

Whole-Genome Assembly of *Klebsiella pneumoniae* Coproducing NDM-1 and OXA-232 Carbapenemases Using Single-Molecule, Real-Time Sequencing

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The whole-genome sequence of a carbapenem-resistant *Klebsiella pneumoniae* strain, PittNDM01, which coproduces NDM-1 and OXA-232 carbapenemases, was determined in this study. The use of single-molecule, real-time (SMRT) sequencing provided a closed genome in a single sequencing run. *K. pneumoniae* PittNDM01 has a single chromosome of 5,348,284 bp and four plasmids: pPKPN1 (283,371 bp), pPKPN2 (103,694 bp), pPKPN3 (70,814 bp), and pPKPN4 (6,141 bp). The contents of the chromosome were similar to that of the *K. pneumoniae* reference genome strain MGH 78578, with the exception of a large inversion spanning 23.3% of the chromosome. In contrast, three of the four plasmids are unique. The plasmid pPKPN1, an IncHI1B-like plasmid, carries the *bla*_{NDM-1}, *armA*, and *qnrB1* genes, along with tellurium and mercury resistance operons. *bla*_{NDM-1} is carried on a unique structure in which Tn125 is further bracketed by IS26 downstream of a class 1 integron. The IncFIIA-like plasmid pPKPN3 also carries an array of resistance elements, including *bla*_{CTX-M-15} and a mercury resistance operon. The ColE-type plasmid pPKPN4 carrying *bla*_{OXA-232} is identical to a plasmid previously reported from France. SMRT sequencing was useful in resolving the complex bacterial genomic structures in the *de novo* assemblies.

Carbapenemase-producing *Enterobacteriaceae* have been a major global public health problem since the late 2000s. Among the various carbapenemases that have been identified to date, the three enzyme groups underpinning the expansion are *Klebsiella pneumoniae* carbapenemases (KPCs), the metallo- β -lactamase NDM-1, and the oxacillinase OXA-48, as well as their variants (1). Among them, NDM-1 was initially reported in 2009 in a *K. pneumoniae* strain from a Swedish patient who had traveled from India (2), and then it rapidly spread worldwide in the following years (3). It is now apparent that NDM-1-producing *Enterobacteriaceae* are endemic in the Indian subcontinent. The *bla*_{NDM-1} gene has been identified on multidrug resistance plasmids belonging to various incompatibility groups, often accompanied by extended-spectrum β -lactamases (ESBL) or plasmid-mediated class C β -lactamase genes (e.g., *bla*_{CTX-M-15} and *bla*_{CMY-2}) and aminoglycoside resistance 16S rRNA methyltransferase genes (e.g., *armA* and *rmtC*) (3). OXA-48 was initially identified in 2004 from a *K. pneumoniae* strain in Turkey (4). Besides Turkey, *bla*_{OXA-48} appears to have spread primarily in *K. pneumoniae* strains in the Middle East and North Africa (5). On the Indian subcontinent, where NDM-1 is endemic, variants of OXA-48, including OXA-181 and OXA-232, also seem to be spreading among *Enterobacteriaceae* (6, 7). As a consequence of the rapid spread of these two groups of carbapenemases, the coproduction of NDM-1 and OXA-48 group carbapenemases is also increasingly reported in *Enterobacteriaceae* (8–16). However, the genetics supporting the coacquisition of multiple carbapenemase genes have not been elucidated.

We recently described a *K. pneumoniae* strain coproducing NDM-1 and OXA-232 that was identified from an inpatient at our hospital (17). The aim of this study was to conduct a whole-genome assembly using a single-molecule, real-time (SMRT) sequencing technology to characterize the genomic structure of this

strain, with a focus on the antimicrobial resistance determinants, including these carbapenemase genes.

MATERIALS AND METHODS

Bacterial strain. *K. pneumoniae* strain PittNDM01 coproducing NDM-1 and OXA-232 was isolated from an inpatient at a hospital in Pittsburgh, PA, in March 2013 (17). In brief, this patient had been transferred from a hospital in India a month earlier for continuing care for a subarachnoid hemorrhage. PittNDM01 was isolated from a urine sample of the patient when she was hospitalized for fever. OXA-232 is a variant of the OXA-48 carbapenemase, which was recently identified from clinical isolates in India (6). The strain belongs to sequence type 14 (ST14) and was found to be resistant to all β -lactams, including carbapenems, aminoglycosides, and fluoroquinolones, but it remained susceptible to fosfomycin and colistin.

