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Molecular genetic analysis of an XDR *Pseudomonas aeruginosa* ST664 clone carrying multiple conjugal plasmids

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Objectives: A group of ST664 XDR *Pseudomonas aeruginosa* strains have been isolated from a burn clinic. Here we decipher their resistomes and likely mechanisms of resistance acquisition.

Methods: The complete nucleotide sequences of representative isolates were determined, by PacBio and Illumina MiSeq sequencing, and analysed for antimicrobial resistance (AMR) genes as well as sequence variations. S1-PFGE was used to determine the sizes and numbers of plasmids harboured by the isolates. Purified plasmid DNA was further sequenced by PacBio technology, closed manually and annotated by RAST. The mobility of plasmids was determined by conjugation assays.

Results: The XDR *P. aeruginosa* ST664 clone carries 11 AMR genes, including a bla_{KPC-2} gene that confers resistance to carbapenems. Most of the ST664 isolates carry three coexisting plasmids. bla_{KPC-2} and a cluster of three AMR genes (*aadB-cmlA1-sul1*) are encoded on a 475 kb megaplasmid pNK546a, which codes for an IncP-3-like replication and partitioning mechanism, but has lost the conjugative transfer system. Interestingly, however, pNK546a is mobilizable and can be transferred to *P. aeruginosa* PAO1 with the help of a co-residing IncP-7 conjugative plasmid. The bla_{KPC-2} gene is carried by an IS*6100*-IS*Kpn27*-bla_{KPC-2}-ΔIS*Kpn6*-Tn*1403* mobile element, which might be brought into the ST664 clone by another co-resident IncP-1α plasmid, which is inclined to be lost. Moreover, pNK546a harbours multiple heavy metal (mercury, tellurite and silver) resistance modules.

Conclusions: To the best of our knowledge, pNK546a is the first fully sequenced bla_{KPC-2}-carrying megaplasmid from *P. aeruginosa*. These results give new insights into bacterial adaptation and evolution during nosocomial infections.

Introduction

Infection caused by XDR *Pseudomonas aeruginosa* with simultaneous resistance to almost all classes of antibiotics is a major concern in burn units throughout the world. It greatly increases patient mortality and medical costs.[1,2] KPC, an Ambler class A β-lactamase, is a powerful carbapenem-hydrolysing enzyme capable of hydrolysing most of the β-lactams.[3] It is encoded by the bla_{KPC} gene and usually located within a Tn*3* family transposon, which is able to insert into plasmids harboured by a large variety of Gram-negative bacteria. Even though it is most prevalent in *Klebsiella pneumoniae*,[4] KPC has been described in other Enterobacterales, such as *Escherichia coli* and in non-fermenters such as *Pseudomonas* or *Acinetobacter* species.[5]

Plasmids, as extrachromosomal genetic elements, are the key vectors mediating the spread of antimicrobial resistance (AMR) genes.[6] Horizontal transfer of plasmids can occur via conjugation, which requires dedicated type 4 secretion systems (T4SSs) encoded on the same replicon in the case of conjugative plasmids (also called self-transmissible plasmids) or elsewhere within the genome or other co-resident plasmid in the cell (called mobilizable plasmids).[7] However, the number and types of different plasmids that can stably coexist within a cell are restricted by plasmid

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incompatibility (Inc type).[8,9] In contrast to the numerous studies of single AMR plasmids in pathogenic bacteria, very little is known about the significance of the coexistence of multiple plasmids in clinical isolates.

In this report, we report an outbreak of an ST664 XDR *P. aeruginosa* clone in a burn clinic. WGS was used to decipher the resistomes of isolates. We identified three co-resident plasmids in one isolate, belonging to Inc groups P-1α, P-3 and P-7. Among these, we specifically describe an IncP-3-like AMR plasmid and characterize the mobile genetic elements that mediate the transmission of AMR genes, as well as this plasmid's mobility and relationship with the other two co-resident plasmids.

Materials and methods

Bacterial isolates and antibiotic susceptibility testing

All *P. aeruginosa* isolates were collected from distinct inpatients attending the same hospital. Species identification and antibiotic susceptibility tests were performed using a VITEK 2 compact system (bioMérieux, France). MICs were determined by broth microdilution as described previously.[10]

Molecular typing of clinical isolates

PFGE of SpeI-digested genomic DNA was performed using a CHEF-DR III system (Bio-Rad Laboratories, USA). Macrorestriction patterns were compared using BioNumerics software v7.1 (Applied Maths, Austin, TX, USA).[11] MLST was performed to investigate the molecular epidemiology of isolates following the method available on the website http://pubmlst.org/paeruginosa/.

WGS and bioinformatics analysis

Genomic DNA from a single colony of each isolate was purified using a Blood and Cell Culture DNA Midi Kit (QIAGEN) and sequenced on a PacBio RS II platform. The full-length genomes of the two isolates were assembled from long reads obtained from the PacBio RS II system using the HGAP3 pipeline. The genomes of the isolates were also sequenced using the Illumina MiSeq sequencing platform to generate paired-end sequences with a read length of 300 bp. Sequence variations of isolates NK389 and NK546 were analysed using the resequencing analysis module in CLC genomics workbench (QIAGEN, https://www.qiagenbioinformatics.com/). The antibiotic resistance genes were analysed using the ResFinder 2.1 server. Core-genome alignment with the *P. aeruginosa* genomes was performed using Parsnp v1.1.2.[12]

Plasmid identification and characterization

S1-PFGE was used to visualize the number and size of plasmids in isolates.[13] Plasmids were purified from isolate NK546 using a large-construct DNA isolation kit (BAC/PAC plasmid DNA extraction kit PL20, Aidlab Biotechnologies). The plasmid DNA was further sequenced by PacBio SMRT Sequencing (Oebiotec, Shanghai, China). The full-length plasmid sequences were assembled using Falcon.[14] Pseudogenes were annotated by the Rapid Annotations using Subsystem Technology (RAST) server,[15] combined with Basic Local Alignment Search Tool (BLAST) searches against the UniProtKB/Swiss-Prot database and the RefSeq database. Gene organization diagrams were drawn using the CGView Server.[16]

Conjugation assays

See the Supplementary data available at *JAC* Online.

