidime-avibactam and tigecycline remain first-line antibiotics for treating multidrug-resistant *Klebsiella pneumoniae*, yet their clinical utility is increasingly threatened by evolving resistance, primarily mediated by KPC variants and the TMexCD-TOprJ efflux system, respectively. Of note, their coexistence in *K. pneumoniae*, particularly on the same mobile genetic platform, represents an emerging antimicrobial resistance concern. In this study, *K. pneumoniae* strain 1640 was obtained as a clinical isolate and investigated using antimicrobial susceptibility testing, conjugation assays, whole-genome sequencing, and bioinformatic analysis. *K. pneumoniae* 1640 exhibited resistance to ceftazidime-avibactam but remained susceptible to carbapenems, aztreonam-avibactam, and tigecycline. Whole-genome sequencing revealed that *bla*$_{\text{KPC-33}}$ and *tmexCD2-toprJ2* were co-located on a 146,193-bp transferable multireplicon plasmid, which harbored a conserved backbone and variable resistance regions. Conjugation assays demonstrated successful transfer of this plasmid to *Escherichia coli* EC600 at a frequency of 1.56×10^{-5}. Plasmid acquisition resulted in decreased susceptibility to several β-lactams, whereas the tigecycline susceptibility remained unaltered. Phylogenetic analysis placed strain 1640 within the Chinese ST15-KL19 lineage; however, none of the other ST15 isolates examined

carried the identical *bla*$_{KPC-33}$/*tmexCD2-toprJ2* combination. These findings revealed that the carbapenem and tigecycline resistance determinants carried by strain 1640 were co-located on the same mobile plasmid and might potentially transmit horizontally to additional strains, warranting continued surveillance.

Keywords: *Klebsiella pneumoniae*, ceftazidime-avibactam resistance, *bla*$_{KPC-33}$, *tmexCD2-toprJ2*, conjugative plasmid

1. Introduction

KPC-type carbapenemases belong to class A β-lactamases and are commonly found in *K. pneumoniae*. The global spread of KPC-producing *K. pneumoniae* has greatly reduced the effectiveness of carbapenems, which were once considered key agents for treating infections caused by multidrug-resistant Gram-negative bacteria. The distribution of KPC enzymes also shows clear geographic differences. *bla*$_{KPC-2}$ is predominant in many parts of Asia, including China, whereas *bla*$_{KPC-3}$ and its derivatives are more frequently reported in Europe and the United States [1]. Most clinically important KPC variants are derived from the globally distributed ancestral enzymes KPC-2 and KPC-3. These variants often carry amino acid substitutions, insertions, or deletions in or around the active-site region, especially within the Ω-loop and nearby structural regions [2]. These changes can alter substrate recognition and catalytic activity of the enzyme. Notably, the number of newly reported KPC variants has increased markedly since 2019. The number reported between 2019 and 2020 exceeded the total reported during the previous 17 years [1]. The introduction and wider clinical use of ceftazidime-avibactam has also created selective pressure that favors the emergence of ceftazidime-avibactam-resistant KPC variants. *bla*$_{KPC-33}$ and *bla*$_{KPC-31}$ are two representative variants that have received considerable attention. Among them, *bla*$_{KPC-33}$ is one of the most frequently reported derivatives of *bla*$_{KPC-2}$.

The *tmexCD-toprJ* cluster encodes a mobilized resistance-nodulation-division (RND) efflux pump and can reduce bacterial susceptibility to several antimicrobial agents, including tigecycline. Multiple *tmexCD-toprJ* variants have now been identified [3]. Of

particular concern, this gene cluster is increasingly being detected in *K. pneumoniae*, an important clinical host of antimicrobial resistance. *K. pneumoniae* is a major host involved in carbapenem resistance and the spread of KPC-type carbapenemases. It also has a strong ability to acquire plasmids and accumulate resistance genes [4]. Therefore, acquisition of *tmexCD-toprJ* may further reduce susceptibility to tigecycline and other important therapeutic agents. This process may further limit treatment options for infections caused by multidrug-resistant bacteria. Molecular epidemiological studies have shown that *tmexCD-toprJ* has already spread among several clinically important Gram-negative bacteria, including *K. pneumoniae* and *Pseudomonas* spp. [5]. The gene cluster can be located on plasmids or integrated into the chromosome. It can also spread through both clonal expansion and horizontal gene transfer. More importantly, *tmexCD-toprJ* has been found together with several important resistance determinants, including carbapenemase genes. These findings suggest that different major resistance mechanisms are increasingly converging in clinically important hosts such as *K. pneumoniae*. In some cases, multiple resistance genes can even coexist on the same mobile genetic platform.