Library preparation and sequencing. Genomic DNA from *K. pneumoniae* PittNDM01 was isolated from 3 ml of lysogenic broth culture using the DNeasy blood and tissue kit (Qiagen, Valencia, CA). Genomic DNA was then sheared to fragments of approximately 10 to 15 kb using ag-TUBE (Covaris, Woburn, MA). SMRTbell sequencing libraries were made as suggested by the manufacturer (Pacific Biosciences, Menlo Park, CA). Briefly, fragmented DNA underwent DNA damage repair, end repair, and ligation to hairpin adapters. Following exonuclease treatment, the SMRTbell library was size selected by BluePippin (Sage Science, Beverly, MA) to retain SMRTbell templates of <5 kb. The SMRTbell libraries were sequenced on the PacBio RS II instrument (Pacific Biosciences) using the recommended protocol for large-insert libraries. The sequence

Received 26 April 2014 Returned for modification 14 June 2014
Accepted 19 July 2014

Published ahead of print 28 July 2014

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doi:10.1128/AAC.03180-14

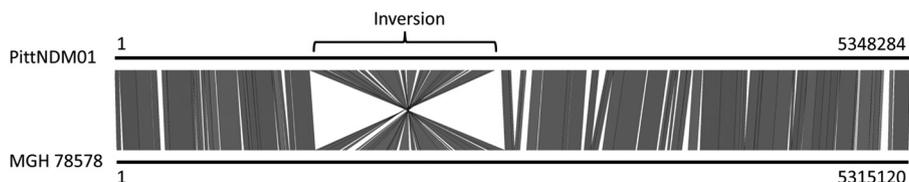


FIG 1 BLAST comparison results for the chromosomes of *K. pneumoniae* strains PittNDM01 and MGH 78578. A large inversion was identified and verified by PCR in PittNDM01 in comparison with MGH 78578, covering 23.3% of the chromosome.

was obtained from a total of 4 SMRT cells using P4-C2 sequencing chemistry, which resulted in an average depth of sequencing coverage of approximately 250-fold. At this coverage, a base-calling accuracy of >99.999% is expected (18).

Genome assembly and annotation. The genome was assembled *de novo* using the hierarchical genome assembly process (HGAP) (19). The assembled sequence was polished using Quiver to obtain a high consensus accuracy (19). One sequence gap was closed manually using the Bridge Mapper tool that is part of the SMRT Analysis software package (version 2.1). With the input of the raw sequence reads, HGAP directly assembled and closed the chromosome and the two largest plasmids (pPKPN1 and pPKPN2). The third plasmid (pPKPN3) did not initially assemble as a closed circular DNA with HGAP but was subsequently closed into a circular plasmid by using the initially unmapped reads and resequencing *in silico* for consensus building by Bridge Mapper. The fourth plasmid (pPKPN4) was initially not detected by HGAP, likely because the pipeline primarily utilizes long reads (>10 kb) to build the consensus sequences. However, pPKPN4 was identified and circularized with the input of the *bla*_{OXA-232} sequence, which was known from our earlier work to exist on the PittNDM01 genome (17). No gap-filling procedures were required.

The following custom primers were designed and used to confirm the chromosomal inversion by PCR and sequencing: KPN-NDM-end3-F (5'-CACCACCAGACGCTACAA-3'), KPN-NDM-end3-R (5'-TCCGGTGCAGATCTGTATG-3'), KPN-NDM-end5-F (5'-CTCGCCTGGCAGACAAGT-3'), KPN-NDM-end5-R (5'-GTTCTTCATCCATGGCCTGT-3'), KPN-NDM-endpre3-F (5'-CAGATGGCTCCGGATATGAT-3'), and KPN-NDM-endpre3-R (5'-AGAACCAGATCCGCTCACAC-3').

Annotation was performed with RAST (<http://rast.nmpdr.org>) and the NCBI Prokaryotic Genome Annotation Pipeline. The sequences were deposited in GenBank under accession no. CP006798 and CP006802. The sequencing project was assigned BioProject no. PRJNA221868 and ID 221868. The DNA methylation data of the *K. pneumoniae* PittNDM01 strain were captured in the process of SMRT sequencing, as previously described (20), and were deposited to the restriction enzyme database (REBASE) (21).