Nucleotide sequence accession numbers

The WGS projects have been deposited at the NCBI Short Read Archive (SRA) database (www.ncbi.nlm.nih.gov/sra) with accession code SRR8571326 (isolate NK546) and SRR8571327 (isolate NK389). The sequences of plasmids pNK546a, pNK546b and pNK546c have been submitted to GenBank under the following accession numbers: MN433457, MN583270 and MN433456, respectively.

Results

Nosocomial outbreak of the XDR P. aeruginosa ST664 clone

During the period of January to May 2018, 21 XDR *P. aeruginosa* isolates were collected from patients with burn wound infections in a burn clinic in Tianjin, China. Clinical specimens were obtained from wounds ($n = 18$) or blood ($n = 3$). The epidemiological information for each isolate is shown in Figure 1. All of the isolates displayed a common antimicrobial susceptibility profile (Table 1), showing resistance to various classes of antibiotics, including aminoglycosides (gentamicin and tobramycin), cephalosporins (cefepime, cefotetan and ceftazidime), carbapenems (imipenem and meropenem) and fluoroquinolones (ciprofloxacin and levofloxacin) but were susceptible to amikacin and polymyxin B. PFGE patterns of the bacterial DNA showed a close relatedness of these XDR *P. aeruginosa* isolates (Figure 1). Cluster analysis primarily divided the isolates into two clonal types (criterion: similarity $\geq$95%); 76% (16/21) of isolates belonged to type A, while 24% (5/21) belonged to type B (Figure 1). MLST revealed that all isolates shared the same ST, i.e. ST664 (Figure 1).

WGS of the ST664 isolates

In order to understand the XDR mechanisms of the ST664 clone, WGS was performed on two representative isolates (NK546 and NK389) by PacBio long-read DNA sequencing and Illumina sequencing (for error correction). Isolate NK546, which belonged to the PFGE type A clone, was isolated from Patient 1 via a wound swab on Day 5 after admission. Isolate NK389 (PFGE type B) was isolated from a blood sample of Patient 2 on Day 26 after admission. The genome sizes of isolates NK389 and NK546 were 7 334 885 bp, with 65.06% GC content, and 7 237 531 bp, with 65.07% GC content, respectively (Table S1, available as Supplementary data at *JAC* Online). Phylogenetic analysis showed that ST664 isolates NK389 and NK546 were clustered in the same clade in the phylogenetic tree and had a relatively close evolutionary relationship with ST549-type *P. aeruginosa* strain PAO1 (Figure 2).

AMR profile of the ST664 isolates

Searching of AMR genes on databases showed that both isolates harboured 11 AMR genes, including three aminoglycoside resistance genes [*aph(3′)-IIb*, *ant(2′′)-Ia* (*aadB*) and *aac(3)-IId*], three β-lactam resistance genes [carbapenemase gene bla_{KPC-2}, cephalosporinase gene bla_{PAO} (*ampC*) and oxacillinase gene bla_{OXA-50}], a potential fluoroquinolone resistance gene (*crpP*), a fosfomycin resistance gene (*fosA*), two phenicol resistance genes (*catB7* and *cmlA1*) and a sulphonamide resistance gene (*sul1*) (Table 1).

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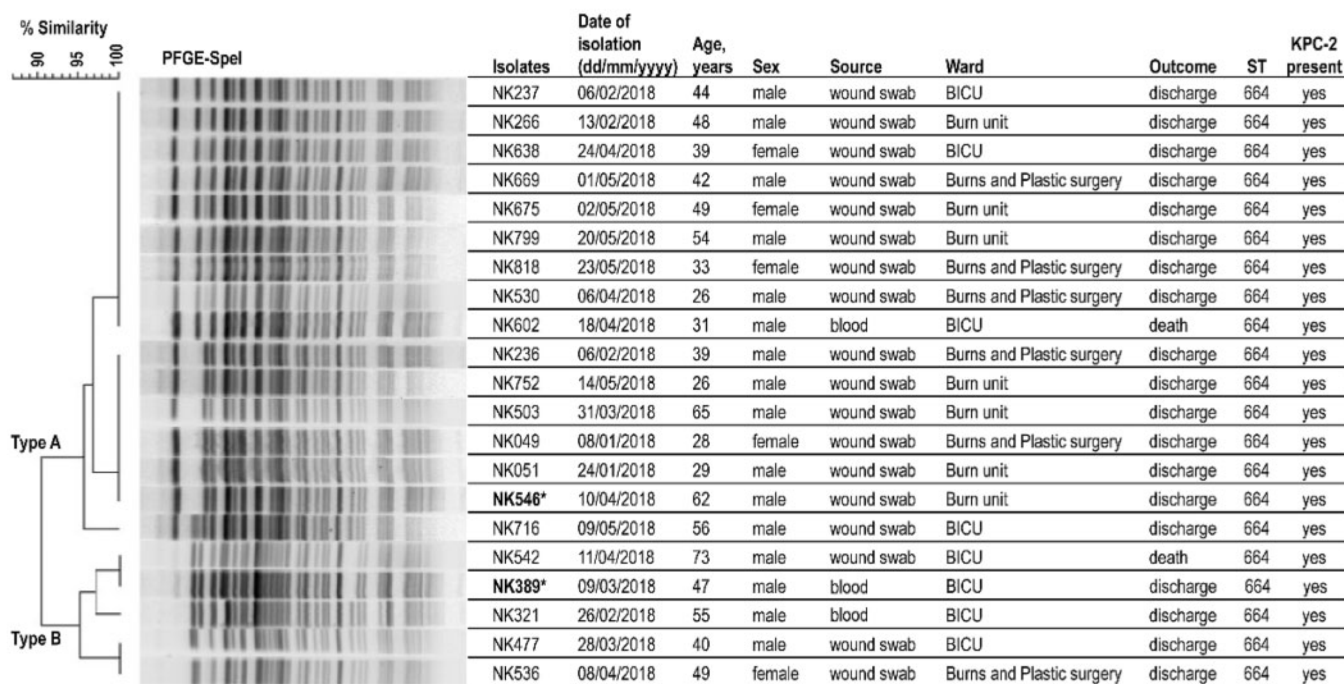