Overall, the continued emergence of KPC variants reflects ongoing changes in resistance mechanisms under the selective pressure of β-lactam treatment. At the same time, the spread of *tmexCD-toprJ* increases the risk of reduced activity of important drugs such as tigecycline. The convergence of these two resistance mechanisms in *K. pneumoniae*, especially their coexistence on the same mobile genetic platform, may further promote the spread of multidrug resistance and associated resistance genes. This convergence therefore deserves close attention.

2. Material and Methods

2.1. Bacterial Isolate

K. pneumoniae isolate 1640 was recovered from a clinical specimen collected at a tertiary teaching hospital in Zhejiang Province, China. The specimen was enriched in LB broth at 37°C overnight and then inoculated onto China blue agar supplemented

with 0.3 mg/L meropenem. Suspected colonies were identified by MALDI-TOF mass spectrometry (Bruker, Bremen, Germany), and carbapenemase production was detected by a colloidal gold immunochromatographic assay (GENESIS (Hangzhou), China).

2.2. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed by the broth microdilution method in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines [6]. The antimicrobial agents tested included imipenem (IPM), meropenem (MEM), aztreonam-avibactam (AZA), cefmetazole (CMZ), ceftazidime (CAZ), cefotaxime (CTX), piperacillin-tazobactam (TZP), cefoperazone-sulbactam (SCF), ceftazidime-avibactam (CZA), cefepime (FEP), polymyxin B (PB), tigecycline (TGC), ciprofloxacin (CIP), amikacin (AK), and aztreonam (ATM). Except for TGC, MIC breakpoints were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) M100, 36th edition (2026) [7]. TGC MICs were interpreted according to the criteria established by the U.S. Food and Drug Administration (FDA) [8].

2.3. Whole-genome sequencing and bioinformatic analysis

Genomic DNA was extracted using the PureLink Genomic DNA Mini Kit (Invitrogen, USA) and sequenced using both short-read sequencing on the Illumina NovaSeq 6000 platform and long-read sequencing on the Oxford Nanopore Technologies MinION platform. Illumina reads were assembled with SPAdes, followed by hybrid assembly with Unicycler [9-10]. The complete genome sequence was annotated using the Proksee platform and manually curated [11]. Genotyping, including species identification, MLST, serotyping and identification of antimicrobial resistance genes and virulence genes, was conducted using Kleborate v3.2.4 [12]. Representative 99 ST15 genomes from Feng et al. were obtained from NCBI [13]. Phylogenetic tree construction was performed using Parsnp v2.1.5 and visualized in iTOL[14]. pBKPC18-1 was identified as the backbone reference by comparison with PLSDB, NCBI BLASTn, and MOB-typer, while NR5632_p1 and pHNNC189-2 were used for comparison of the *bla*$_{\text{KPC-33}}$ and *tmexCD-toprJ* regions, respectively. Plasmid comparison was performed with

BRIG, and regions around *bla*$_{KPC-33}$ and tnfxB2-tmexCD2-toprJ2 were compared by BLASTn [15].

2.4 Conjugation

The transferability of p1640-tmexKPC was evaluated by a conjugation experiment using the filter-mating method [16]. *E. coli* EC600 were used as recipients. Transconjugants were then selected on screening plates containing 600 μg/mL rifampicin and 2/4 μg/mL CZA and further verified by MALDI-TOF MS and PCR for interspecies conjugation. Putative transconjugants were confirmed by PCR using previously reported primers [17][18], followed by Sanger sequencing for further verification.

3. Results

3.1. Antimicrobial Susceptibility of Strain 1640

Antimicrobial susceptibility testing showed that strain 1640 was resistant to ceftazidime, cefepime, ciprofloxacin, and ceftazidime-avibactam, with a ceftazidime-avibactam MIC of 16/4 mg/L. In contrast, the isolate remained susceptible to imipenem and meropenem (MICs ≤1 mg/L), as well as aztreonam-avibactam. Susceptibility to tigecycline, polymyxin B, and amikacin was also retained (Tab. 1).

Overall, strain 1640 showed a distinct susceptibility profile. It was resistant to ceftazidime-avibactam but remained susceptible to carbapenems and tigecycline. These results indicate that the observed resistance phenotype was not fully consistent with the resistance determinants detected in the genome.

3.2. Genomic Characteristics of Strain 1640

Whole-genome sequencing and hybrid assembly showed that strain 1640 contained one circular chromosome of 5,453,194 bp and one circular plasmid of 146,193 bp, with a total genome size of 5,599,387 bp (Fig. 1A–B). The strain was assigned to KL19 (*wzi19*) and OL2α.2 (O1αβ/O2β). It carried a truncated ICEKp12-associated ybt14 locus. No typical hypervirulence-associated genes were detected.

