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Mechanisms Involved in Acquisition of bla_{NDM} Genes by IncA/C_2 and IncFII$_Y$ Plasmids

Alexander M. Wailan,[a] Hanna E. Sidjabat,[a] Wan Keat Yam,[a]* Nabil-Fareed Alikhan,[b]* Nicola K. Petty,[b,c]* Anna L. Sartor,[a]* Deborah A. Williamson,[d]* Brian M. Forde,[b,c] Mark A. Schembri,[b,c] Scott A. Beatson,[b,c] David L. Paterson,[b,e,f] Timothy R. Walsh,[a]* Sally R. Partridge[g]

The University of Queensland, UQ Centre for Clinical Research, Herston, Queensland, Australia[a]; Australian Infectious Diseases Research Centre, Brisbane, Australia[b]; School of Chemistry and Molecular Biosciences, The University of Queensland, Brisbane, Australia[c]; Institute of Environmental Science and Research, Wellington, New Zealand[d]; Infectious Diseases Unit, Royal Brisbane and Women's Hospital, Brisbane, Australia[e]; Pathology Queensland, Brisbane, Australia[f]; Centre for Infectious Diseases and Microbiology, The Westmead Institute for Medical Research, The University of Sydney, Westmead Hospital, New South Wales, Australia[g]

bla_{NDM} 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_{NDM} 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_{NDM} 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_2 plasmids with almost identical backbones. Different regions carrying bla_{NDM} are inserted in different locations in the antibiotic resistance island known as ARI-A, and IS*CR1* may have been involved in the acquisition of bla_{NDM-3} by pEC2-NDM-3. pECL3-NDM-1 and pEC4-NDM-6, from *Enterobacter cloacae* and *E. coli*, respectively, have similar IncFII$_Y$ backbones, but different regions carrying bla_{NDM} are found in different locations. Tn*3*-derived inverted-repeat transposable elements (TIME) appear to have been involved in the acquisition of bla_{NDM-6} by pEC4-NDM-6 and the *rmtC* 16S rRNA methylase gene by IncFII$_Y$ plasmids. Characterization of these plasmids further demonstrates that even very closely related plasmids may have acquired bla_{NDM} 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_{NDM} genes among species of the *Enterobacteriaceae* family.

In Gram-negative bacteria, especially the *Enterobacteriaceae* family, β-lactamases are the major mechanism of resistance against β-lactams. In particular, β-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_{NDM} 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_{NDM} 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 β-lactamase genes (such as bla_{CMY-2} and variants and $bla_{CTX-M-15}$) (6).

The original source of bla_{NDM} 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_{NDM} genes have often been observed within the 10,099-bp composite transposon Tn*125* that is bounded by two copies of IS*Aba125* (9–12). The bla_{NDM} gene starts 93 bp downstream of the right-hand end (IR_R) of IS*Aba125*, which provides the −35 region of a promoter (13, 14), and is followed by several genes, including ble_{MBL} (bleomycin resistance), *trpF* (involved in tryptophan biosynthesis), and the mobile element IS*CR27*. In several *Acinetobacter* species plasmids (e.g., pNDM-BJ01; GenBank accession no.

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Address correspondence to Alexander M. Wailan, alexander.wailan@uqconnect.edu.au, or Sally R. Partridge, sally.partridge@health.nsw.gov.au.

* Present address: Wan Keat Yam, Institute of Molecular and Cell Biology, A*STAR, Singapore; Nabil-Fareed Alikhan, Microbiology and Infection Unit, Warwick Medical School, University of Warwick, Coventry, United Kingdom; Nicola K. Petty, The ithree institute, University of Technology Sydney, Sydney, Australia; Anna L. Sartor, Health Protection Branch, Department of Health, Queensland Government, Brisbane, Queensland, Australia; Deborah A. Williamson, Microbiological Diagnostic Unit Public Health Laboratory, Doherty Institute, Melbourne, Australia; Timothy R. Walsh, Department of Medical Microbiology and Infectious Disease, Institute of Infection & Immunity, Heath Park Hospital, Cardiff, United Kingdom, and School of Medicine, Cardiff University, Health Park, Cardiff, United Kingdom.