Isolates	Date of isolation (dd/mm/yyyy)	Age, years	Sex	Source	Ward	Outcome	ST	KPC-2 present
NK237	06/02/2018	44	male	wound swab	BICU	discharge	664	yes
NK266	13/02/2018	48	male	wound swab	Burn unit	discharge	664	yes
NK638	24/04/2018	39	female	wound swab	BICU	discharge	664	yes
NK669	01/05/2018	42	male	wound swab	Burns and Plastic surgery	discharge	664	yes
NK675	02/05/2018	49	female	wound swab	Burn unit	discharge	664	yes
NK799	20/05/2018	54	male	wound swab	Burn unit	discharge	664	yes
NK818	23/05/2018	33	female	wound swab	Burns and Plastic surgery	discharge	664	yes
NK530	06/04/2018	26	male	wound swab	Burns and Plastic surgery	discharge	664	yes
NK602	18/04/2018	31	male	blood	BICU	death	664	yes
NK236	06/02/2018	39	male	wound swab	Burns and Plastic surgery	discharge	664	yes
NK752	14/05/2018	26	male	wound swab	Burn unit	discharge	664	yes
NK503	31/03/2018	65	male	wound swab	Burn unit	discharge	664	yes
NK049	08/01/2018	28	female	wound swab	Burns and Plastic surgery	discharge	664	yes
NK051	24/01/2018	29	male	wound swab	Burn unit	discharge	664	yes
NK546*	10/04/2018	62	male	wound swab	Burn unit	discharge	664	yes
NK716	09/05/2018	56	male	wound swab	BICU	discharge	664	yes
NK542	11/04/2018	73	male	wound swab	BICU	death	664	yes
NK389*	09/03/2018	47	male	blood	BICU	discharge	664	yes
NK321	26/02/2018	55	male	blood	BICU	discharge	664	yes
NK477	28/03/2018	40	male	wound swab	BICU	discharge	664	yes
NK536	08/04/2018	49	female	wound swab	Burns and Plastic surgery	discharge	664	yes

Figure 1. Dendrogram of PFGE patterns of 21 XDR *P. aeruginosa* isolates. *Isolates subjected to WGS. BICU, burn intensive care unit.

Table 1. Summary of antimicrobial susceptibility and resistance mechanisms of the ST664 XDR *P. aeruginosa* clone

Antimicrobial category	Antimicrobial agent	MIC (mg/L)[a]	Mutations	AMR genes
Aminoglycosides	amikacin	≤2, S		*aph(3')-IIb*, *ant(2'')-Ia (addB)*[b], *aac(3)-IId*
	gentamicin	≥16, R		
	tobramycin	≥16, R		
Cephalosporins	cefepime	≥64, R		bla_{KPC-2}[b], bla_{PAO} (*ampC*), bla_{OXA-50}
	cefotetan	≥64, R		
	ceftazidime	≥64, R		
Monobactam	aztreonam	≥64, R		
Carbapenems	imipenem	≥16, R	*oprD* (E336 frameshift insertion)	
	meropenem	≥16, R		
Fluoroquinolones	ciprofloxacin	≥4, R	*gyrA* (T83I), *gyrB* (E468D), *parC* (S87L)	*crpP*
	levofloxacin	≥8, R		
Polymyxin	polymyxin B	≤2, S		

[a]R, resistant; S, susceptible (as recommended by CLSI guidelines).
[b]Genes encoded on a plasmid.

The presence of these AMR genes can largely explain the XDR phenotype of the isolates. The presence of the bla_{KPC-2} gene in all of the 21 isolates was further tested by PCR using primers listed in Table S2 and the results showed that all of the ST664 *P. aeruginosa* isolates harboured bla_{KPC-2} (Figure 1). Plasmids usually play an important role in the dissemination of bla_{KPC-2} genes. BLAST analysis and average GC% differences of assembled contigs revealed that both isolates harboured large plasmid content (Table S1); bla_{KPC-2} and three other AMR genes (*aadB*, *cmlA1* and *sul1*) were encoded on plasmid contigs and the seven remaining AMR genes were encoded on the chromosome.