Chromosomal resistance-associated alterations were identified in *ompK35*, *ompK36*, *gyrA*, and *parC*. Additional resistance genes included *bla*$_{SHV-28}$, *fosA6, oqxAB*, and **aac(3)-IId*. *bla*$_{KPC-33}$ and the *tnfxB2-tmexC2-tmexD2-toprJ2* cluster were co-located on a multireplicon plasmid, which we designated p1640-tmexKPC. This plasmid carried three replicons, IncU, IncFIA(pBK30683), and IncFII(Yp), together with multiple mobile genetic elements.

Overall, p1640-tmexKPC showed a complex resistance-region structure, providing a genetic basis for the potential co-dissemination of *bla*$_{KPC-33}$ and *tmexCD2-toprJ2*.

3.3. Phylogenetic Analysis

A core-genome SNP-based phylogenetic tree was constructed for strain 1640 and 99 representative ST15 *K. pneumoniae* isolates, including 60 from China and 39 from other countries or regions (Fig. 2). Isolates from different regions were interspersed across the tree, with no clear geographic clustering. This indicates substantial genetic diversity within the ST15 population.

Strain 1640 clustered within a branch mainly composed of Chinese KL19 isolates. Pairwise SNP analysis showed that the closest isolate was BioSample SAMN16770105, differing by only 36 SNPs. This was followed by SAMN10600538 (40 SNPs), SAMN25087049 (44 SNPs), SAMN18042954 (45 SNPs), and SAMN24668372 (45 SNPs). These closely related isolates were all from China, and most belonged to KL19. This suggests a close chromosomal relationship between strain 1640 and the Chinese ST15-KL19 sublineage.

Despite their close core-genome relationships, clear differences were observed in resistance-associated features. Most closely related isolates carried *bla*$_{KPC-2}$, whereas strain 1640 carried both *bla*$_{KPC-33}$ and *tmexCD2-toprJ2*. No other isolate among the 99 reference genomes showed the same *bla*$_{KPC-33}$/*tmexCD2-toprJ2* combination. These findings suggest that the distinctive resistance genotype of strain 1640 is more likely related to changes in the accessory genome, including acquisition or remodeling of resistance plasmids, rather than major divergence of the chromosomal background. Its exact evolutionary origin and transmission route remain unclear.

3.4. Comparative Genetic Analysis of the p1640-tmexKPC Plasmid

Comparative plasmid analysis showed extensive sequence similarity between p1640-tmexKPC and the reference plasmids. MOB-typer identified pBKPC18-1 (CP022275.1) as the closest plasmid neighbor (Fig. 3). Most of the plasmid backbone was highly conserved, while sequence variation was mainly located around resistance-associated regions.

In the *bla*$_{KPC}$ region, p1640-tmexKPC showed a Tn*3*-like – IS*Kpn27* – *bla*$_{KPC-33}$ – ΔIS*Kpn6* – *korC* arrangement (Fig. 4). A similar structure was observed in pBKPC18-1, which carried *bla*$_{KPC-2}$ instead of *bla*$_{KPC-33}$. p1640-tmexKPC, pBKPC18-1, and pB_KPC all contained ΔIS*Kpn6*. In comparison, pB_KPC lacked the upstream IS*Kpn27*/Tn*3*-like region, while NR5632_p1 contained an intact IS*Kpn6* rather than ΔIS*Kpn6*.

The *tmexCD2-toprJ2* region was highly conserved among the compared plasmids (Fig. 5). All four plasmids shared the *tnfxB2* – IS*5-family element* – *tmexC2* – *tmexD2* – *toprJ2* arrangement. Differences were mainly observed in the flanking regions. Notably, a Tn*5403*-like element was located upstream of this cluster in p1640-tmexKPC.

Overall, p1640-tmexKPC contained a conserved plasmid backbone with distinct local structures surrounding *bla*$_{KPC-33}$ and *tmexCD2-toprJ2*. The co-location of these two resistance determinants on the same plasmid represents the main genetic feature of p1640-tmexKPC.

3.5. Conjugative Transfer of p1640-tmexKPC Plasmid

Filter-mating experiments yielded confirmed transconjugants, designated J1640. The transconjugants were identified as *E. coli* and retained the EC600 recipient background. PCR detected both *bla*$_{KPC-33}$ and *tmexCD2-toprJ2*, confirming horizontal transfer of the resistance plasmid from strain 1640 to *E. coli* EC600.