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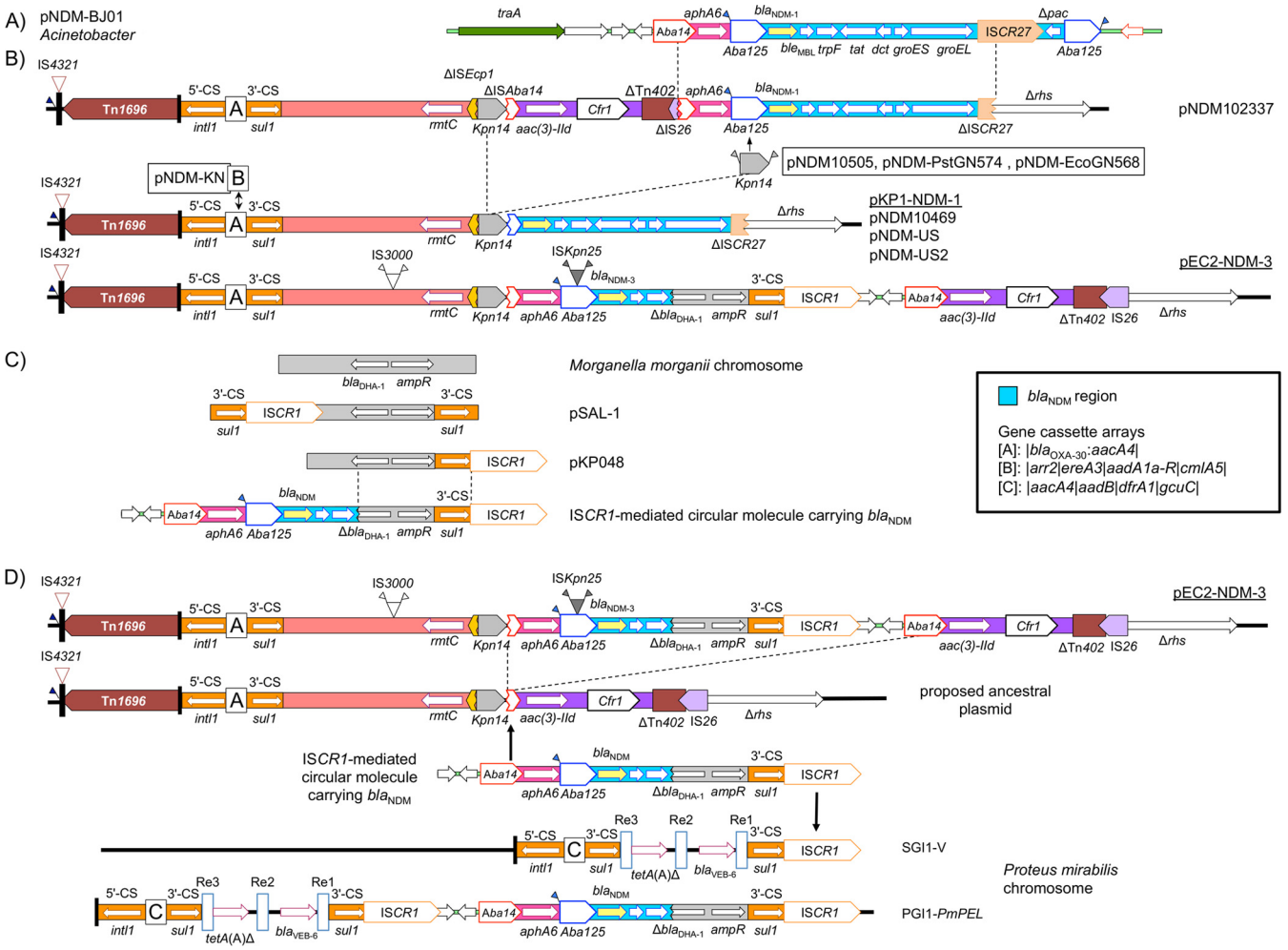

FIG 1 ARI-A of type 1 IncA/C_2 plasmids carrying bla_{NDM} and potential routes for bla_{NDM} insertion. IS are shown as block arrows labeled with their name or number. DR are represented by flags of the same color. Triangles indicate the insertion sites of IS elements flanked by DR. Vertical black bars represent the transposon IR of ARI-A and IRi of class 1 In/Tn. Horizontal green and black lines represent *Acinetobacter* and IncA/C_2 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) Tn*125* in *Acinetobacter lwoffii* plasmid pNDM-BJ01. (B) ARI-A of type 1 IncA/C_2 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_{NDM} 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-*Pm*PEL, KF856624.

