

# Mechanisms Involved in Acquisition of *bla*<sub>NDM</sub> Genes by IncA/C<sub>2</sub> and IncFII<sub>Y</sub> Plasmids

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*bla*<sub>NDM</sub> genes confer carbapenem resistance and have been identified on transferable plasmids belonging to different incompatibility (Inc) groups. Here we present the complete sequences of four plasmids carrying a *bla*<sub>NDM</sub> gene, pKP1-NDM-1, pEC2-NDM-3, pECL3-NDM-1, and pEC4-NDM-6, from four clinical samples originating from four different patients. Different plasmids carry segments that align to different parts of the *bla*<sub>NDM</sub> region found on *Acinetobacter* plasmids. pKP1-NDM-1 and pEC2-NDM-3, from *Klebsiella pneumoniae* and *Escherichia coli*, respectively, were identified as type 1 IncA/C<sub>2</sub> plasmids with almost identical backbones. Different regions carrying *bla*<sub>NDM</sub> are inserted in different locations in the antibiotic resistance island known as ARI-A, and *ISCR1* may have been involved in the acquisition of *bla*<sub>NDM-3</sub> by pEC2-NDM-3. pECL3-NDM-1 and pEC4-NDM-6, from *Enterobacter cloacae* and *E. coli*, respectively, have similar IncFII<sub>Y</sub> backbones, but different regions carrying *bla*<sub>NDM</sub> are found in different locations. Tn3-derived inverted-repeat transposable elements (TIME) appear to have been involved in the acquisition of *bla*<sub>NDM-6</sub> by pEC4-NDM-6 and the *rmtC* 16S rRNA methylase gene by IncFII<sub>Y</sub> plasmids. Characterization of these plasmids further demonstrates that even very closely related plasmids may have acquired *bla*<sub>NDM</sub> genes by different mechanisms. These findings also illustrate the complex relationships between antimicrobial resistance genes, transposable elements, and plasmids and provide insights into the possible routes for transmission of *bla*<sub>NDM</sub> genes among species of the *Enterobacteriaceae* family.

In Gram-negative bacteria, especially the *Enterobacteriaceae* family,  $\beta$ -lactamases are the major mechanism of resistance against  $\beta$ -lactams. In particular,  $\beta$ -lactamases known as carbapenemases are becoming a key concern in the effective administration of antimicrobial therapy, as they can confer resistance to carbapenems, a major last-line antimicrobial. The NDM carbapenemase was first reported in 2009, produced by a *Klebsiella pneumoniae* isolated from a Swedish patient recently returned from India (1). There are currently 16 known NDM variants, and *bla*<sub>NDM</sub> genes have now been reported in strains sourced from every inhabitable continent and in multiple species of *Enterobacteriaceae*, including *Escherichia coli*, *K. pneumoniae*, and *Enterobacter cloacae* (2).

Plasmids are important vehicles for the capture, accumulation, and spread of various antimicrobial resistance determinants. Several different types of plasmids associated with the *Enterobacteriaceae* family have been reported to harbor *bla*<sub>NDM</sub> genes, including IncA/C, IncFII subtypes, IncH types, IncL/M, IncN (2–4), and IncX (5). Some of these plasmids coharbor additional antimicrobial resistance genes, including the 16S rRNA methylase genes *armA* and *rmtC* (conferring high-level aminoglycoside resistance), quinolone resistance genes (*qnrB1* and *qnrS1*), and/or other  $\beta$ -lactamase genes (such as *bla*<sub>CMY-2</sub> and variants and *bla*<sub>CTX-M-15</sub>) (6).

The original source of *bla*<sub>NDM</sub> is not known, but *Acinetobacter* spp. may have acted as an intermediate between this organism and the *Enterobacteriaceae* family (7–9). In *Acinetobacter* spp., *bla*<sub>NDM</sub> genes have often been observed within the 10,099-bp composite transposon Tn125 that is bounded by two copies of ISAb125 (9–12). The *bla*<sub>NDM</sub> gene starts 93 bp downstream of the right-

hand end (IR<sub>R</sub>) of ISAb125, which provides the –35 region of a promoter (13, 14), and is followed by several genes, including *ble*<sub>MBL</sub> (bleomycin resistance), *trpF* (involved in tryptophan biosynthesis), and the mobile element *ISCR27*. In several *Acinetobacter* species plasmids (e.g., pNDM-BJ01; GenBank accession no.