The conjugation frequency was 1.56×10^{-5} transconjugants per recipient cell.

Compared with EC600, J1640 showed reduced susceptibility to CAZ, CZA, and FEP. In contrast, the MICs of IPM, MEM, AZA, and TGC showed no obvious change (Table 1).

4. Discussion

In this study, we characterized a clinical ST15 *K. pneumoniae* strain, 1640, with an unusual resistance phenotype. The strain was resistant to CZA but remained susceptible to carbapenems, AZA, and TGC. Notably, $bla_{\text{KPC-33}}$ and *tmexCD2-toprJ2* were located on the same transferable multireplicon plasmid.

Strain 1640 showed CZA resistance but retained carbapenem susceptibility, which is consistent with the known features of KPC-33. KPC-33 differs from KPC-2 by the D179Y substitution in the Ω-loop. This change can reduce the inhibitory effect of avibactam and is associated with CZA resistance [19]. D179Y-type KPC variants have also been linked to the "see-saw effect", in which CZA resistance is accompanied by restored carbapenem susceptibility [20]. In this study, the MICs of IPM and MEM were both ≤1 mg/L, which was consistent with this pattern. Strain 1640 also carried OmpK35 p.Met83fs and OmpK36 p.134_135insGlyAsp. Therefore, its β-lactam susceptibility profile may be affected by both KPC-33 and changes in outer-membrane permeability.

Strain 1640 also remained susceptible to AZA. Wise et al. reported that AZA showed good in vitro activity against most KPC-positive Enterobacterales [21]. In our study, CZA resistance was not accompanied by reduced AZA susceptibility, which was generally consistent with previous reports. However, only one clinical isolate was included in this study. Therefore, the reason for the AZA-susceptible phenotype cannot be clearly determined.

A genotype–phenotype mismatch was also observed for *tmexCD2-toprJ2*. This gene cluster encodes a transferable RND-family efflux system and is associated with reduced TGC susceptibility. Wang et al. showed that expression of *tmexCD2-toprJ2* in *Escherichia coli* increased the TGC MIC by about eightfold [22]. Although strain 1640 carried this gene cluster, and its local genetic environment was highly similar to that of

pHNNC189-2 [23], its TGC MIC remained ≤1 mg/L. The TGC MIC also remained unchanged after the plasmid was transferred to EC600. These results suggest that carriage of *tmexCD2-toprJ2* alone does not always lead to an obvious TGC-resistant phenotype. Previous studies have suggested that *tnfxB2* regulation, promoter activity, gene expression level, gene dosage, and host background may affect its phenotype [24]. Further expression and regulatory studies are therefore needed.

The conjugation assay further confirmed the horizontal transferability of the plasmid. After transfer to EC600, susceptibility to CAZ, CZA, and FEP decreased, while the TGC MIC remained unchanged. These results show that the plasmid can affect β-lactam susceptibility. They also support that the presence of *tmexCD2-toprJ2* does not have a simple one-to-one relationship with TGC resistance.

From a plasmid structure perspective, the co-location of bla_{KPC-33} and *tmexCD2-toprJ2* was the main genetic feature of p1640-tmexKPC. Previous studies have shown that *tmexCD-toprJ* variants can be carried by different plasmid backbones and spread among different bacterial hosts [25]. p1640-tmexKPC showed high backbone similarity to several KPC-associated plasmids, with most differences located in resistance-associated regions. IS*Kpn27*, ΔIS*Kpn6, korC*, and a Tn3-like element were identified around bla_{KPC-33}, while a complete classical Tn4401 structure was not detected. In contrast, the core *tmexCD2-toprJ2* region was relatively conserved among related plasmids, with differences mainly found in the flanking regions.

Phylogenetic analysis showed that strain 1640 was closely related to Chinese ST15-KL19 isolates and was not a highly divergent ST15 isolate. ST15 is a globally distributed high-risk clone with a strong ability to acquire antimicrobial resistance genes [26]. Most closely related isolates carried bla_{KPC-2}, while none of the other 99 ST15 isolates carried the same bla_{KPC-33}/*tmexCD2-toprJ2* combination. This suggests that the distinct resistance profile of strain 1640 may be more closely related to changes in the accessory genome than to major chromosomal divergence. However, the current data cannot determine whether bla_{KPC-33} evolved locally from bla_{KPC-2} or was acquired through horizontal transfer. The origin and acquisition time of *tmexCD2-toprJ2* also remain unclear.