Coexistence of three plasmids in the ST664 isolates

To determine the sizes of the large plasmids, S1-PFGE analyses were performed on all of the 21 isolates. Most of the isolates showed three plasmid bands, with estimated sizes of 450, 230 and 50 kb (Figure 3). We purified the plasmid DNA from isolate NK546 and subjected it to PacBio long-read DNA sequencing again. The plasmid sequences were assembled into three circularly closed replicons, designated as pNK546a, pNK546b and pNK546c, with sizes of 475 027, 232 884 and 51 113 bp, respectively (Figure 3). Plasmid pNK546a (475 kb), containing 604 ORFs with an overall GC content of 57.2%, carried all the plasmid-encoding AMR genes,

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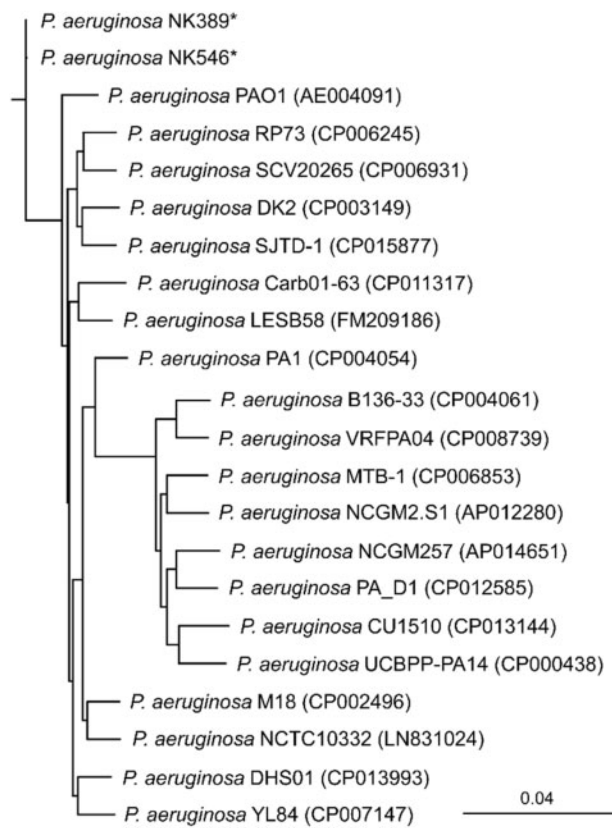

Figure 2. Phylogenetic analysis of NK389 and NK546 with 20 other *P. aeruginosa* genomes. The phylogenetic tree was constructed based on detected variant sites of core-genome alignment using the approximate maximum-likelihood algorithm, with clade confidence estimated with SH-like support values.[51] The scale bar shows substitutions per core-genome site. *Isolates studied. Accession numbers of genomes are shown in parentheses.

including bla_{KPC-2} and the *aadB-cmlA1-sul1* gene cluster (Figure 4a). Plasmid pNK546b (233 kb, 358 ORFs and 57.1% GC content) was an IncP-7 plasmid and also carried the *aadB-cmlA1-sul1* gene cluster (Figure 5a). Plasmid pNK546c (51 kb, 62 ORFs and 59.4% GC content) was an IncP-1α-like plasmid and did not carry any known AMR genes (Figure 5b). Since carbapenems are considered to be the last line of defence for serious infections caused by MDR Gram-negative bacilli,[3] we subjected the KPC-2-carbapenemase-encoding plasmid pNK546a to further analysis.

KPC-2 carbapenemase-carrying megaplasmid

Replication and transfer

pNK546a was highly related to two reported carbapenemase-carrying megaplasmids, pOZ176 (GenBank accession number KC543497; 79% query cover and 100% nucleotide similarity) and pJB37 (GenBank accession number KY494864; 85% query cover and 99% nucleotide similarity) (Figure 4a). pOZ176 and pJB37 carry IMP-9-type and VIM-2-type carbapenemases, respectively,[17,18] while pNK546a harboured the KPC-2-type carbapenemase. Comparative analysis of the plasmid maintenance regions revealed that the maintenance nature of pNK546a was significantly different from that of pOZ176 and pJB37. According to amino acid sequence homology of the replication initiator protein RepA, both pOZ176 and pJB37 belong to plasmid incompatibility group IncP-2, which is one of the most common plasmid types encountered in clinical isolates of *P. aeruginosa*, typically having sizes of >300 kb.[17,18] However, we did not detect the IncP-2 replication region (*rep-par*) in pNK546a; instead, we found a putative RepA (encoded by bp 52 149 to 53 336) that shared 81% cover and 52% amino acid sequence similarity with the RepA of IncP-3 plasmid pQBR103 (GenBank accession number NC_009444) in *Pseudomonas fluorescens* SBW25.[19] Partitioning proteins ParA (encoded by bp 88 752 to 89 651) and ParB (encoded by bp 89 651 to 90 850) exhibited 54% to 63% similarity with the partition proteins of the IncP-3 plasmid pQBR103. In consideration of similar replication and partition proteins, we classified pNK546a as an IncP-3-like plasmid. Both pJB37 and pOZ176 are conjugative plasmids and self-transmissible between *Pseudomonas* species, consistent with the presence of a conjugative transfer region (*traF*, *traG*, *virD2* and *trbBCDEJLFGI* genes).[17,18] However, the conjugal transfer region was not found in pNK546a.

AMR genes and their mobile elements

The bla_{KPC-2} gene was located on an 11 kb mobile genetic element IS*6100*-IS*Kpn27*-bla_{KPC-2}-ΔIS*Kpn6*-Tn*1403*, which had been identified on a recently reported plasmid p14057-KPC (GenBank accession number KY296095, with identity of 99.6%) from a clinical *P. aeruginosa* isolate.[20] As shown in Figure 4(b), the ΔTn*3*:IS*Kpn27* to Δ*repB* region represents a core bla_{KPC-2} platform, the similar modules of which had been found in the KPC-encoding plasmids p628-KPC from *K. pneumoniae*[21] and p10265-KPC from *P. aeruginosa*.[22] The core bla_{KPC-2} platform was flanked by an IS*6100*-based transposon upstream and a Tn*1403* transposon downstream. Tn*1403*, belonging to the Tn*21* subgroup of the Tn*3* family, is an MDR transposon involved in acquisition of the carbapenemase gene.[23,24] Interestingly, the coexisting 51 kb plasmid pNK546c showed a high similarity with p14057-KPC (76% query cover and 97.7% nucleotide identity), excluding the 11 kb bla_{KPC-2}-carrying region (Figure 5b). Plasmid pNK546c carried an intact set of conjugative transfer genes (*traI-mobC-traML-ssb-topB-virD4-virBs*; bp 12 077 to 39 605), similar to those described in p14057-KPC,[20] indicating it is likely self-transmissible. It could be seen from the S1-PFGE map that the 51 kb plasmid pNK546c had been lost in isolates NK321 and NK389 (Figure 3), although it contained the HigB/A toxin/antitoxin (TA) system for post-segregational killing of plasmid-free daughter cells (Figure 5b).[25] The loss of plasmid pNK546c in isolates was confirmed by colony PCR against the *higBA* genes, which are exclusively encoded on plasmid pNK546c. The whole-genome sequence of the isolate NK389 further confirmed the loss of pNK546c.