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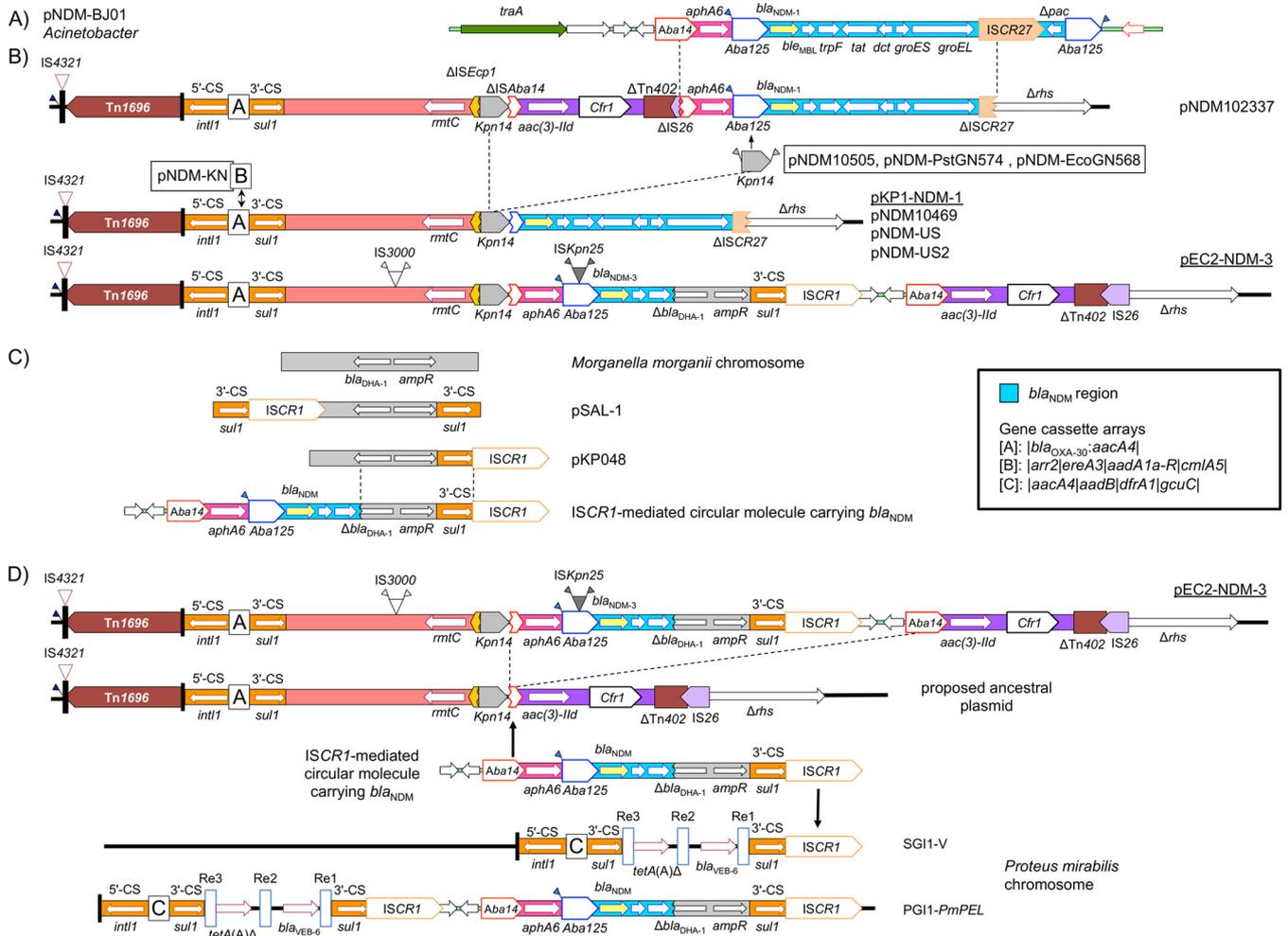
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**FIG 1** ARI-A of type 1 IncA/C<sub>2</sub> plasmids carrying *bla*<sub>NDM</sub> and potential routes for *bla*<sub>NDM</sub> insertion. IS are shown as block arrows labeled with their name or number. DR are represented by flags of the same color. Triangles indicate the insertion sites of IS elements flanked by DR. Vertical black bars represent the transposed IR of ARI-A and IRI of class 1 In/Tn. Horizontal green and black lines represent *Acinetobacter* and IncA/C<sub>2</sub> plasmid backbones, respectively. Vertical dotted lines indicate the boundaries of closely related sequences. Vertical black arrows and dotted diagonal lines indicate possible deletion and insertion events. (A) Tn125 in *Acinetobacter lwoffii* plasmid pNDM-BJ01. (B) ARI-A of type 1 IncA/C<sub>2</sub> plasmids closely related to pKP1-NDM-1 and pEC2-NDM-3. (C) Possible derivation of the circular molecule inserted in pEC2-NDM-3. (D) Insertion of circular molecule carrying *bla*<sub>NDM</sub> into pEC2-NDM-3 and a *P. mirabilis* genomic island. The sequences used to draw these diagrams are from the GenBank accession numbers listed in Table 1 plus the following: pNDM-BJ01, NC\_019268; pSAL-1, AJ237702; pKP048, NC\_014312; SGI1-V, HQ888851; PGI1-PmPEL, KF856624.