Overall, this study identified the co-existence of *bla*$_{KPC-33}$ and *tmexCD2-toprJ2* on the same transferable plasmid in an ST15-KL19 *K. pneumoniae* background. The resistance genotype was not fully reflected by the antimicrobial phenotype, especially for carbapenems and TGC. The plasmid was successfully transferred to *E. coli* EC600, confirming its horizontal transfer potential. These findings suggest that genomic analysis, antimicrobial susceptibility testing, and functional transfer assays should be combined when evaluating composite resistance plasmids.

Declarations

Ethics approval and consent to participate

The study was reviewed and approved by the Ethics Committee of The Second Affiliated Hospital of Zhejiang University School of Medicine (ethical approval no. 2026-0307). The study was conducted in accordance with relevant institutional, national, and international ethical guidelines and regulations, including the applicable principles of the Declaration of Helsinki. This retrospective study involved analysis of an anonymized bacterial isolate recovered during routine clinical diagnostics; no additional human specimens were collected, no experiments were performed directly on human participants, and no identifiable patient information was accessed or included. The need for informed consent was waived by the Ethics Committee of The Second Affiliated Hospital of Zhejiang University School of Medicine. All authors have read and approved the final version of this manuscript and consent to its publication.

Availability of Data and Materials

The genome assemblies of 1640 have been deposited in the National Center for Biotechnology information (NCBI) and are registered under BioProject accession NO. PRJNA1548813. All data are available from the corresponding authors upon reasonable request.

Consent for publication

Not Applicable.

Competing interests

The authors declare that they have no competing interests.

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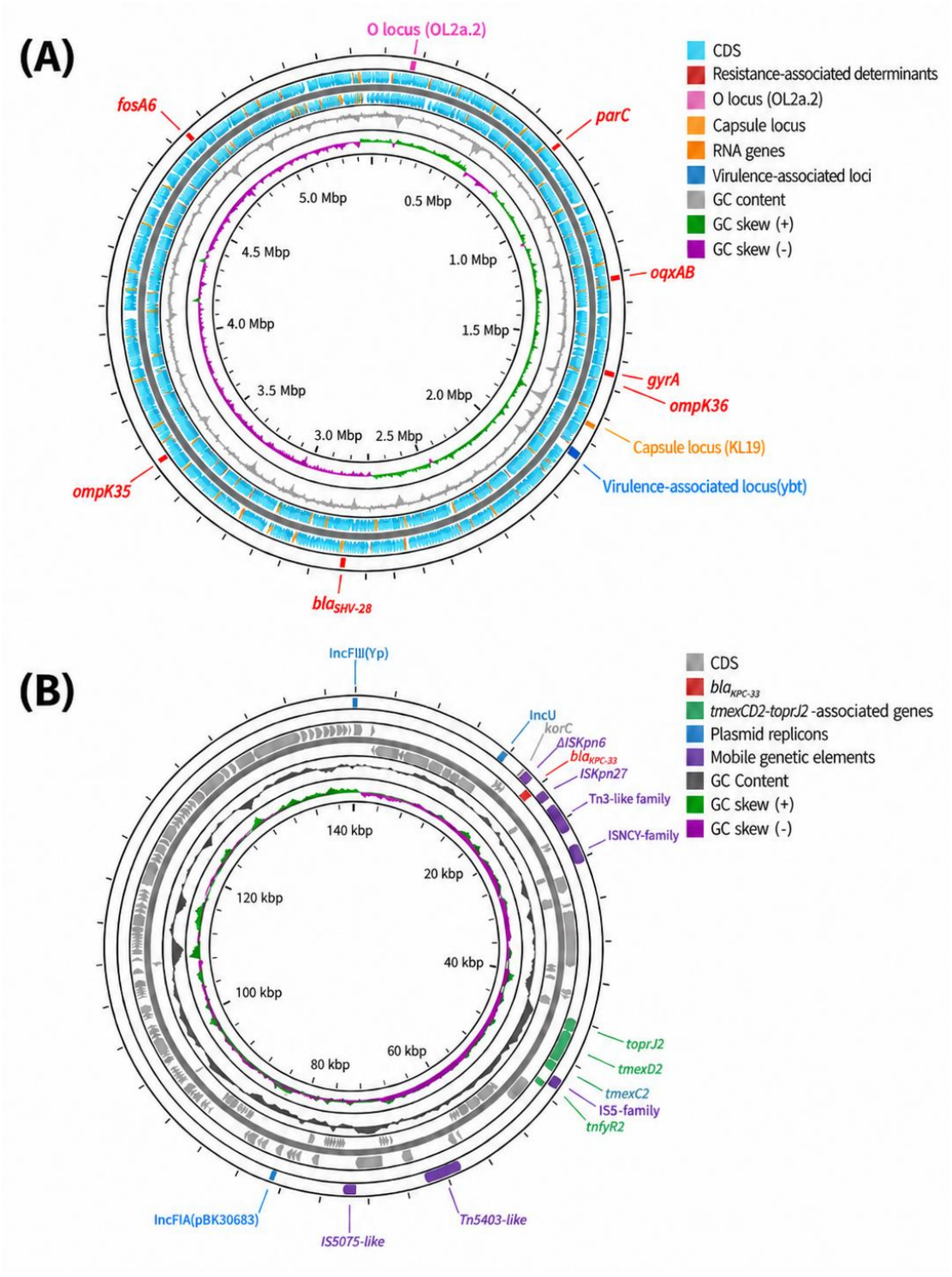