The other three AMR genes on the plasmid were clustered into a 10 kb Tn*3*-*aadB-cmlA1-sul1*-TniBA genetic element (bp 320 026 to 334 872; Figure 4c) and inserted into a Tn*5041*-mercury resistance transposon backbone (from Tn*5041* to Tn*4652*; Figure 4a).[18,26] This genetic element confers resistance to aminoglycosides, chloramphenicol and sulphonamides, as well as quaternary ammonium disinfectants due to the presence of the *qacE*Δ*1* gene (Figure 4c), which is usually located in class 1 integrons with an adjoining *sul1* gene and widespread in MDR

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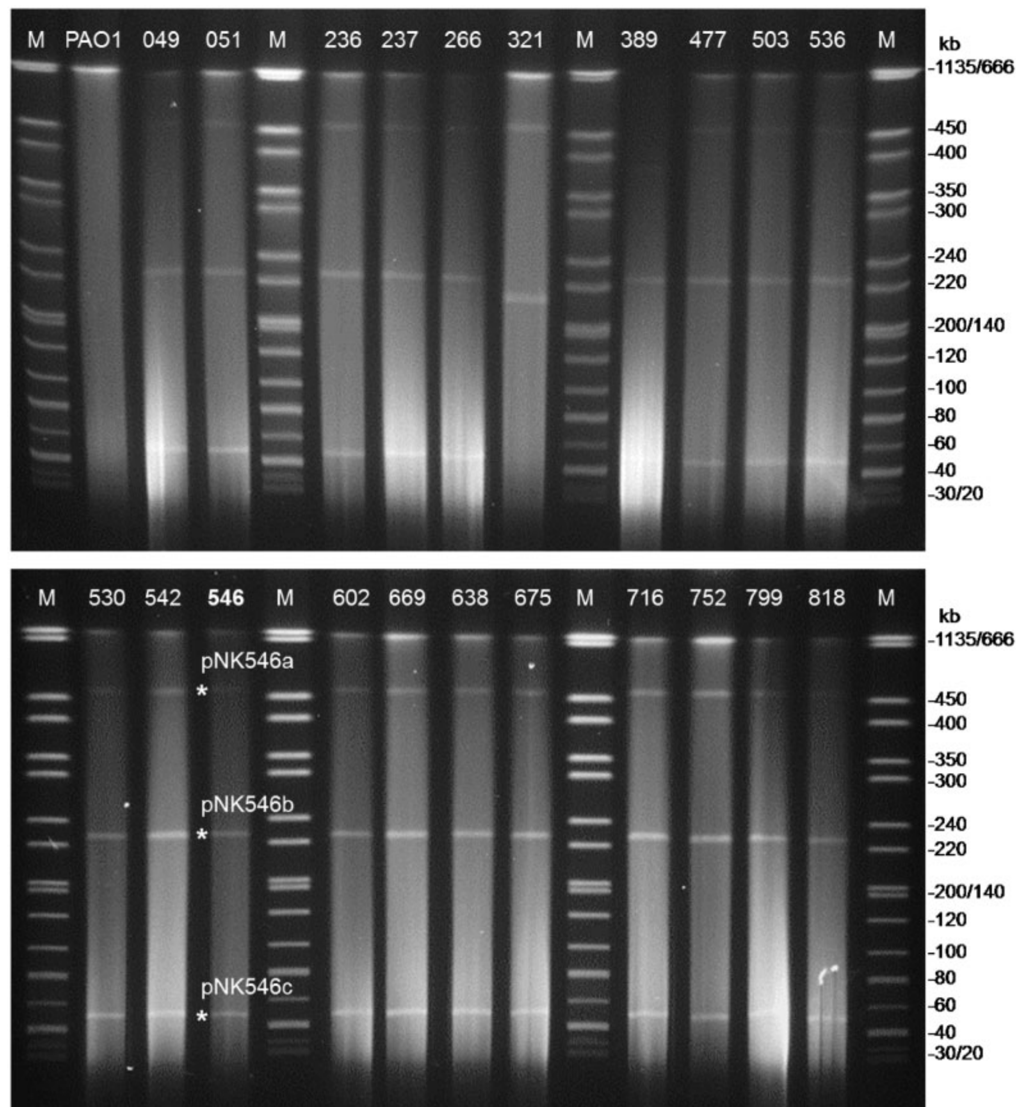

Figure 3. PFGE of S1 nuclease-digested plasmid DNA from 21 ST664 *P. aeruginosa* isolates. The bands with an asterisk indicate the 475 kb plasmid pNK546a, the 233 kb plasmid pNK546b and the 51 kb plasmid pNK546c. M = *Salmonella* serotype Braenderup strain H9812 (restricted with XbaI) as a molecular marker. The clinical isolate numbers are listed above each lane (with 'NK' omitted). *P. aeruginosa* PAO1 was used as a control.