Phylogenetic analysis. Phylogenomic analysis of PittNDM01 was performed as previously described (22) by aligning the whole genome of PittNDM01 with the whole genomes of other representative *Klebsiella* and *Enterobacter* strains available in the public domain. The genomic data were aligned using Mugsy (23), and the homologous blocks from each genome that aligned were concatenated with the bx-python tool kit (https://bitbucket.org/james_taylor/bx-python). A maximum-likelihood phylogeny with 100 bootstrap replicates was generated for the concatenated blocks of the aligned sequence using RAxML version 7.2.8 (24) and was visualized using FigTree version 1.3.1 (<http://tree.bio.ed.ac.uk/software/figtree/>).

RESULTS

Overall features of the genome. *K. pneumoniae* PittNDM01 has a single chromosome of 5,348,284 bp and four plasmids: pPKPN1 (283,371 bp), pPKPN2 (103,694 bp), pPKPN3 (70,814 bp), and pPKPN4 (6,141 bp). The chromosome has a G+C content of 57.5%, with a total of 4,940 predicted open reading frames identified by the NCBI Prokaryotic Genome Annotation Pipeline. It

shares 99% identity with the chromosome of the reference genome *K. pneumoniae* strain MGH 78578 (GenBank accession no. CP000647), with a large inverted region spanning 23.3% of the entire chromosome (Fig. 1). This inversion was confirmed with PCR and sequencing using custom primers designed to cover the positions at which it occurred.

A phylogenetic comparison of the PittNDM01 strain with other sequenced *K. pneumoniae* strains and closely related species identified that this strain is highly similar to other *K. pneumoniae* strains (Fig. 2). This measure of genome similarity did not take into account the inversion identified above.

The antimicrobial resistance genes identified on the chromosome include the β -lactamase genes *bla*_{SHV-28} and *bla*_{OXA-1}, the aminoglycoside and fluoroquinolone acetyltransferase gene *aac(6')-Ib-cr*, the chloramphenicol acetyltransferase gene *catB4*, the macrolide efflux genes *macA* and *macB*, and the fosfomycin resistance gene *fosA*. *bla*_{SHV}, *catB4*, *macA*, *macB*, and *fosA* are frequently present on the *K. pneumoniae* chromosomes (GenBank accession no. CP000647) (25, 26).

IncHI1B-like plasmid pPKPN1 carrying *bla*_{NDM-1}. pPKPN1 was found to be the largest of the plasmids, carries *bla*_{NDM-1}, and contains 291 predicted open reading frames (Fig. 3). It possesses three replicon genes: *repAciN*, recently identified in plasmid pNDM-CIT carrying *bla*_{NDM-1} and speculated to have originated from a plasmid in *Acinetobacter* spp. (27), and *repA* encoding an FIB-like replicon (predicted) and *repA* encoding an HI1B-like replicon, both recently identified in pNDM-MAR, a *bla*_{NDM-1}-carrying plasmid in a *K. pneumoniae* strain from Morocco (28). Overall, 99% identity was observed in >87% of the pPKPN1 sequence with the pNDM-MAR sequence. However, the region surrounding *bla*_{NDM-1} differed substantially between these two plasmids (Fig. 4). The genetic context of the *bla*_{NDM-1} gene in pPKPN1 resembled that of pNDM-BJ01 from *Acinetobacter lwoffii* (29), in which the *bla*_{NDM-1} region is bracketed by two copies of insertion sequence IS_{Aba125} and is located downstream of *aphA6* (encoding aminoglycoside phosphotransferase for amikacin and neomycin resistance). However, this entire IS_{Aba125}-bracketed structure is further bracketed by two tandem copies of insertion sequence IS₂₆, commonly found in *Enterobacteriaceae* (Fig. 4). Therefore, it may be inferred that the region was first mobilized onto a plasmid in *Acinetobacter* spp. by an IS_{Aba125}-mediated event, followed by a transfer to *K. pneumoniae* and capture of this element onto this IncHI1B-like plasmid by an IS₂₆-mediated transpositional event.

pPKPN1 also carries *aac(6')-Ib-cr*, *bla*_{OXA-1}, and *catB4* (encoding chloramphenicol acetyltransferase) on a class 1 integron, *dfrA12* (encoding dihydrofolate reductase for trimethoprim resistance), *aadA2* (encoding aminoglycoside adenylyltransferase for streptomycin resistance), and *sul1* (encoding dihydropteroate