NC_019268 [15]), IS*Aba14* and an *aphA6* gene (amikacin resistance) are present upstream of the IS*Aba125* adjacent to bla_{NDM-1} (Fig. 1A). In plasmids from the *Enterobacteriaceae*, bla_{NDM} 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_{NDM-1}, *E. coli* carrying bla_{NDM-3} (G283A, Asp95Asn) (23), and *E. coli* carrying bla_{NDM-6} (C698T, Ala233Val) (24). The bla_{NDM} 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_{NDM} with those in closely related plasmids.

MATERIALS AND METHODS

Bacterial isolates and plasmids. *K. pneumoniae* KP1 (22) and *E. cloacae* ECL3 carrying bla_{NDM-1} (23) were isolated in Australia, as was *E. coli* EC2 carrying bla_{NDM-3} (23), while *E. coli* EC4 carrying bla_{NDM-6} (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^r 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_2 and IncFII$_Y$ plasmids studied here and close relatives[a]

Plasmid	NDM	Size (bp)	Species	ST	Country[b]	Yr	Source	GenBank accession no.	Reference
A/C_2									
pKP1-NDM-1	1	137,552	*K. pneumoniae*	147	India/Australia	2010	Human	KF992018	This study
pEC2-NDM-3	3	160,989	*E. coli*	443	India/Australia	2010	Human	KC999035	This study
pNDM-EcoGN568	1	166,750	*E. coli*	1289	India/Canada	na	Human	KJ802404	50
pNDM-PstGN576	1	147,886	*P. stuartii*	N/A	India/Canada	na	Human	KJ802405	50
pNDM102337	1	165,974	*E. coli*	na	Canada	na	na	NC_019045	
pNDM10505	1	166,744	*E. coli*	na	Canada	na	na	NC_019069	
pNDM10469	1	137,813	*K. pneumoniae*	na	Canada	na	na	NC_019158	
pNDM-KN	1	162,746	*K. pneumoniae*	14	Kenya	2009	Human	JN157804	51, 52
pNDM-US	1	140,825	*K. pneumoniae*	11	India/USA	2010	Human	CP006661	53
pNDM-US-2	1	140,821	*K. pneumoniae*	na				KJ588779[c]	
FII$_Y$									
pECL3-NDM-1	1	99,435	*E. cloacae*	265	India/Australia	2011	Human	KC887917	This study
pEC4-NDM-6	6	110,786	*E. coli*	101	India/New Zealand	2010	Human	KC887916	This study
pKOX_NDM1	1	110,781	*Klebsiella oxytoca*	na	China/Taiwan	2010	Human	NC_021501	18
pNDM1_EC14653	1	109,353	*E. cloacae*	177	China	2014	Human	KP868647	43
pNDM-EclGN574	1	110,786	*E. cloacae*	na	India/Canada	na	Human	KJ812998	50
pP10164-NDM	1	99,276	*Leclercia adecarboxylata*	N/A	China	2012	Human	KP900016	19
pRJF866	1	110,786	*K. pneumoniae*	11	China	2011	Human	NC_025184	54
pK351	1	106,844	*K. pneumoniae*	147	Iran/USA	2014	Human	KR351290	41

[a] 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.
[b] Travel history is given if available, e.g., India/Australia indicates isolation in Australia from a patient recently returned from India.
[c] 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_{NDM} 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 (Biomatters 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_{NDM}. Isolates KP1, EC2, ECL3, and EC4 each transferred a plasmid carrying bla_{NDM} to *E. coli* J53Azi^r by conjugation. Plasmids carrying bla_{NDM} 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_2 (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_2 plasmids (see Table S1 in the supplemental material) and include characteristic IncA/C_2 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 IS*Ecp1* transposition unit carrying a bla_{CMY-2} variant, in this case bla_{CMY-6}, inserted in the same location as in many other type 1 IncA/C_2 plasmids, between *traA* and *traC*, flanked by 5-bp direct repeats (DR). Neither carries Tn*6170*, present in some type 1 IncA/C_2 plasmids (31).