Fig 1. Genomic architecture of *Klebsiella pneumoniae* strain 1640 and plasmid p1640-tmexKPC.

(A) Circular chromosome map showing major resistance- and virulence-associated features. (B) Circular map of p1640-tmexKPC showing *bla*KPC-33, the *tnfxB2-tmexCD2-toprJ2* cluster, plasmid replicons, and representative mobile genetic elements.

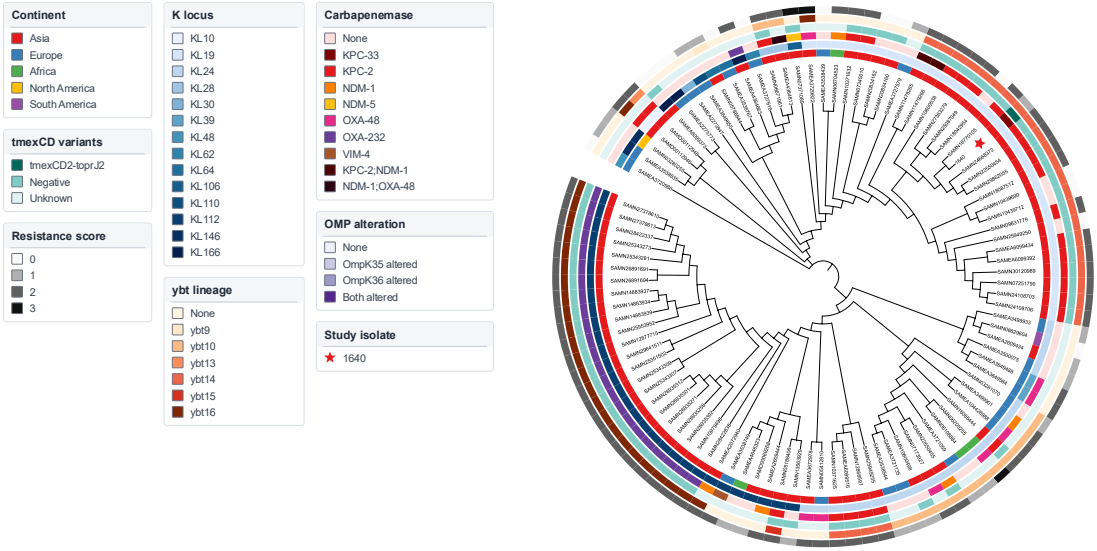


Fig 2. Core-genome phylogeny of ST15 *Klebsiella pneumoniae* isolates and the genomic context of strain 1640.