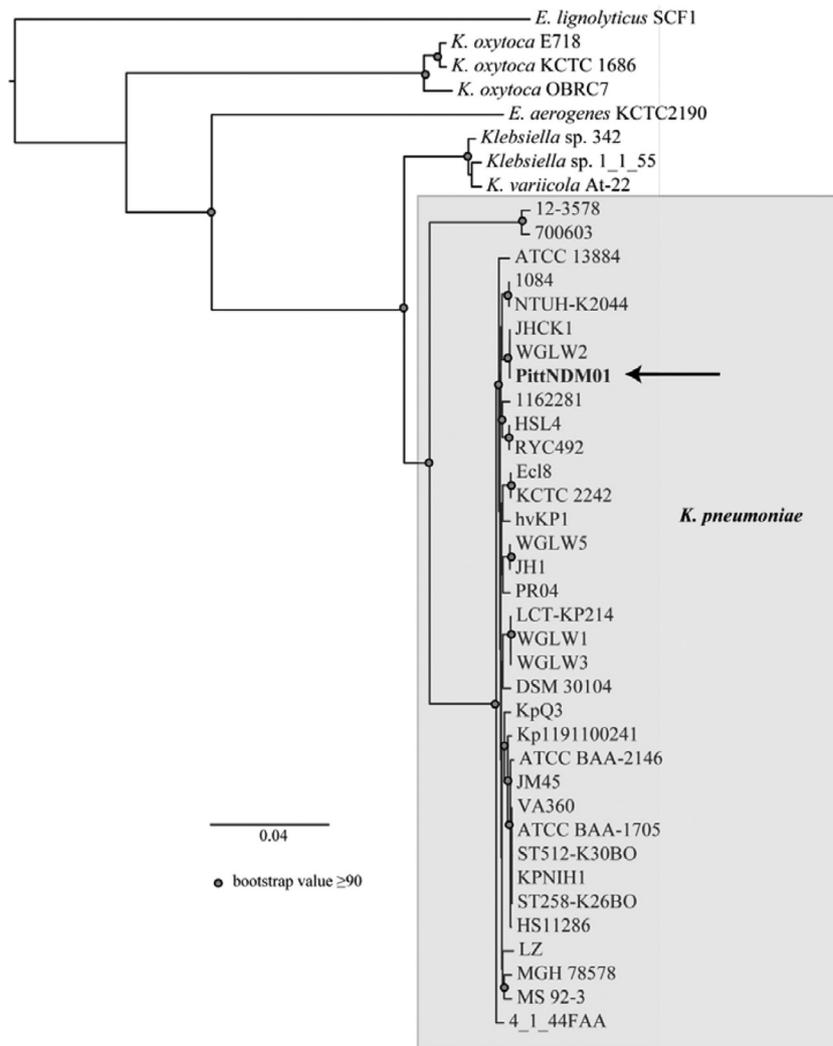


FIG 2 Whole-genome phylogeny of *K. pneumoniae* PittNDM01 relative to other *Klebsiella* strains. Bootstrap values of ≥ 50 are shown. The scale bar indicates the distance of 0.04 nucleotide substitutions per site. The tree demonstrates that the core genome of *K. pneumoniae* PittNDM01 is similar to that of other *K. pneumoniae* isolates that have been sequenced.

synthase for sulfonamide resistance) on another class 1 integron, *armA* (encoding 16S rRNA methyltransferase for high-level aminoglycoside resistance), *mel* (encoding macrolide efflux), *mph2* (encoding macrolide phosphotransferase), *ble_{MBL}* (encoding bleomycin resistance), and *qnrB1* (encoding ciprofloxacin resistance). As such, the acquisition of this plasmid would compromise the activities of all β -lactams (with the exception of aztreonam), aminoglycosides, fluoroquinolones, and sulfonamides.

IncFIB-like plasmid pPKPN2. pPKPN2, with 101 predicted open reading frames, possesses an IncFIB-like replicon previously reported in pKPN-IT from a carbapenem-resistant KPC-3-producing *K. pneumoniae* ST258 strain in Italy (30), and it contains loci for plasmid partition, conjugal transfer, and a type I restriction/modification system. Approximately 78% of the pPKPN2 sequence aligned at 99% identity with that of pKPN-IT. However, pPKPN2 (103.7 kb) is much smaller than pKPN-IT (208.2 kb) and lacks all of the heavy metal resistance loci that characterize pKPN-IT-type plasmids. In addition, antimicrobial resistance determinants were completely absent on pPKPN2.