Both pECL3-NDM-1 (99,435 bp) and pEC4-NDM-6 (110,786 bp) are IncFII$_Y$ 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$_Y$ plasmids carrying bla_{NDM} (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_2 plasmids carry bla_{NDM} in antibiotic resistance island ARI-A. In both IncA/C_2 plasmids sequenced here, the

bla_{NDM} 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_2 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 IS*4321* 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 IS*26*-*aphA1*-IS*26* interrupting the Tn*402 tni* region, and other resistance genes. Islands carrying bla_{NDM} 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_R end of IS*Ecp1*. IS*Ecp1* is truncated by IS*Kpn14*, 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 ISC*fr1* (34). The adjacent fragment of the Tn*402 tni* region has the same boundary with IS*26* as in ARI-A of pRMH760, but only 217 bp of IS*26* 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_{NDM-1}, and a fragment of ISC*R27*.

pNDM10505, pNDM-PstGN576, and pNDM-EcoGN568 (Table 1) have a variant of the pNDM102337 ARI-A with a second IS*Kpn14* inserted 130 bp upstream of the left end of IS*Aba125* (Fig. 1B). IS*Kpn14*-mediated deletion may have been responsible for creating the ARI-A variant present in the other closely related type 1 IncA/C_2 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 IS*Kpn14* upstream of bla_{NDM}. This difference was confirmed by reexamining raw reads and has been seen in other partial sequences (17, 35), and IS*Kpn14* is ~89% identical to IS*1*, which is known to cause adjacent deletions (34). All of these type 1 IncA/C_2 plasmids except pNDM-KN have the same cassette array, consisting of a single fused cassette comprised of the first 87 bp of the bla_{OXA-30} 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_{NDM} region into the proposed pNDM102337-like progenitor plasmid is unclear, but it is possible that it involved ISC*R27* and/or IS*26* 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 IS*Ec23* 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 IS*3000* is inserted upstream of *rmtC*, flanked by characteristic 5-bp DR. The region containing bla_{NDM}, however, is different from the one in the other IncA/C_2 plasmids and is inserted between IS*Kpn14* and the *aac(3)-IId*/ISC*fr1*/tni_{402} region. The region matching pNDM-BJ01 encompasses 198 bp of IS*Aba14*, *aphA6*, one copy of IS*Aba125*, bla_{NDM}, ble_{MBL}, and *trpF*. IS*Kpn25*, 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_{NDM} gene has the single nucleotide change giving bla_{NDM-3} rather than bla_{NDM-1}, and *trpF* is followed by a truncated bla_{DHA} gene and the associated *ampR* gene, nucleotides 180 to 1,313 of the 3′-CS and ISC*R1*. 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*-ISC*fr1*-tni_{402} region, but a complete copy of IS*26* truncates the *rhs* gene in the IncA/C_2 backbone. The only other known location of the bla_{NDM-3} variant is on an IncFII plasmid (37) associated with ISC*R1* but not with the truncated bla_{DHA}-*ampR* region present in pEC2-NDM-3.

This context in pEC2-NDM-3 suggests insertion of bla_{NDM} from a circular molecule mediated by ISC*R1*. ISC*R1* is proposed to transpose by a rolling-circle mechanism, similar to the related IS*91* family elements (38), in which replication proceeds from the *ori*IS end, located downstream of *rcr* (rolling-circle replicase gene), toward the *ter*IS upstream and can continue into and capture an adjacent region. ISC*R1* 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 ISC*R1* (38). ISC*R1* has previously been suggested to be associated with movement of bla_{NDM} (39) and was recently shown to be responsible for mobilizing a region containing bla_{NDM} and part of the 3′-CS, but without the bla_{DHA}Δ-*ampR* region, between plasmids (20).