NC\_019268 [15], IS*Aba14* and an *aphA6* gene (amikacin resistance) are present upstream of the IS*Aba125* adjacent to *bla*<sub>NDM-1</sub> (Fig. 1A). In plasmids from the *Enterobacteriaceae*, *bla*<sub>NDM</sub> genes are generally found in this immediate genetic context, with at least a fragment of IS*Aba125* containing the -35 promoter region present upstream, within different length fragments matching *Acinetobacter* plasmids and associated with different mobile elements (3, 16–21).

We previously reported locally identified *K. pneumoniae* (22) and *E. cloacae* (23) clinical isolates carrying *bla*<sub>NDM-1</sub>, *E. coli* carrying *bla*<sub>NDM-3</sub> (G283A, Asp95Asn) (23), and *E. coli* carrying *bla*<sub>NDM-6</sub> (C698T, Ala233Val) (24). The *bla*<sub>NDM</sub> gene could be transferred from all four isolates by transformation and/or conjugation, indicating a plasmid location in each case, but replicon types were not determined (22–24). In this study, we present the complete sequences of these four plasmids and a comparison of

the genetic contexts of *bla*<sub>NDM</sub> with those in closely related plasmids.

**MATERIALS AND METHODS**

**Bacterial isolates and plasmids.** *K. pneumoniae* KP1 (22) and *E. cloacae* ECL3 carrying *bla*<sub>NDM-1</sub> (23) were isolated in Australia, as was *E. coli* EC2 carrying *bla*<sub>NDM-3</sub> (23), while *E. coli* EC4 carrying *bla*<sub>NDM-6</sub> (previously designated ARL10/167 [24]) was isolated in New Zealand. All isolates were from patients recently returned from India. Transconjugants in sodium azide-resistant *E. coli* J53Azi<sup>r</sup> were available and/or were obtained by conjugation on solid medium, as previously described (17).

**DNA preparation and sequencing.** Genomic DNA (gDNA) was extracted from all four isolates using the UltraClean Microbial DNA isolation kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA). DNA from KP1, ECL3, and EC4 was sequenced by Illumina HiSeq 2000 technology (Illumina, San Diego, CA, USA). Illumina sequences were *de novo* assembled using CLC genomic workbench v8.0 (CLC Bio, Aarhus, Denmark). Initial

TABLE 1 General features of IncA/C<sub>2</sub> and IncFII<sub>Y</sub> plasmids studied here and close relatives<sup>a</sup>

Plasmid	NDM	Size (bp)	Species	ST	Country <sup>b</sup>	Yr	Source	GenBank accession no.	Reference
<b>A/C<sub>2</sub></b>									
<b>pKP1-NDM-1</b>	1	137,552	<i>K. pneumoniae</i>	147	India/Australia	2010	Human	KF992018	This study
<b>pEC2-NDM-3</b>	3	160,989	<i>E. coli</i>	443	India/Australia	2010	Human	KC999035	This study
pNDM-EcoGN568	1	166,750	<i>E. coli</i>	1289	India/Canada	na	Human	KJ802404	50
pNDM-PstGN576	1	147,886	<i>P. stuartii</i>	N/A	India/Canada	na	Human	KJ802405	50
pNDM102337	1	165,974	<i>E. coli</i>	na	Canada	na	na	NC_019045	
pNDM10505	1	166,744	<i>E. coli</i>	na	Canada	na	na	NC_019069	
pNDM10469	1	137,813	<i>K. pneumoniae</i>	na	Canada	na	na	NC_019158	
pNDM-KN	1	162,746	<i>K. pneumoniae</i>	14	Kenya	2009	Human	JN157804	51, 52
pNDM-US	1	140,825	<i>K. pneumoniae</i>	11	India/USA	2010	Human	CP006661	53
pNDM-US-2	1	140,821	<i>K. pneumoniae</i>	na				KJ588779 <sup>c</sup>	
<b>FII<sub>Y</sub></b>									
<b>pECL3-NDM-1</b>	1	99,435	<i>E. cloacae</i>	265	India/Australia	2011	Human	KC887917	This study
<b>pEC4-NDM-6</b>	6	110,786	<i>E. coli</i>	101	India/New Zealand	2010	Human	KC887916	This study
pKOX_NDM1	1	110,781	<i>Klebsiella oxytoca</i>	na	China/Taiwan	2010	Human	NC_021501	18
pNDM1_EC14653	1	109,353	<i>E. cloacae</i>	177	China	2014	Human	KP868647	43
pNDM-EclGN574	1	110,786	<i>E. cloacae</i>	na	India/Canada	na	Human	KJ812998	50
pP10164-NDM	1	99,276	<i>Leclercia adecarboxylata</i>	N/A	China	2012	Human	KP900016	19
pRJF866	1	110,786	<i>K. pneumoniae</i>	11	China	2011	Human	NC_025184	54
pK351	1	106,844	<i>K. pneumoniae</i>	147	Iran/USA	2014	Human	KR351290	41

<sup>a</sup> Plasmids with names in bold typeface were sequenced in this study. Abbreviations: na, not available; N/A, not applicable (no multilocus typing schemes for these species); ST, sequence type.