IncFIA-like plasmid pPKPN3. pPKPN3, with 80 predicted open reading frames, has an IncFIA-like replicon reported in pEK499, which is a multidrug resistance *bla_{CTX-M-15}*-carrying plasmid found in an *Escherichia coli* ST131 strain from the United Kingdom (31). It also possesses a *repB* gene that is commonly observed in *bla_{KPC}*-carrying plasmids, including pKPN101-IT from Italy (32). pPKPN3 possesses a large array of antimicrobial resistance genes (Table 1). A class 1 integron contains the gene cassettes *arr-2* (encoding rifampin ADP-ribosyl transferase for rifampin resistance), *ereC* (encoding erythromycin esterase for erythromycin resistance), *aadA1* (encoding aminoglycoside adenyltransferase for streptomycin resistance), *cmlA5* (encoding an efflux pump for chloramphenicol resistance), and *sul1* (encoding sulfonamide resistance). Other resistance genes are scattered across the plasmid and include *bla_{CTX-M-15}* (encoding ESBL), *bla_{SHV-11}* and *bla_{TEM-1}* (encoding non-ESBL), *aacC2* (encoding aminoglycoside acetyltransferase for gentamicin resistance), *strB* (encoding aminoglycoside phosphotransferase for streptomycin resistance), and *sul2* (encoding dihydropteroate synthase for sulfonamide resistance).