ISC*R1* appears to have been responsible for capturing the bla_{DHA}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 ISC*R1* could create the arrangement seen in, e.g., pKP048 (GenBank accession no. NC_014312), with ISC*R1* downstream of the bla_{DHA}Δ-*ampR* region and the 3′-CS, and the usual 3′-CS/ISC*R1* boundary (Fig. 1C). ISC*R1* may then have mobilized this 3′-CS segment and the bla_{DHA}Δ-*ampR* region and inserted them downstream of bla_{NDM}, before picking up the bla_{NDM} 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_{NDM} also appears to have inserted in a *Proteus mirabilis* genomic island to create PGI-*Pm*PEL (39) but in this case by recombination in ISC*R1* (Fig. 1D), supporting the proposed mechanism of ISC*R1*-mediated capture of bla_{NDM}. Regions containing the same ISC*R1*, 3′-CS, bla_{DHA}Δ-*ampR* region, but adjacent to shorter fragments of the bla_{NDM} region, are found in the original bla_{NDM-1} 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_{NDM} regions and/or subsequent deletions.

IncFII$_Y$ plasmids carry bla_{NDM} flanked by TIMEs. Several very closely related IncFII$_Y$ plasmids carrying a bla_{NDM} 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 IS*1* (41). In all of these plasmids, bla_{NDM} lies within a 5,945-bp region matching Tn*125* that includes 101 bp of IS*Aba125* and a fragment of ISC*R27*. This