<sup>b</sup> Travel history is given if available, e.g., India/Australia indicates isolation in Australia from a patient recently returned from India.

<sup>c</sup> GenBank accession no. KJ588779 implies that pNDM-US-2 was extracted in China from the same strain (ATCC BAA-2146) as pNDM-US.

annotation of contigs was performed using RAST (25). IS finder (<https://www-is.biotoul.fr/>) and the Repository of Antibiotic-resistance Cassettes (RAC; <http://rac.aihi.mq.edu.au/rac/>) were used to identify insertion elements (IS) and integron components, respectively. BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) searches were used to identify related plasmids carrying *bla*<sub>NDM</sub> to guide PCR-based gap closure and Sanger sequencing (Macrogen, South Korea) to assemble contigs into complete plasmids.

gDNA from EC2 was sheared using a g-TUBE (Covaris) into fragment sizes targeted at 20 kb. Following purification, SMRTbell template libraries were prepared using the commercial Template Preparation kit (Pacific Biosciences Inc., Menlo Park, CA, USA) and sequenced on a Pacific Biosciences (PacBio) RSII instrument (University of Queensland Centre for Clinical Genomics [UQCCG]) using the P6 polymerase and C4 sequencing chemistry. The raw PacBio sequence data were assembled *de novo* using the hierarchical genome assembly process (HGAP version 2) and Quiver (26) from the SMRT Analysis software suite (version 2.3.0; PacBio) with default parameters and a seed read cutoff of 17,000 bp. Following assembly, contigs were examined for overlapping 5' and 3' ends (a characteristic feature of the HGAP assembly process) using Contiguity (27) and were manually trimmed to generate circular contigs. Raw sequence reads were then mapped back onto the assembled circular plasmid contig (BLASR [28] and Quiver) to validate the assembly and resolve any remaining errors.

RAST, IS finder, RAC, CLC genomic workbench v8.0, Geneious R9 (Bio-matters Ltd., New Zealand, including Mauve [29]), and BLAST were used for manual annotation, alignment, single nucleotide polymorphism (SNP) detection, and other analysis and comparisons of complete plasmid sequences.

**Nucleotide sequence accession numbers.** Existing GenBank entries for partial sequences of all four plasmids were updated to include the complete sequences, as follows: pKP1-NDM-1, KF992018; pEC2-NDM-3, KC999035; pECL3-NDM-1, KC887917; pEC4-NDM-6, KC887916.

## RESULTS AND DISCUSSION

**General features of plasmids carrying *bla*<sub>NDM</sub>.** Isolates KP1, EC2, ECL3, and EC4 each transferred a plasmid carrying *bla*<sub>NDM</sub> to *E. coli* J53Azi<sup>r</sup> by conjugation. Plasmids carrying *bla*<sub>NDM</sub> assembled from whole-genome sequences (at least 50-fold coverage) were designated pKP1-NDM-1, pEC2-NDM-3, pECL3-NDM-1, and pEC4-NDM-6, respectively. pKP1-NDM-1 (137,552 bp) and pEC2-NDM-3 (160,989 bp) were identified as type 1 IncA/C<sub>2</sub> (Table 1). The backbones of pKP1-NDM-1 and pEC2-NDM-3 are very closely related to those of several other type 1 IncA/C<sub>2</sub> plasmids (see Table S1 in the supplemental material) and include characteristic IncA/C<sub>2</sub> core regions, such as the conjugative transfer (*tra*) region and *parA-parB*, required for plasmid partitioning (30). They have identical replication regions, with a *repA* gene and 14 19-bp direct repeat sequences (iterons), which are binding sites for the RepA protein (30). Both pKP1-NDM-1 and pEC2-NDM-3 have the same *ISEcp1* transposition unit carrying a *bla*<sub>CMY-2</sub> variant, in this case *bla*<sub>CMY-6</sub>, inserted in the same location as in many other type 1 IncA/C<sub>2</sub> plasmids, between *traA* and *traC*, flanked by 5-bp direct repeats (DR). Neither carries Tn6170, present in some type 1 IncA/C<sub>2</sub> plasmids (31).

Both pECL3-NDM-1 (99,435 bp) and pEC4-NDM-6 (110,786 bp) are IncFII<sub>Y</sub> type plasmids (Table 1) carrying two replicons, classified as Y4 (*repA*) and FIB36 (*repB*) by the replicon sequence typing (RST) scheme (32). The backbones of both plasmids are closely related to those of other IncFII<sub>Y</sub> plasmids carrying *bla*<sub>NDM</sub> (Table 1), which have not been well studied but include a conjugation (*tra*) region and stability (*psi*, *parAB*) and maintenance (*ccdAB*) genes (18, 19).