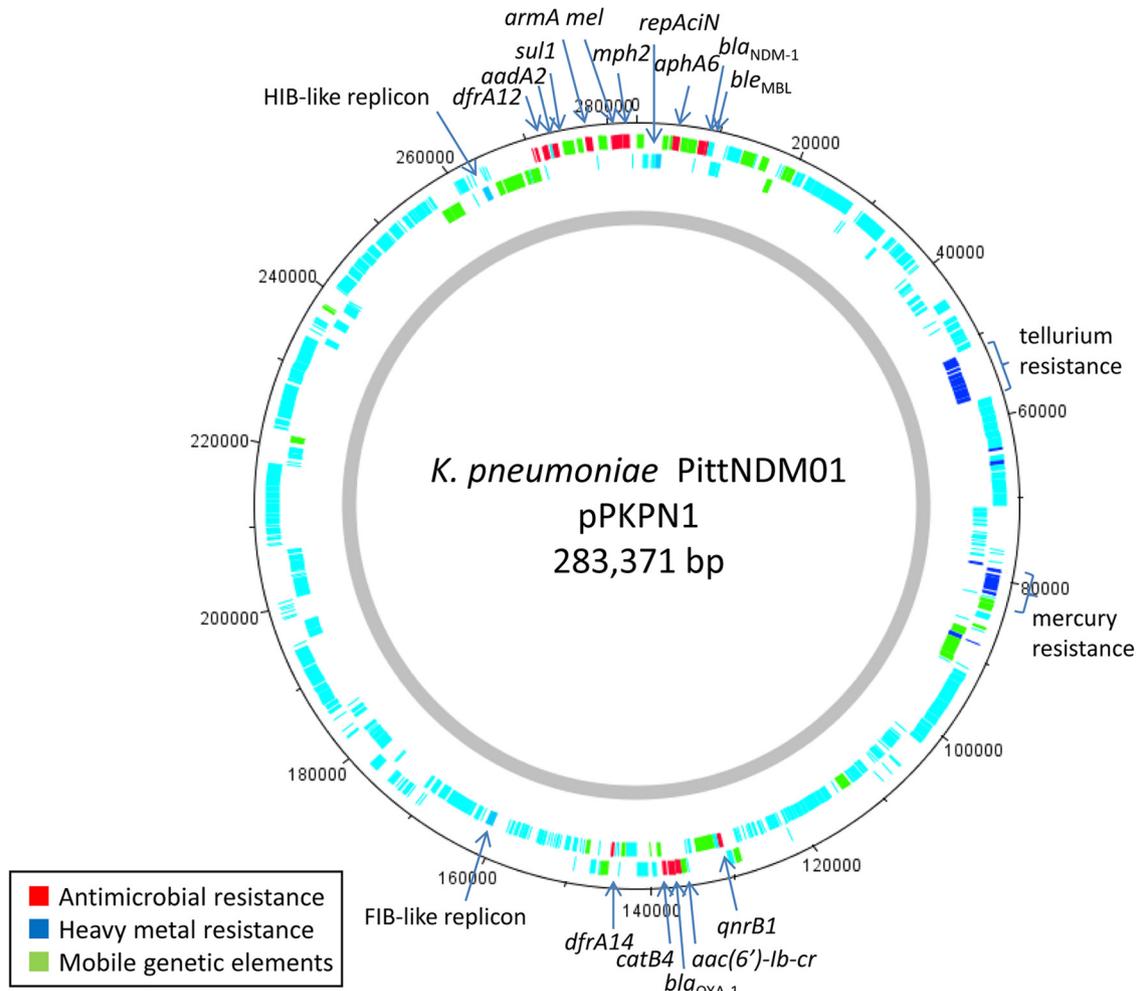


FIG 3 Overview of pPKPN1. The plasmid carries 12 resistance genes, including *bla*_{NDM-1}, *armA*, and *qnrB1*, which are clustered in two regions of the plasmid containing class 1 integrons. pPKPN1 therefore confers resistance to the three key antimicrobial classes (carbapenems, aminoglycosides, and fluoroquinolones). The numbers indicate the positions on the plasmid.

***bla*_{OXA-232}-carrying ColE-like plasmid pPKPN4.** pPKPN4 is nearly identical to the fully sequenced plasmid carrying *bla*_{OXA-232} with eight predicted open reading frames, including the carbapenemase gene, which was identified in *K. pneumoniae* and *E. coli* isolates recovered from patients who were transferred from India to France (6). Other than the antimicrobial resistance gene, the remainder of the plasmid appears to be cryptic in nature.

Methylome of PittNDM01. There were five active methyltransferases (MTases) that were detected, as shown in Table 2. In addition to the *dam* methyltransferase, which modifies 5'-GATC-3' sequences (with the base in bold type as the site of methylation), there are 3 different active type I MTases that also modify their recognition sequences with N⁶-methyladenine (6mA). 5-Methylcytosine (5mC) modifications at the 5'-CCWGG-3' sequences imparted by a homolog of the *dcm* gene were also detected. As described previously (33), the direct detection of 5mC during SMRT sequencing without enhancement techniques is limited, because the signal is more subtle and dispersed than in other modifications. This explains why only 27.5% of the sites were detected, even though the expectation is that the majority of the 5'-CCWGG-3' motifs are modified.

DISCUSSION

NDM-1 and OXA-48 group carbapenemases are rapidly emerging worldwide in *Enterobacteriaceae*, in particular in *K. pneumoniae*. In the United States, however, KPC remains by far the most common carbapenemase in *Enterobacteriaceae*, and clinical strains producing NDM-1 or OXA-48 group carbapenemases are still rare. The finding of NDM-1 and OXA-232 coproduction in *K. pneumoniae* PittNDM01 was therefore surprising. This strain was also highly drug resistant, only sparing fosfomycin and colistin. We therefore set out to characterize the genomic features of this unique clinical strain in order to obtain insights into the evolution of multidrug resistance.

While *K. pneumoniae* PittNDM01 originated in India, its chromosome shares 99% identity with that of the reference genome *K. pneumoniae* MGH 78578, a strain that was isolated in the United States in 1994. In contrast, three of the four plasmids are unique to *Klebsiella* isolates in strain PittNDM01. pPKPN1, a multidrug resistance plasmid, has a hybrid structure of two *bla*_{NDM-1}-carrying plasmids, pNDM-MAR and pNDM-CIT. Its *bla*_{NDM-1} region revealed a distinctive arrangement in which the original IS*Aba125*-