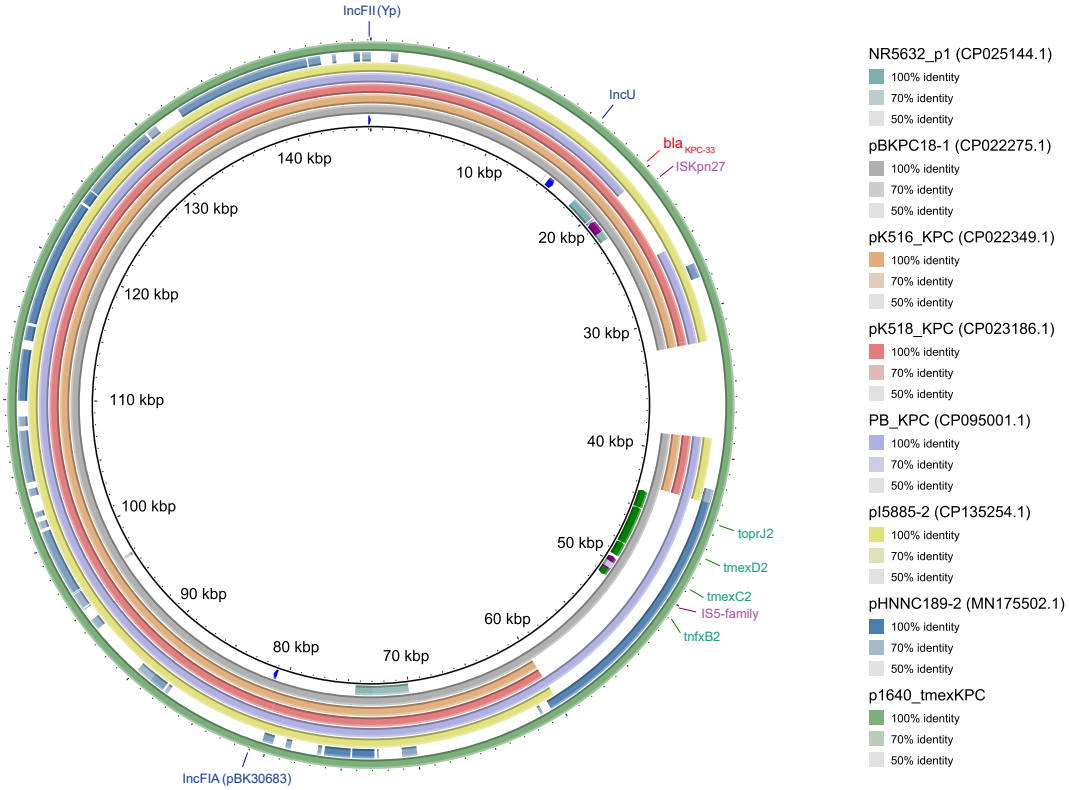
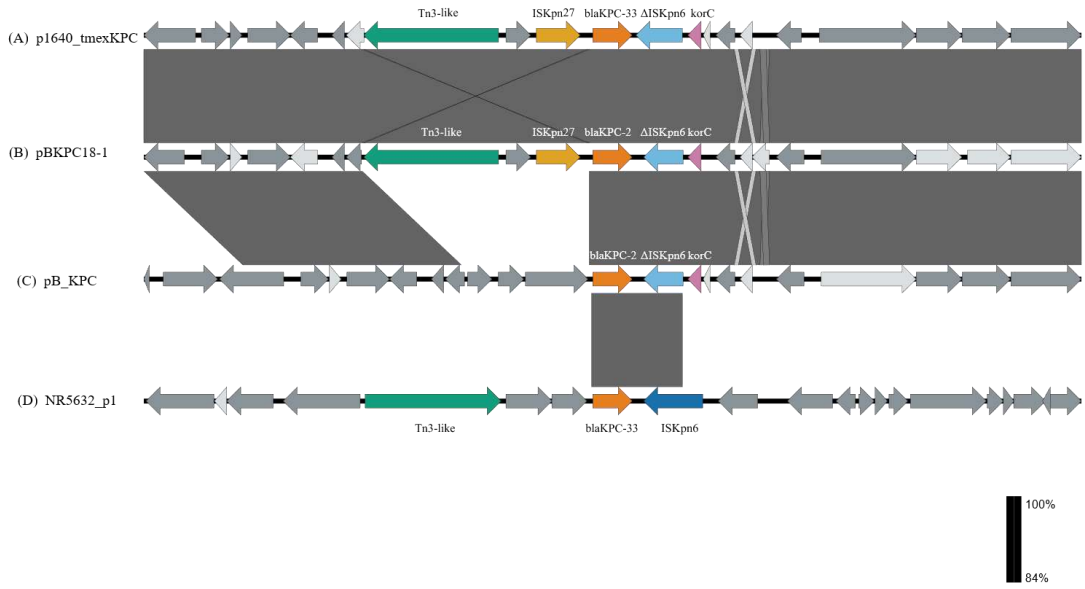


Fig 3. Circular comparative analysis of p1640-tmexKPC and six related plasmids.

Color intensity represents nucleotide sequence identity, whereas blank regions indicate low or absent sequence homology. The locations of plasmid replicons, *bla*$_{KPC-33}$, the *tmexCD2-toprJ2* gene cluster, and representative mobile genetic elements are indicated around the outermost ring.



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421 **Fig 4. Comparison of the *bla*$_{KPC}$-associated genetic environments in p1640-**
422 **tmexKPC and related plasmids.**

423 (A) p1640-tmexKPC, (B) pBKPC18-1 (CP022275.1), (C) pB_KPC (CP095001.1), and (D) NR5632_p1
424 (CP025144.1). The *bla*$_{KPC}$ regions differ in the arrangement of IS*Kpn27*, ΔIS*Kpn6*/IS*Kpn6*, korC, and Tn3-like
425 elements. In p1640-tmexKPC, *bla*$_{KPC-33}$ is flanked by IS*Kpn27* and ΔIS*Kpn6*, with korC and a Tn3-like element
426 nearby. Gray arrows indicate other coding sequences and hypothetical proteins. Shaded regions indicate BLASTn
427 similarity, with darker shading representing higher nucleotide identity.