**Both IncA/C<sub>2</sub> plasmids carry *bla*<sub>NDM</sub> in antibiotic resistance island ARI-A.** In both IncA/C<sub>2</sub> plasmids sequenced here, the

bla<sub>NDM</sub> gene is located within an antibiotic resistance island known as ARI-A, which is found in exactly the same location in different type 1 IncA/C<sub>2</sub> plasmids, between two *tra* regions (30, 31). The prototype ARI-A, found in pRMH760, is a complex hybrid transposon structure bounded by 38-bp inverted repeats (IR) interrupted by IS4321 and is inserted upstream of the *rhs* gene (unknown function) flanked by 5-bp DR (TTGTA) (31, 33). ARI-A in pRMH760 carries a class 1 In/Tn, with IS26-*aphA1*-IS26 interrupting the Tn402 *tni* region, and other resistance genes. Islands carrying bla<sub>NDM</sub> appear to be derived from this structure, with deletions of part of the adjacent *rhs* gene in some cases (3). In pNDM102337 (Table 1; Fig. 1B), nucleotides 1 to 1,617 of the 3'-conserved segment (3'-CS) of the class 1 integron are followed by a 3,562-bp region carrying a type III restriction-modification system and the *rmtC* 16S rRNA methylase gene and then 224 bp of the IR<sub>R</sub> end of *ISEcp1*. *ISEcp1* is truncated by ISKpn14, which is followed by a 198-bp fragment of IS*Aba14* and then a region found on a number of different plasmids that contains the *aac(3)-IId* (gentamicin resistance) gene and IS*Cfr1* (34). The adjacent fragment of the Tn402 *tni* region has the same boundary with IS26 as in ARI-A of pRMH760, but only 217 bp of IS26 is present. This is followed by an 8,913-bp region matching *Acinetobacter* plasmids such as pNDM-BJ01, which includes 662 bp of the right end of IS*Aba14*, *aphA6*, one copy of IS*Aba125*, bla<sub>NDM-1</sub>, and a fragment of ISCR27.

pNDM10505, pNDM-PstGN576, and pNDM-EcoGN568 (Table 1) have a variant of the pNDM102337 ARI-A with a second ISKpn14 inserted 130 bp upstream of the left end of IS*Aba125* (Fig. 1B). ISKpn14-mediated deletion may have been responsible for creating the ARI-A variant present in the other closely related type 1 IncA/C<sub>2</sub> plasmids pNDM-US, pNDM-US-2, pNDM-KN, and pNDM10469, which lack the *aac(3)-IId* region (Table 1; Fig. 1B) (3). pKP1-NDM-1, sequenced here, has an almost identical ARI-A, except that only 89 bp of IS*Aba125* is present adjacent to ISKpn14 upstream of bla<sub>NDM</sub>. This difference was confirmed by reexamining raw reads and has been seen in other partial sequences (17, 35), and ISKpn14 is ~89% identical to IS1, which is known to cause adjacent deletions (34). All of these type 1 IncA/C<sub>2</sub> plasmids except pNDM-KN have the same cassette array, consisting of a single fused cassette comprised of the first 87 bp of the bla<sub>OXA-30</sub> cassette and position 17 to the end of the *aacA4* cassette, overlapping by a single A (36). The mechanism(s) responsible for insertion of the bla<sub>NDM</sub> region into the proposed pNDM102337-like progenitor plasmid is unclear, but it is possible that it involved ISCR27 and/or IS26 and subsequent deletion(s).

The backbone of pEC2-NDM-3 is almost identical to the pNDM102337-like plasmids described above (see Table S1 in the supplemental material), but *ISEc23* is inserted 222 bp upstream of ARI-A, flanked by 8-bp DR characteristic of this element. ARI-A of pEC2-NDM-3 includes the same *rmtC* region as the one described above except that IS3000 is inserted upstream of *rmtC*, flanked by characteristic 5-bp DR. The region containing bla<sub>NDM-3</sub> however, is different from the one in the other IncA/C<sub>2</sub> plasmids and is inserted between ISKpn14 and the *aac(3)-IId*/IS*Cfr1*/*tni*<sub>402</sub> region. The region matching pNDM-BJ01 encompasses 198 bp of IS*Aba14*, *aphA6*, one copy of IS*Aba125*, bla<sub>NDM-1</sub>, ble<sub>MBL</sub>, and *trpF*. ISKpn25, carrying a restriction-modification system, is inserted in IS*Aba125* upstream of the -35 promoter region, flanked by characteristic 8-bp DR (Fig. 1B). The bla<sub>NDM</sub> gene has the single nucleotide change giving bla<sub>NDM-3</sub> rather than bla<sub>NDM-1</sub>, and *trpF* is