Gram-negative bacteria.[27,28] Several reports pinpointed a strong correlation between the presence of *qacE*Δ*1-sul1* genes with aminoglycoside-modifying enzyme (AME) genes and ESBL genes.[27–29] Here, *qacE*Δ*1-sul1* was associated with the AME gene *aadB* and the chloramphenicol resistance gene *cmlA1* (Figure 4c). BLAST analysis showed that the 233 kb coexisting megaplasmid pNK546b also contained this Tn*5041*-Tn*3*-*aadB*-*cmlA1*-*sul1*-TniBA-*mers*-*czcs*-Tn*4652* genetic element with 99.9% nucleotide identity. pNK546b shared a similar plasmid backbone with the IncP-7-type plasmid pDK1 (GenBank accession number AB434906.1) from *Pseudomonas putida*[30] and pCAR1 (GenBank accession number AB088420) from *Pseudomonas resinovorans* (Figure 5a),[31] including the replication/partition region (bp 169 775 to 175 152) as well as the conjugative transfer system (bp 77 538 to 98 346, consisting of *TraDLEKBVCW* genes) (Figure 5a). This conjugative transfer region encoded a set of F-plasmid-type T4SS,[7] including the membrane-associated mating channel (TraLEKBVW), major ATPase (TraC) and type IV coupling protein (TraD), indicating that pNK546b was a conjugative plasmid.

Heavy metal resistance modules

Megaplasmid pNK546a harboured multiple heavy metal resistance operons (Figure 4a), including: (i) a mercury resistance (*mer*) operon 1 (bp 247 705 to 250 988) consisting of *merRTPCA* genes and a Tn*5041*-based *mer* operon 2 (bp 306 362 to 339 446) consisting of *merRTPCADE* genes, which was interrupted by a Tn*3*-*aadB*-*cmlA1*-*sul1*-carrying mobile element; (ii) a tellurite resistance (*ter*) operon (bp 177 033 to 187 104) consisting of *terZABCDE* genes, which is widely spread among pathogenic bacterial species and involved in tellurite resistance, phage inhibition, colicin resistance and pathogenicity;[32] and

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Figure 4. Schematic maps of the bla_{KPC-2}-harbouring megaplasmid and its accessory modules. (a) Genetic organization of pNK546a and comparison with similar plasmid backbones. Coding sequences (CDS) from pNK546a are represented by arrowheads in the outermost circles, comprising both the forward and reverse frames. Homology regions are represented in dark green for the bla_{IMP-9}-harbouring pOZ176 plasmid from *P. aeruginosa* and in light blue for the bla_{VIM-2}-carrying pJB37 plasmid from *P. aeruginosa*. The inner multi-peak circle illustrates GC skew+/−. The innermost circle indicates the scale. (b) The bla_{KPC-2}-carrying genetic element. White arrows represent transposase (*tnpA*) and resolvase (*tnpR*) genes. Grey rectangles represent the inverted repeats. IRR, inverted repeat right; IRL, inverted repeat left; *hyp*, gene encoding a hypothetical protein. (c) The *aadB-cmlA1-sul1*-carrying genetic element. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Figure 5. Schematic maps of two co-resident plasmids. Coding sequences (CDS) from pNK546b and pNK546c are represented by arrowheads in the outermost two circles, comprising both the forward and reverse frames. (a) Genetic organization of pNK546b and comparison with similar plasmid backbones. Homology regions are represented in dark green for the pCAR1 plasmid (GenBank accession number AB088420) from *P. resinovorans* and in light blue for the pDK1 plasmid (GenBank accession number AB434906) from *P. putida*. (b) Genetic organization of pNK546c and comparison with similar plasmid pCR1 (outer circle, GenBank accession number CP020561.2) and plasmid p14057-KPC (inner circle, GenBank accession number KY296095) from *P. aeruginosa*. The inner multi-peak circle illustrates GC skew+/−. The innermost circle indicates the scale. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

(iii) a *czc* operon (bp 350 132 to 355 124) consisting of *czcCBA* genes involved in the efflux of metal ions such as Ag^{2+}, Cu^{2+}, Co^{2+}, Cd^{2+} and Zn^{2+}.[33,34]

Mobility of the plasmids

To test whether the bla_{KPC-2}-carrying megaplasmid was able to self-transfer into susceptible strains, we conducted a conjugation assay by using a polymyxin B-resistant mutant derivative of *P. aeruginosa* PAO1 as the recipient and isolate NK546 as the donor. Conjugal transfer of the plasmid pNK546a into PAO1 was successful when selected for with meropenem and polymyxin B, as confirmed by PCR of bla_{KPC-2} for pNK546a (Figure S1a). All transconjugants were identified as the PAO1 strain by housekeeping gene *acsA* allele sequencing. The bla_{KPC-2}-positive transconjugants (PAO1/pNK546a) exhibited 16- to 32-fold increases in the MICs of carbapenems and ceftazidime compared with PAO1 (Table 2). Interestingly, the majority of bla_{KPC-2}-positive transconjugants also carried pNK546b (Figure S1a), but none contained pNK546c

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Table 2. Antimicrobial susceptibility of transconjugants

Strain[a]	MIC (mg/L)			
	meropenem	imipenem	ceftazidime	gentamicin
PAO1	0.25	1	0.125	4
PAO1/pNK546a	4–8	16	4–8	>16
PAO1/pNK546b	0.25	1	0.125	>16

[a]Plasmids pNK546a and pNK546b were transferred from clinical isolate NK546 by conjugation.

(Figure S1a), indicating that pNK546b frequently accompanies pNK546a in the transfer to susceptible recipient strains.