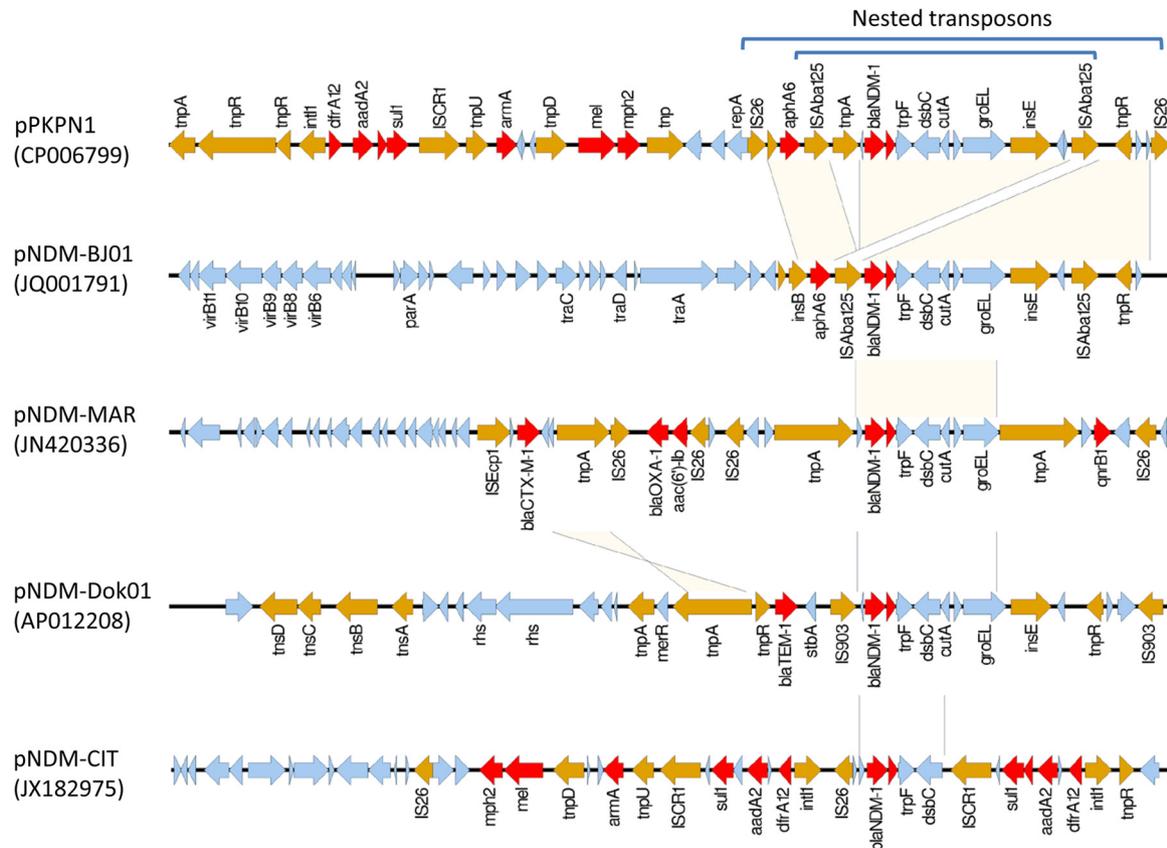


FIG 4 Alignment of the bla_{NDM-1} region of pPKPN1 and representative bla_{NDM-1} -carrying plasmids. The antimicrobial resistance genes and mobile genetic elements are colored in red and brown, respectively. The region surrounding bla_{NDM-1} differed substantially among the plasmids, with pPKPN1 being especially rich in antimicrobial resistance genes, along with pNDM-CIT.

bracketed bla_{NDM-1} seems to have been further mobilized by an IS26-mediated event downstream of a class 1 integron, generating an extended multidrug resistance scaffold. Also, among the genes carried by pPKPN1 are *armA* and *qnrB1*, which compromise the activity of aminoglycosides and fluoroquinolones, respectively. pPKPN2 is somewhat unusual for its lack of striking functional features, but it might be considered the progenitor of the series of heavy metal resistance plasmids represented by pKPN-IT. Finally, pPKPN3 is the second multidrug resistance plasmid after pPKPN1 and carries a variety of genes conferring resistance to expanded-spectrum cephalosporins, gentamicin, sulfonamides, and chloramphenicol. Together, these plasmids provide an extraordinary array of antimicrobial and heavy metal resistance determinants.

Given that this was an isolated imported case from India, we

were not able to identify an adequate reference genome prior to this study. Another consideration was the high variability of bla_{NDM-1} -carrying plasmids reported to date, which may confound the assembly of the bla_{NDM-1} -carrying plasmid in *K. pneumoniae* PittNDM01. For these reasons, we conducted a *de novo* assembly of the genome of this strain using the SMRT sequencing technology, whose reads of >10 kb facilitated scaffold building. The assembly was conducted on the Celera-based HGAP pipeline (7). This allowed for closure of the chromosome and the two largest plasmids directly from the raw sequence read input. The third plasmid (pPKPN3) was initially assembled as an approximately 67-kb linear element. The identification of the missing 3 kb and closure was achieved by remapping the SMRT sequencing data to the assembled contigs and identifying long sequencing