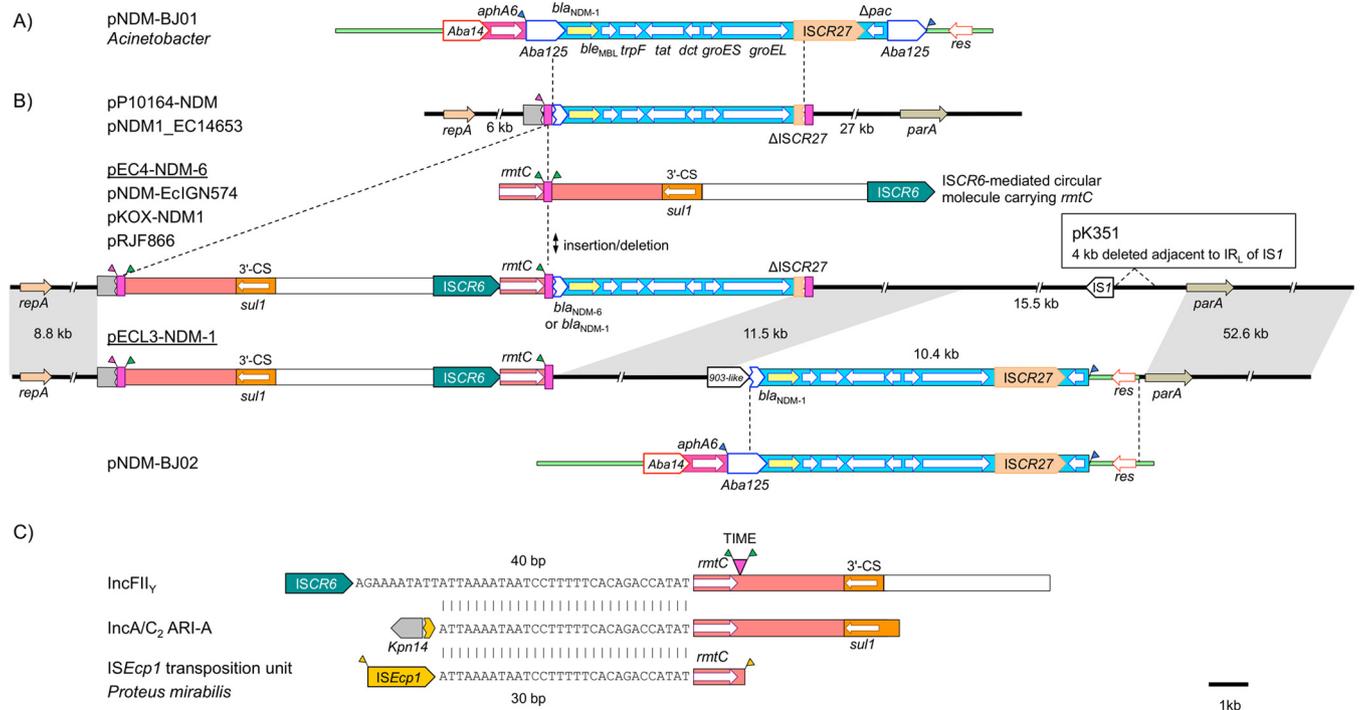
followed by a truncated bla<sub>DHA</sub> gene and the associated *ampR* gene, nucleotides 180 to 1,313 of the 3'-CS and ISCR1. This region is separated from a complete IS*Aba14* by 934 bp matching the region upstream of IS*Aba14* in pNDM-BJ01. ARI-A in pEC2-NDM-3 ends with the *aac(3)-IId*-IS*Cfr1*-*tni*<sub>402</sub> region, but a complete copy of IS26 truncates the *rhs* gene in the IncA/C<sub>2</sub> backbone. The only other known location of the bla<sub>NDM-3</sub> variant is on an IncFII plasmid (37) associated with ISCR1 but not with the truncated bla<sub>DHA</sub>-*ampR* region present in pEC2-NDM-3.

This context in pEC2-NDM-3 suggests insertion of bla<sub>NDM</sub> from a circular molecule mediated by ISCR1. ISCR1 is proposed to transpose by a rolling-circle mechanism, similar to the related IS91 family elements (38), in which replication proceeds from the *oriS* end, located downstream of *rcr* (rolling-circle replicase gene), toward the *terIS* upstream and can continue into and capture an adjacent region. ISCR1 has generally been found associated with class 1 integrons, after position 1,313 of the 3'-CS, suggesting integration of circular molecules by recombination in either the 3'-CS or an existing ISCR1 (38). ISCR1 has previously been suggested to be associated with movement of bla<sub>NDM</sub> (39) and was recently shown to be responsible for mobilizing a region containing bla<sub>NDM</sub> and part of the 3'-CS, but without the bla<sub>DHA</sub>Δ-*ampR* region, between plasmids (20).