In the above conjugation assay, we selected transconjugants with gentamicin (gentamicin resistance gene *aadB* was present on both pNK546a and pNK546b). Among six randomly picked transconjugants, five were *aadB* positive (one false positive; Figure S1b), of which four were pNK546b positive and pNK546a negative (Figure S1b), indicating conjugal transfer of pNK546b alone can confer a >16-fold increase in the MIC of gentamicin for PAO1 (Table 2; PAO1/pNK546b). One of the five *aadB*-positive transconjugants was positive for both pNK546a and pNK546b (Figure S1b), again showing a high incidence of co-transfer of megaplasmids pNK546a and pNK546b (∼20%).

In further tests, in which the donor strain contained pNK546a only (Figure S1a) while the recipient was a ciprofloxacin-resistant PAO1 derivative, the conjugal transfer of pNK546a was unsuccessful, indicating that the megaplasmid pNK546a is not self-transmissible. This agrees with the sequence analysis results, which revealed no functional T4SS genes on pNK546a. Successful conjugation of pNK546a apparently requires pNK546b, which provides the functional T4SS for both megaplasmids.

Chromosomally encoded resistance mechanisms in the ST664 isolates

In addition to the acquired AMR genes, sequence variation of 146 chromosomal genes related to intrinsic or adaptive AMR in *P. aeruginosa* was analysed by mapping the MiSeq reads of two isolates (NK389 and NK546) to the reference PAO1 genome (GenBank accession number NC_002516.2). A set of 146 genes was initially established, based on literature review, in order to correlate the documented resistance genotypes with the observed resistance phenotypes.[35] The genes showing non-synonymous SNPs or insertion/deletion events (indels) in both sequenced isolates are listed in Table S3. As many as 59 (40.4%) of the 146 genes showed mutations in both of the isolates and most of the mutations were documented in other available *P. aeruginosa* genomes (www.pseudomonas.com), indicating high frequency polymorphism among *P. aeruginosa* strains, and were not thought to be involved in resistance phenotypes.

For β-lactam resistance, both isolates shared non-synonymous SNPs in genes related to *ampC* expression and β-lactam resistance, such as *ampD* (D183Y and G148A), *ampE* (S69P), *ampG* (A583T), *ampP* (R171C, T172A and M87I), *mpl* (R322Q) or *ampDh3* (A219T) (Table S3) but, in both isolates, *ampC* expression levels were similar or lower than that in WT PAO1 (Figure S2). Therefore, AmpC seems not to be involved in the high-level β-lactam resistance in ST664 isolates. Mutations or loss of OprD, a porin for amino acids and carbapenems, is a 'classical' imipenem resistance mechanism of *P. aeruginosa*.[36] Both isolates contained an E336 frameshift insertion (4 bp) in the *oprD* gene (Table 1). The frameshift insertion at position 336 resulted in the destruction of β-sheets 18–21 and loops 7–8 of the OprD porin,[37] which would decrease the uptake of carbapenem antibiotics. For fluoroquinolone resistance, both isolates showed high-level fluoroquinolone resistance (ciprofloxacin MIC = 32 mg/L) and contained three mutations: T83I in *gyrA*, E468D in *gyrB* and S87L in *parC* (Table 1), all of which correlate with quinolone resistance.[38,39] Additionally, a chromosomally encoded CrpP may also confer fluoroquinolone resistance (Table 1).[40] Another major relevant consideration in the analysis of *P. aeruginosa* resistance profiles is the expression of four major efflux pumps, namely MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM. Although many mutations occurred in genes that are involved in the regulation of efflux pump expression (*mexR*, *mexZ*, *nalC* and *armZ*) (Table S3), in both ST664 isolates studied, none of the efflux pumps exhibited significantly higher levels of expression compared with PAO1 (Figure S2).

Discussion

The present study provides a first insight into the nosocomial outbreak of XDR *P. aeruginosa* isolates in a burn clinic due to the clonal expansion of ST664. In 2017, ST664 had been identified as the most common type of *P. aeruginosa* isolate in a referral burn hospital of Isfahan, Iran.[41] More recently, a nationwide epidemiology report from India stated that ST664-type *P. aeruginosa* is one of the high-risk carbapenem-resistant clones associated with VIM carbapenemase.[42] Here, the ST664 XDR *P. aeruginosa* in China encoded a KPC-2 carbapenemase. Up to now, six fully sequenced bla_{KPC}-carrying plasmids from *P. aeruginosa* have been available in public databases, including two IncP-6 plasmids, pCOL-1 and p10265-KPC,[22,43] two IncU plasmids, pPA-2 and pD5170990,[43] an IncP-1α-like plasmid, p14057-KPC,[20] and plasmid pBH6 (Table 3).[44] The sizes of the above plasmids are all less than 60 kb. However, pNK546a is a 475 kb megaplasmid and carries multiple AMR genes, as well as multiple heavy metal resistance modules, conferring the fitness of the ST664 *P. aeruginosa* clone in a nosocomial infection environment. Recent studies demonstrated a high association between metal resistance genes and MDR in bacteria;[45–47] thus increased vigilance is needed when using heavy metal agents in hospitals and the environment.