TABLE 1 Overall features of the *K. pneumoniae* PittNDM01 genome

Parameter	Chromosome	pPKPN01	pPKPN02	pPKPN03	pPKPN04
Size (bp)	5,348,284	283,371	103,694	70,814	6,141
No. of reads	391,218	19,936	1,505	1,315	99
G+C %	57.50	46.41	51.00	52.92	52.19
No. of predicted open reading frames	4,940	291	101	80	8
Resistance gene(s)	<i>bla</i> _{SHV-28} , <i>bla</i> _{OXA-1} , <i>aac</i> (6')-Ib-cr, <i>cat</i> , <i>macA/macB</i> , <i>fosA</i>	<i>bla</i> _{NDM-1} , <i>bla</i> _{OXA-1} , <i>armA</i> , <i>aac</i> (6')-Ib-cr, <i>aadA2</i> , <i>aphA6</i> , <i>qnrB1</i> , <i>catB4</i> , <i>mel</i> , <i>mph2</i> , <i>dfrA12</i> , <i>sul1</i>	None	<i>bla</i> _{CTX-M-15} , <i>bla</i> _{SHV-11} , <i>bla</i> _{TEM-1} , <i>aadA1</i> , <i>aacC2</i> , <i>strB</i> , <i>ereC</i> , <i>cmlA5</i> , <i>sul1</i> , <i>sul2</i> , <i>arr-2</i>	<i>bla</i> _{OXA-232}

TABLE 2 Detection of methylation in *K. pneumoniae* PittNDM01

Motif ^a	Modification type	No. of motifs detected	No. of motifs in genome	% motifs detected
5'-GATC-3'	6mA	63,453	63,698	99.6
3'-CTAG-5'				
5'-CAACNNNNNCGT-3'	6mA	756	758	99.7
3'-GTTGNNNNNGCA-5'		756	758	99.7
5'-GATGNNNNNNTTGT-3'	6mA	1,040	1,043	99.7
3'-CTACNNNNNNAAC-5'		1,037	1,043	99.4
5'-GTCANNNNNNNTGT-3'	6mA	322	325	99.1
3'-CAGTNNNNNNAACA-5'		324	325	99.7
5'-CCWGG-3'	5mC	7,296	26,524	27.5
3'-GGWCC-5'				

^a The methylated positions in the methyltransferase recognition sequence motifs are in bold type.

reads that connected the 5' and 3' ends of the linear element. The linear element sequence was manually extended using the identified reads and polished with the Quiver consensus tool (19). Finally, the smallest plasmid carrying *bla*_{OXA-232} (pKPN4) was initially not recognized by the SMRT Analysis software as the BluePippin size selection of fragments of >7 kb was carried out. Therefore, with the presence of very small plasmids, a lower size selection cutoff or the alternative use of the AMPure purification method would likely facilitate the automated assembly of these smaller plasmids from the SMRT sequencing data. A whole-genome mapping-assisted *de novo* assembly method was recently proposed and was used in the full-genome assembly of an NDM-1-producing *Providencia stuartii* strain (34). The SMRT sequencing used in our study has the advantage of achieving closure of the genome using a single platform. The cost of SMRT sequencing per bacterial strain is slightly higher than those of the other popular sequencing platforms that yield shorter reads, but the total cost required to finish a bacterial genome is substantially lower with SMRT sequencing (18). Therefore, the most cost-effective use of this technology likely lies in the *de novo* sequencing of highly plastic bacterial genomes.

In conclusion, we characterized the genomic features of a double carbapenemase-producing *K. pneumoniae* clinical isolate through the use of a long-read sequencing platform and semiautomated *de novo* assembly process. The approach provided fully assembled genome sequences without the need for gap-filling procedures, and it was particularly useful in elucidating the complex structures of the large multidrug resistance plasmids.

ACKNOWLEDGMENTS

We thank K. Luong for assistance with data analysis and Rich Roberts (New England BioLabs) for entering the methylome information into REBASE.

The whole-genome sequencing was supported by Pacific Biosciences. The effort of Y.D. was supported in part by research grants from the National Institutes of Health (grants R21AI107302 and R01AI104895).

M.B., Y.-C.T., T.A.C., and J.K. are employees of Pacific Biosciences.

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