ISCR1 appears to have been responsible for capturing the bla<sub>DHA</sub>Δ-*ampR* region from the *Morganella morganii* chromosome and inserting it into a class 1 integron (40) (Fig. 1C). Generation of a circular molecule by recombination between the two flanking 3'-CS and reintegration at ISCR1 could create the arrangement seen in, e.g., pKP048 (GenBank accession no. NC\_014312), with ISCR1 downstream of the bla<sub>DHA</sub>Δ-*ampR* region and the 3'-CS, and the usual 3'-CS/ISCR1 boundary (Fig. 1C). ISCR1 may then have mobilized this 3'-CS segment and the bla<sub>DHA</sub>Δ-*ampR* region and inserted them downstream of bla<sub>NDM</sub>, before picking up the bla<sub>NDM</sub> region as part of a circular molecule (Fig. 1C).

The complete IS*Aba14* in pEC2-NDM-3 has the same boundary with the *aac(3)-IId* region as the IS*Aba14* fragment in pNDM102337, suggesting that homologous recombination between the complete and partial copies of IS*Aba14* could have been responsible for the insertion of this circular molecule into pEC2-NDM-3 (Fig. 1D). The same circular molecule carrying bla<sub>NDM</sub> also appears to have inserted in a *Proteus mirabilis* genomic island to create PGI-*PmPEL* (39) but in this case by recombination in ISCR1 (Fig. 1D), supporting the proposed mechanism of ISCR1-mediated capture of bla<sub>NDM</sub>. Regions containing the same ISCR1, 3'-CS, bla<sub>DHA</sub>Δ-*ampR* region, but adjacent to shorter fragments of the bla<sub>NDM</sub> region, are found in the original bla<sub>NDM-1</sub> plasmid pKpANDM-1 (FN396876.1) (1) and in plasmids of other Inc types (3) (e.g., the IncL/M plasmid pNDM-HK) (21), suggesting capture of shorter bla<sub>NDM</sub> regions and/or subsequent deletions.

**IncFII<sub>Y</sub> plasmids carry bla<sub>NDM</sub> flanked by TIMEs.** Several very closely related IncFII<sub>Y</sub> plasmids carrying a bla<sub>NDM</sub> gene have now been identified (Table 1). They all have almost identical backbones with the same insertions of multiple IS elements in the same places, mostly between the replication (*repA*) and plasmid stability (*parA*) regions (Fig. 2) and minor sequence differences (see Table S2 in the supplemental material). pKP351 alone appears to have a deletion adjacent to one copy of IS1 (41). In all of these plasmids, bla<sub>NDM</sub> lies within a 5,945-bp region matching Tn125 that includes 101 bp of IS*Aba125* and a fragment of ISCR27. This



**FIG 2** Contexts of *bla*<sub>NDM</sub> on IncFII<sub>Y</sub> plasmids. Features are generally shown as described for Fig. 1. Solid black lines represent the IncFII<sub>Y</sub> plasmid backbone. Gray-shaded areas indicate matching plasmid backbone regions, with their sizes given. (A) Tn125 in *Acinetobacter lwoffii* plasmid pNDM-BJ01. (B) Comparison of IncFII<sub>Y</sub> plasmids. (C) Comparison of *rmtC* contexts in IncFII<sub>Y</sub> plasmids, IncA/C<sub>2</sub> ARI-A, and *Proteus mirabilis*. The sequence shown is the spacer between *rmtC* and the associated transposable element. The pink triangle indicates the insertion site of the TIME. The sequences used to draw these diagrams are from the GenBank accession numbers listed in Table 1 plus the following: pNDM-BJ01, NC\_019268; pNDM-BJ02, NC\_019281.1; ISEcp1 transposon unit in *P. mirabilis*, AB194779.

region is flanked by two copies of a 256-bp Tn3-derived inverted-repeat transposable element (TIME), each bounded by 38-bp IRs (42). These TIMES, previously described as miniature inverted-repeat transposable elements (MITEs), may have been responsible for capturing the *bla*<sub>NDM</sub> region from a pNDM-BJ01-like plasmid (18, 19, 43). pEC4-NDM-6 is very closely related to these plasmids (see Table S2 in the supplemental material) but has the single nucleotide change giving *bla*<sub>NDM-6</sub> (44) rather than *bla*<sub>NDM-1</sub>, suggesting mutation in this context.

In most of these IncFII<sub>Y</sub> plasmids carrying *bla*<sub>NDM</sub>, an 11,029-bp region that includes the *rmtC* gene and an ISCR6-like element separates the TIME upstream of *bla*<sub>NDM-1</sub> from a third copy of this TIME. TIMES create a 5- to 6-bp DR on transposition like the Tn3 transposons from which they appear to be derived (42). In these plasmids, the 5-bp sequences adjacent to the “inside” of each TIME flanking the *rmtC* region are identical (TAT AA). This configuration could be explained by insertion of a circular molecule, consisting of this region plus one copy of the TIME (flanked by these 5-bp sequences as DR), into the TIME upstream of *bla*<sub>NDM-1</sub> (Fig. 2B). Gain and loss of the *rmtC* region in this way are supported by the sequences of the IncFII<sub>Y</sub> plasmids pP10164-NDM and pNDM-EC14653 (Table 1; Fig. 2B), which lack the *rmtC* region. Removing the TIME and one DR from this circular molecule also gives a region that matches the *rmtC* region found in ARI-A of IncA/C<sub>2</sub> plasmids, also supporting this hypothesis. *rmtC* was originally identified in a transposition unit flanked by a DR with a complete copy of ISEcp1 that also matches part of this structure (Fig. 2C) (45). The same 30 bp separate *rmtC* from

this complete ISEcp1 and the ISEcp1 fragment in IncA/C<sub>2</sub> plasmids, while an additional 10 bp are present between ISCR6 and *rmtC*. While these contexts are clearly related, without additional examples of *rmtC* contexts it is difficult to say exactly how each arose.

