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1 Mechanisms involved in acquisition of bla_{NDM} genes by IncA/C_2 and $IncFII_Y$ plasmids

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15 Running Head: bla_{NDM} acquistion by IncA/C_2 and $IncFII_Y$ plasmids

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28 **ABSTRACT**

29 *bla*$_{NDM}$ genes confer carbapenem resistance and have been identified on transferable plasmids
30 belonging to different incompatibility (Inc) groups. Here we present the complete sequences
31 of four plasmids carrying a *bla*$_{NDM}$ gene, pKP1-NDM-1, pEC2-NDM-3, pECL3-NDM-1 and
32 pEC4-NDM-6, from four clinical samples originating from four different patients. Different
33 plasmids carry segments that align to different parts of the *bla*$_{NDM}$ region found on
34 *Acinetobacter* plasmids. pKP1-NDM-1 and pEC2-NDM-3, from *Klebsiella pneumoniae* and
35 *Escherichia coli*, respectively, were identified as type 1 IncA/C_2 plasmids with almost
36 identical backbones. Different regions carrying *bla*$_{NDM}$ are inserted in different locations in
37 the antibiotic resistance island known as ARI-A and IS*CR1* may have been involved in
38 acquisition of *bla*$_{NDM-3}$ by pEC2-NDM-3. pECL3-NDM-1 and pEC4-NDM-6, from
39 *Enterobacter cloacae* and *E. coli*, respectively, have similar IncFII$_Y$ backbones but different
40 regions carrying *bla*$_{NDM}$ are found in different locations. Tn*3*-derived Inverted-repeat
41 Transposable Elements (TIME) appear to have been involved in acquisition of *bla*$_{NDM-6}$ by
42 pEC4-NDM-6 and the *rmtC* 16S rRNA methylase gene by IncFII$_Y$ plasmids. Characterisation
43 of these plasmids further demonstrates that even very closely related plasmids may have
44 acquired *bla*$_{NDM}$ genes by different mechanisms. These findings also illustrate the complex
45 relationships between antimicrobial resistance genes, transposable elements and plasmids and
46 provide insights into the possible routes for transmission of *bla*$_{NDM}$ genes amongst species of
47 the *Enterobacteriaceae* family.

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48 In Gram-negative bacteria, especially the *Enterobacteriaceae* family, β-lactamases are the
49 major mechanism of resistance against β-lactams. In particular, β-lactamases known as
50 carbapenemases are becoming a key concern in the effective administration of antimicrobial
51 therapy, as they can confer resistance to carbapenems, a major last-line antimicrobial. The
52 NDM carbapenemase was first reported in 2009, produced by a *Klebsiella pneumoniae*
53 isolated from a Swedish patient recently returned from India (1). There are currently 16
54 known NDM variants (http://www.lahey.org/Studies/other.asp#table1, accessed April 2016)
55 and *bla*$_{NDM}$ genes have now been reported in strains sourced from every inhabitable continent
56 and in multiple species of *Enterobacteriaceae*, including *Escherichia coli*, *K. pneumoniae*
57 and *Enterobacter cloacae* (2).

58 Plasmids are important vehicles for the capture, accumulation and spread of various
59 antimicrobial resistance determinants. Several different types of plasmids associated with the
60 *Enterobacteriaceae* family have been reported to harbor *bla*$_{NDM}$ genes, including IncA/C,
61 IncFII sub-types, IncH types, IncL/M, IncN (2-4), and IncX (5). Some of these plasmids co-
62 harbour additional antimicrobial resistance genes, including the 16S rRNA methylase genes
63 *armA* and *rmtC* (conferring high-level aminoglycoside resistance), quinolone resistance genes
64 (*qnrB1* and *qnrS1*) and/or other β-lactamase genes (such as *bla*$_{CMY-2}$ and variants, *bla*$_{CTX-M-15}$)
65 (6).