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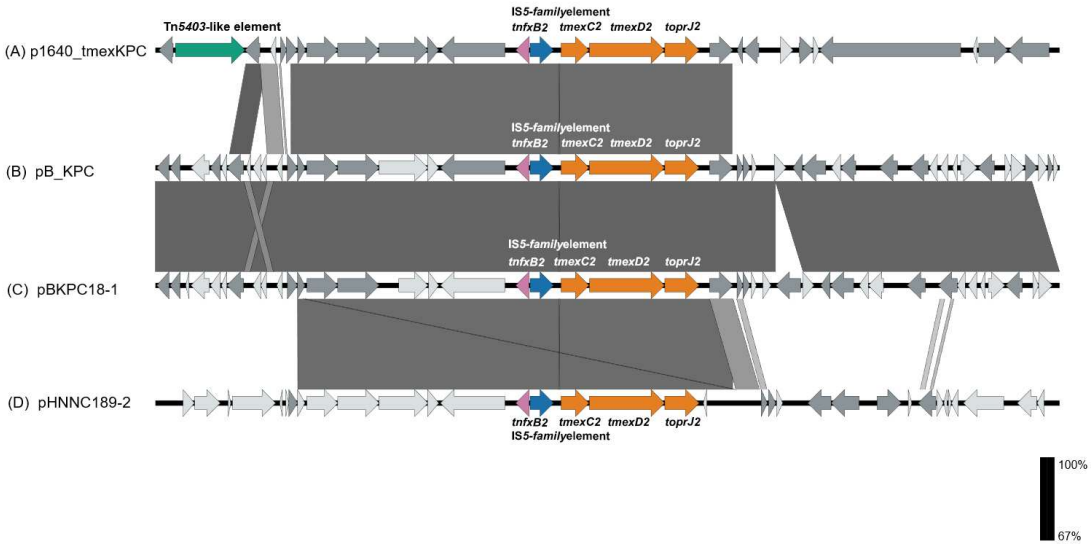
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437 **Fig 5. Comparative genetic environments of the *tmexCD2-toprJ2* cluster in p1640-**

438 **tmexKPC and related plasmids.**

439 (A) p1640-tmexKPC, (B) pB_KPC (CP095001.1), (C) pBKPC18-1 (CP022275.1), and (D) pHNNC189-2

440 (MN175502.1). The *tnfxB2-tmexC2-tmexD2-toprJ2* region is highly conserved, with an IS5-family element located

441 between *tnfxB2* and *tmexC2*. A Tn5403-like element is present upstream in p1640-tmexKPC. Grey arrows indicate

442 other coding sequences or hypothetical proteins. Shaded regions represent BLASTn similarity; darker shading

443 indicates higher nucleotide identity.

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Antibiotic class	Antibiotic	MIC (μg/mL)		
		1640 (donor)	EC600 (recipient)	J1640 (transconjugant)
Carbapenems	IPM	≤1	≤1	≤1
	MEM	≤1	≤1	≤1
Cephalosporins and cephamycins	CMZ	4	≤2	≤2
	CAZ	128	≤2	64
	CTX	≤4	≤4	≤4
	FEP	16	≤4	8
β-Lactam	AZA	≤2/4	≤2/4	≤2/4
	TZP	≤8/4	≤8/4	≤8/4
	SCF	≤8/4	≤8/4	≤8/4
	CZA	16/4	≤0.5/4	4/4
Monobactam	ATM	≤4	≤4	≤4
Polymyxin	PB	≤1	≤1	≤1
Glycylcycline	TGC	≤1	≤1	≤1
Fluoroquinolone	CIP	>32	≤1	2
Aminoglycoside	AK	≤4	≤4	≤4

Table 1. Antimicrobial susceptibility profiles of strain 1640, EC600, and transconjugant J1640.

Abbreviations: IPM, imipenem; MEM, meropenem; AZA, aztreonam-avibactam; CMZ, cefmetazole; CAZ, ceftazidime; CTX, cefotaxime; TZP, piperacillin-tazobactam; SCF, cefoperazone-sulbactam; CZA, ceftazidime-avibactam; FEP, cefepime; PB, polymyxin B; TGC, tigecycline; CIP, ciprofloxacin; AK, amikacin; ATM, aztreonam.