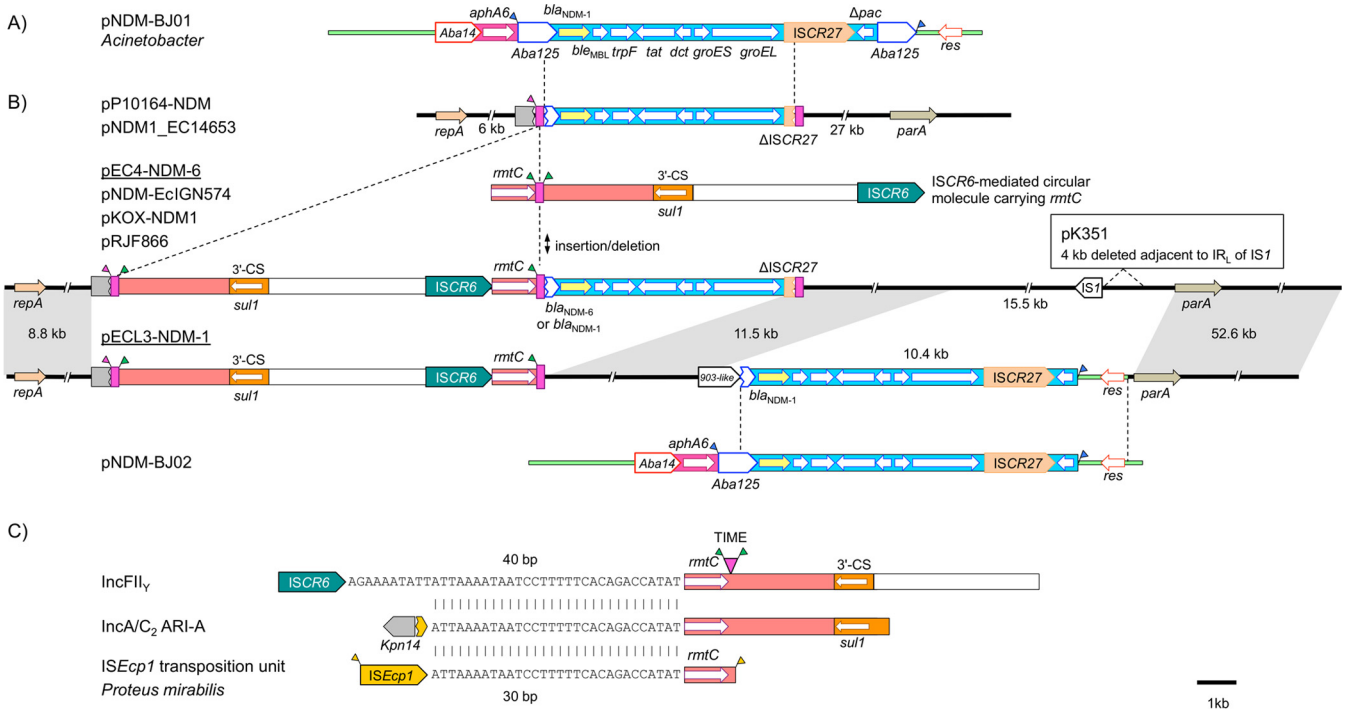

FIG 2 Contexts of bla_{NDM} on IncFII$_Y$ plasmids. Features are generally shown as described for Fig. 1. Solid black lines represent the IncFII$_Y$ plasmid backbone. Gray-shaded areas indicate matching plasmid backbone regions, with their sizes given. (A) Tn*125* in *Acinetobacter lwoffii* plasmid pNDM-BJ01. (B) Comparison of IncFII$_Y$ plasmids. (C) Comparison of *rmtC* contexts in IncFII$_Y$, plasmids, IncA/C$_2$ 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; IS*Ecp1* transposition unit in *P. mirabilis*, AB194779.

region is flanked by two copies of a 256-bp Tn*3*-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_{NDM} 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_{NDM-6} (44) rather than bla_{NDM-1}, suggesting mutation in this context.

In most of these IncFII$_Y$ plasmids carrying bla_{NDM}, an 11,029-bp region that includes the *rmtC* gene and an IS*CR6*-like element separates the TIME upstream of bla_{NDM-1} from a third copy of this TIME. TIMEs create a 5- to 6-bp DR on transposition like the Tn*3* 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_{NDM-1} (Fig. 2B). Gain and loss of the *rmtC* region in this way are supported by the sequences of the IncFII$_Y$ 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$_2$ plasmids, also supporting this hypothesis. *rmtC* was originally identified in a transposition unit flanked by a DR with a complete copy of IS*Ecp1* that also matches part of this structure (Fig. 2C) (45). The same 30 bp separate *rmtC* from this complete IS*Ecp1* and the IS*Ecp1* fragment in IncA/C$_2$ plasmids, while an additional 10 bp are present between IS*CR6* 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$_Y$ plasmids, but its backbone has a number of confirmed nucleotide differences (see Table S2 in the supplemental material) and a different region carrying bla_{NDM-1} has been inserted in a different location (Fig. 2B). This region matches pNDM-BJ02, which lacks the copy of IS*Aba125* downstream of bla_{NDM} (3), rather than pNDM-BJ01, and also includes 1,369 bp of pNDM-BJ02 backbone. An IS*903*-like element truncates IS*Aba125*, leaving 83 bp upstream of bla_{NDM-1}. This 10,411-bp region replaces a 15,560-bp region present in the other IncFII$_Y$ plasmids, and it is possible that the IS*903*-like element was involved in the insertion of this bla_{NDM} region into pECL3-NDM-1.

Conclusions. In summary, the analysis presented in this study supplements and complements the catalogue of previously characterized IncA/C$_2$ and IncFII$_Y$ plasmids carrying bla_{NDM}. All four plasmids studied here carry segments that align to different parts of the bla_{NDM} regions found on *Acinetobacter* plasmids. Different mechanisms appear to have been responsible for independently transferring different segments of Tn*125* into ARI-A in the same IncA/C$_2$ plasmid backbone (giving pKP1-NDM-1-type plasmids or pEC2-NDM-3). Other less closely related type 1 IncA/C$_2$ plasmids, e.g., pNDM-1_Dok01 from *E. coli* (46) and pMR0211 from *Providencia stuartii* (47), also carry segments matching different

parts of Tn*125* and the adjacent *Acinetobacter* plasmid backbone within ARI-A, illustrating further variation in the ways in which bla_{NDM} genes appear to have been acquired by similar plasmids. Different mechanisms also appear to have transferred different segments matching bla_{NDM} contexts found in *Acinetobacter baumannii* to slightly different IncFII$_Y$ backbones (giving pEC4-NDM-1-type plasmids or pECL3-NDM-1).

At least theoretically, the transfer of bla_{NDM} 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_{NDM} into *E. coli* J53Az^r 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_{NDM} 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_{NDM} contexts matching pKP1-NDM-1 (with the 89-bp IS*Aba125* 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$_Y$ plasmids in isolates from multiple Australian health care facilities (16). The other related IncA/C_2 and IncFII$_Y$ plasmids harboring bla_{NDM} genes discussed here were also isolated in several different countries (Table 1). This distribution illustrates the geographical spread of bla_{NDM} genes on these particular plasmid types.

There appears to be an underlying complex network of interactions between bla_{NDM}, 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_{NDM} 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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