66 The original source of *bla*$_{NDM}$ is not known, but *Acinetobacter* spp. may have acted as an
67 intermediate between this organism and the *Enterobacteriacae* family (7-9). In *Acinetobacter*
68 spp. *bla*$_{NDM}$ genes have often been observed within the 10,099 bp composite transposon
69 Tn*125* that is bounded by two copies of IS*Aba125* (9-12). The *bla*$_{NDM}$ gene starts 93 bp
70 downstream of the right-hand end (IR$_R$) of IS*Aba125*, which provides the -35 region of a
71 promoter (13, 14), and is followed by several genes, including *ble*$_{MBL}$ (bleomycin resistance),
72 *trpF* (involved in tryptophan biosynthesis), and the mobile element IS*CR27*. In several

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73 *Acinetobacter* spp. plasmids (e.g. pNDM-BJ01; GenBank accession no. JQ001791 (15)),
74 IS*Aba14* and an *aphA6* gene (amikacin resistance) are present upstream of the IS*Aba125*
75 adjacent to *bla*$_{NDM-1}$ (Fig. 1A). In plasmids from the *Enterobacteriaceae*, *bla*$_{NDM}$ genes are
76 generally found in this immediate genetic context, with at least a fragment of IS*Aba125*
77 containing the -35 promoter region present upstream, within different length fragments
78 matching *Acinetobacter* plasmids and associated with different mobile elements (3, 16-21).

79 We previously reported locally-identified *K. pneumoniae* (22) and *E. cloacae* (23) clinical
80 isolates carrying *bla*$_{NDM-1}$, *E. coli* carrying *bla*$_{NDM-3}$ (G283A, Asp95Asn) (23) and *E. coli*
81 carrying *bla*$_{NDM-6}$ (C698T, Ala233Val) (24). The *bla*$_{NDM}$ gene could be transferred from all
82 four isolates by transformation and/or conjugation, indicating a plasmid location in each case,
83 but replicon types were not determined (22-24). In this study, we present the complete
84 sequences of these four plasmids and a comparison of the genetic contexts of *bla*$_{NDM}$ with
85 those in closely related plasmids.

86

87 **MATERIALS AND METHODS**

88 **Bacterial isolates and plasmids.** *K. pneumoniae* KP1 (22) and *E. cloacae* ECL3 carrying
89 *bla*$_{NDM-1}$ (23) were isolated in Australia, as was *E. coli* EC2 carrying *bla*$_{NDM-3}$ (23), while *E.*
90 *coli* EC4 carrying *bla*$_{NDM-6}$ (previously designated ARL10/167 (24)) was isolated in New
91 Zealand. All isolates were from patients recently returned from India. Transconjugants in
92 sodium-azide resistant *E. coli* J53Azi^{r} were available and/or were obtained by conjugation on
93 solid media, as previously described (17).

94 **DNA preparation and sequencing.** Genomic DNA (gDNA) was extracted from all four
95 isolates using the UltraClean Microbial DNA Isolation kit (Mo Bio Laboratories, Inc.,
96 Carlsbad, California, USA). DNA from KP1, ECL3 and EC4 was sequenced by Illumina
97 HiSeq 2000 technology (Illumina, San Diego, USA). Illumina sequences were *de novo*

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98 assembled using CLC genomic workbench v8.0 (CLC Bio, Aarhus, Denmark). Initial
99 annotation of contigs was performed using RAST (25). IS finder (https://www-is.biotoul.fr/)
100 and the Repository of Antibiotic-resistance Cassettes (RAC; http://rac.aihi.mq.edu.au/rac/)
101 were used to identify IS and integron components, respectively. BLAST
102 (http://blast.ncbi.nlm.nih.gov/Blast.cgi) searches were used to identify related plasmids
103 carrying *bla*$_{NDM}$ to guide PCR-based gap closure and Sanger sequencing (Macrogen, Korea)
104 to assemble contigs into complete plasmids.
105 gDNA from EC2 was sheared using a g-TUBE (Covaris®) into fragment sizes targeted at 20
106 Kb. Following purification, SMRTbell template libraries were prepared using the commercial
107 Template Preparation kit (Pacific Biosciences Inc., Menlo Park, California, USA) and
108 sequenced on a Pacific Biosciences (PacBio) RSII instrument (University of Queensland
109 Centre for Clinical Genomics; UQCCG) using the P6 polymerase and C4 sequencing
110 chemistry. The raw PacBio sequence data were assembled *de novo* using the hierarchical
111 genome assembly process (HGAP version 2) and Quiver (26) from the SMRT Analysis
112 software suite (version 2.3.0; http://www.pacb.com/devnet/) with default parameters and a
113 seed read cut-off of 17,000 bp. Following assembly, contigs were examined for overlapping
114 5' and 3' ends (a characteristic feature of the HGAP assembly process) using Contiguity
115 (https://peerj.com/preprints/1037/) and were manually trimmed to generate circular contigs.
116 Raw sequence reads were then mapped back onto the assembled circular plasmid contig
117 (BLASR (27) and Quiver) to validate the assembly and resolve any remaining errors.
118 RAST, IS finder, RAC, CLC genomic workbench v8.0, Geneious R9 (Biomatters Ltd, New
119 Zealand, including Mauve (28)) and BLAST were used for manual annotation, alignment,
120 SNP detection, and other analysis and comparisons of complete plasmid sequences.
121 **Nucleotide sequence accession numbers.** Existing GenBank entries for partial sequences of
122 all four plasmids were updated to include the complete sequences, as follows: pKP1-NDM-1,