Horizontal gene transfer is recognized as an important driving force for bacterial evolution, in which the conjugal plasmids are actively moving around bacterial strains to acquire and/or donate useful gene cassettes, rendering new hosts better able to adapt to the growth environment.[48] Next-generation sequencing technology enabled us to examine the emergence of resistance *in situ*. In the case of the ST664 clone, we observed three co-resident plasmids, which interacted with each other to increase the adaptability of the host during nosocomial infections. Based on our studies, the relationship among the co-resident plasmids can be summarized into the following five possible events (Figure 6): (i) Loss of IncP-2 replication (*rep-par* genes) and conjugation (*tra* genes) region in megaplasmid pNK546a. Conjugation is an energetically expensive process and an important source of plasmid cost for the bacterial

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Table 3. Fully sequenced *bla*$_{KPC}$-carrying plasmids from *P. aeruginosa*

Plasmid	Inc group	Mobility	Size (bp) of plasmid	GenBank accession number	AMR genes	Genetic context of *bla*$_{KPC-2}$	ST of host *P. aeruginosa*	Isolated in
pCOL-1	IncP-6	mobilizable	31 529	KC609323	*bla*$_{KPC-2}$	Tn*4401a*-IS*Kpn7*-*bla*$_{KPC-2}$-IS*Kpn6*	ST308	Colombia
p10265-KPC	IncP-6	mobilizable	38 939	KU578314	*bla*$_{KPC-2}$	IS*Kpn27*-Δ*bla*$_{TEM-1}$-*bla*$_{KPC-2}$-ΔIS*Kpn6*	unknown	China
pPA-2	IncU	non-mobilizable	7995	KC609322	*bla*$_{KPC-2}$	Tn*4401b*-*bla*$_{KPC-2}$-IS*Kpn6*	ST1006	Colombia
pD5170990	IncU	unknown	32 424	KX169264	*bla*$_{KPC-2}$, *strAB*, *cmx*, *sul1*	IS*6100*-*bla*$_{KPC-2}$-IS*Kpn6*	unknown	Brazil
pBH6	unknown	non-mobilizable	3652	CM003767	*bla*$_{KPC-2}$	Tn*3*-*bla*$_{KPC-2}$-IS*Kpn6*	ST244	Brazil
p14057-KPC	IncP-1α-like	conjugative	51 663	KY296095	*bla*$_{KPC-2}$	IS*6100*-IS*Kpn27*-*bla*$_{KPC-2}$-ΔIS*Kpn6*-Tn*1403*	unknown	China
pNK546a	IncP-3-like (IncA/C)	mobilizable	475 027	MN433457	*bla*$_{KPC-2}$, *aadB*, *cmlA1*, *sul1*	IS*6100*-IS*Kpn27*-*bla*$_{KPC-2}$-ΔIS*Kpn6*-Tn*1403*	ST664	China

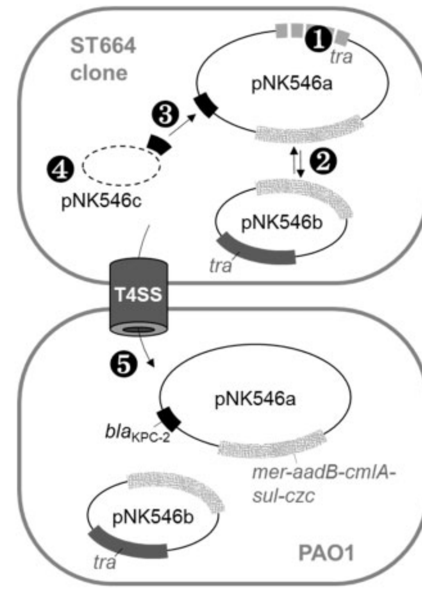

Figure 6. Schematic view of five hypothetical events occurring in the ST664 *P. aeruginosa* clone with coexistence of three plasmids. (1) Loss of IncP-2 replication and conjugation region in pNK546a. (2) Tn*5041*-Tn*4652*-mediated exchange of *mer-aadB-cmlA1-sul1-czc* gene cassettes between pNK546a and pNK546b. (3) Tn*1430*-IS*6100*-mediated *bla*$_{KPC-2}$ gene transposition from pNK546c to pNK546a. (4) Loss of pNK546c. (5) Co-transfer of plasmids pNK546a and pNK546b by conjugation using the T4SS encoded on pNK546b (*tra* genes).

host.[49] To minimize this cost, plasmids either tightly control the expression of conjugal transfer systems or simply discard them, as observed in the plasmid pNK546a. Alternatively, this loss may be forced by the plasmid's incompatibility with the other megaplasmid pNK546b, although we are unable to determine which one entered the ST664 clone first.

(ii) Tn*5041*-Tn*4652*-mediated duplication of the *mer-aadB-cmlA1-sul1-czc* gene cluster between pNK546a and pNK546b, although the donor–recipient relationship is not clear.

(iii) Tn*1430*-IS*6100*-mediated *bla*$_{KPC-2}$ gene transposition from pNK546c to pNK546a. The 51 kb plasmid pNK546c is a self-transmissible IncP-1α-like plasmid; these usually have a broad host range and are known to mediate horizontal gene transfer.[50] The *bla*$_{KPC-2}$ gene cassette may have initially been transmitted from *K. pneumoniae* to *Pseudomonas* species by this broad-host-range plasmid and then translocated onto the more stable megaplasmid pNK546a in the new host.

(iv) Loss of pNK546c in some ST664 isolates. This could be a passive loss due to the non-essential nature of the plasmid, especially after donation of the *bla*$_{KPC-2}$ gene to the megaplasmid pNK546a. However, we are unable to rule out the possibility of an active abandonment by the host to maintain competence to acquire a new plasmid with additional useful genes.

(v) Co-transfer of plasmids pNK546a and pNK546b by conjugation. Although pNK546a does not encode its own conjugative system, it can still transfer using the mating pair channel (T4SS) encoded by the other conjugative plasmids. Both pNK546b and pNK546c are conjugative plasmids, encoding F-type mating pair and Ti-plasmid-type T4SSs, respectively.[7] From our conjugation

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assays, the KPC-2-carrying megaplasmid pNK546a seemed to utilize the F-type mating pair channel from pNK546b for conjugal transfer.

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Transparency declarations

None to declare.

Supplementary data

Tables S1 to S3 and Figures S1 and S2 are available as Supplementary data at *JAC* Online.

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