pECL3-NDM-1 carries the same *rmtC* region as the other IncFII<sub>Y</sub> plasmids, but its backbone has a number of confirmed nucleotide differences (see Table S2 in the supplemental material) and a different region carrying *bla*<sub>NDM-1</sub> has been inserted in a different location (Fig. 2B). This region matches pNDM-BJ02, which lacks the copy of IS*Aba125* downstream of *bla*<sub>NDM</sub> (3), rather than pNDM-BJ01, and also includes 1,369 bp of pNDM-BJ02 backbone. An IS903-like element truncates IS*Aba125*, leaving 83 bp upstream of *bla*<sub>NDM-1</sub>. This 10,411-bp region replaces a 15,560-bp region present in the other IncFII<sub>Y</sub> plasmids, and it is possible that the IS903-like element was involved in the insertion of this *bla*<sub>NDM</sub> region into pECL3-NDM-1.

**Conclusions.** In summary, the analysis presented in this study supplements and complements the catalogue of previously characterized IncA/C<sub>2</sub> and IncFII<sub>Y</sub> plasmids carrying *bla*<sub>NDM</sub>. All four plasmids studied here carry segments that align to different parts of the *bla*<sub>NDM</sub> regions found on *Acinetobacter* plasmids. Different mechanisms appear to have been responsible for independently transferring different segments of Tn125 into ARI-A in the same IncA/C<sub>2</sub> plasmid backbone (giving pKP1-NDM-1-type plasmids or pEC2-NDM-3). Other less closely related type 1 IncA/C<sub>2</sub> plasmids, e.g., pNDM-1\_Dok01 from *E. coli* (46) and pMR0211 from *Providencia stuartii* (47), also carry segments matching different

parts of Tn125 and the adjacent *Acinetobacter* plasmid backbone within ARI-A, illustrating further variation in the ways in which bla<sub>NDM</sub> genes appear to have been acquired by similar plasmids. Different mechanisms also appear to have transferred different segments matching bla<sub>NDM</sub> contexts found in *Acinetobacter baumannii* to slightly different IncFII<sub>Y</sub> backbones (giving pEC4-NDM-1-type plasmids or pECL3-NDM-1).

At least theoretically, the transfer of bla<sub>NDM</sub> segments between *Acinetobacter* and *Enterobacteriaceae* plasmids could have occurred either in *Acinetobacter* or in one or more of the *Enterobacteriaceae*. Transfer of *Acinetobacter* plasmids carrying bla<sub>NDM</sub> into *E. coli* J53Az<sup>r</sup> by conjugation has been demonstrated (12, 13), and recently a pNDM-BJ01-like plasmid (p3SP-NDM) was found in an *Enterobacter aerogenes* isolate (48). IncA/C plasmids have also been reported in a few *A. baumannii* clinical isolates on the basis of PCR assays (49). While independent transfer from *Acinetobacter* plasmids to different types of plasmids found in the *Enterobacteriaceae* is possible, it may be more likely that bla<sub>NDM</sub> regions have subsequently moved between these plasmids in the *Enterobacteriaceae*.

The four plasmids in this study were carried by clinical isolates from Australia or New Zealand, from different patients recently returning from India. We have also recently reported partial sequences of bla<sub>NDM</sub> contexts matching pKP1-NDM-1 (with the 89-bp ISAbal25 fragment) in IncA/C plasmids harbored by isolates from a hospital in Pakistan (17) and those matching pECL3-NDM-1 or pEC4-NDM-6 in IncFII<sub>Y</sub> plasmids in isolates from multiple Australian health care facilities (16). The other related IncA/C<sub>2</sub> and IncFII<sub>Y</sub> plasmids harboring bla<sub>NDM</sub> genes discussed here were also isolated in several different countries (Table 1). This distribution illustrates the geographical spread of bla<sub>NDM</sub> genes on these particular plasmid types.

There appears to be an underlying complex network of interactions between bla<sub>NDM</sub>, different mobile elements, and different plasmids, but without access to the sequences of additional intermediate and progenitor plasmids it is difficult to fully understand the contributions that different factors make to the transmission of bla<sub>NDM</sub> genes. The different mechanisms observed here to capture relevant genes onto different plasmid types emphasize the capability of *Enterobacteriaceae* to adapt to their environment, especially when antimicrobial pressure is present.

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