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123 KF992018; pEC2-NDM-3, KC999035; pECL3-NDM-1, KC887917; pEC4-NDM-6,
124 KC887916.
125
126 **RESULTS AND DISCUSSION**
127 **General features of plasmids carrying *bla*$_{NDM}$.** Isolates KP1, EC2, ECL3, EC4 each
128 transferred a plasmid carrying *bla*$_{NDM}$ to *E. coli* J53Azi^r by conjugation. Plasmids carrying
129 *bla*$_{NDM}$ assembled from whole genome sequences (at least 50 fold coverage) were designated
130 pKP1-NDM-1, pEC2-NDM-3, pECL3-NDM-1 and pEC4-NDM-6, respectively. pKP1-
131 NDM-1 (137,552 bp) and pEC2-NDM-3 (160,989 bp) were identified as type 1 IncA/C_2
132 (Table 1). The backbones of pKP1-NDM-1 and pEC2-NDM-3 are very closely related to
133 those of several other type 1 IncA/C_2 plasmids (Table S1) and include characteristic IncA/C_2
134 core regions, such as the conjugative transfer (*tra*) region and *parA*-*parB* required for plasmid
135 partitioning (29). They have identical replication regions, with a *repA* gene and fourteen 19
136 bp direct repeat sequences (iterons), which are binding sites for the RepA protein (29). pKP1-
137 NDM-1 and pEC2-NDM-3 both have the same IS*Ecp1* transposition unit carrying a *bla*$_{CMY-2}$
138 variant, in this case *bla*$_{CMY-6}$, inserted in the same location as in many other type 1 IncA/C_2
139 plasmids, between *traA* and *traC*, flanked by 5 bp direct repeats (DR). Neither carries
140 Tn*6170* present in some type 1 IncA/C_2 plasmids (30).

141 pECL3-NDM-1 (99,435 bp) and pEC4-NDM-6 (110,786 bp) are both IncFII$_Y$ type plasmids
142 (Table 1) carrying two replicons, classified as Y4 (*repA*) and FIB36 (*repB*) by the replicon
143 sequence typing (RST) scheme (31). The backbones of both plasmids are closely related to
144 those of other IncFII$_Y$ plasmids carrying *bla*$_{NDM}$ (Table 1), which have not been well studied
145 but include a conjugation (*tra)* region and stability (*psi, parAB*) and maintenance (*ccdAB*)
146 genes (18, 19).

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147 **Both IncA/C_2 plasmids carry *bla*$_{NDM}$ in antibiotic resistance island ARI-A.** In both
148 IncA/C_2 plasmids sequenced here the *bla*$_{NDM}$ gene is located within an antibiotic resistance
149 island known as ARI-A, which is found in exactly the same location in different type 1
150 IncA/C_2 plasmids, between two *tra* regions (29, 30). The prototype ARI-A, found in
151 pRMH760, is a complex hybrid transposon structure bounded by 38 bp inverted repeats (IR)
152 interrupted by IS*4321* and is inserted upstream of the *rhs* gene (unknown function) flanked
153 by 5 bp DR (TTGTA) (30, 32). ARI-A in pRMH760 carries a class 1 In/Tn, with IS*26*-
154 *aphA1*-IS*26* interrupting the Tn*402 tni* region, and other resistance genes. Islands carrying
155 *bla*$_{NDM}$ appear to be derived from this structure, with deletions of part of the adjacent *rhs*
156 gene in some cases (3). In pNDM102337 (Table 1; Fig. 1B) nucleotides 1-1,616 of the 3′-CS
157 of the class 1 integron are followed by a 3,562 bp region carrying a type III restriction-
158 modification system and the *rmtC* 16S rRNA methylase gene, then 224 bp of the IR$_R$ end of
159 IS*Ecp1*. IS*Ecp1* is truncated by IS*Kpn14*, which is followed by a 198 bp fragment of
160 IS*Aba14*, then a region found on a number of different plasmids that contains the *aac(3)-IId*
161 (gentamicin resistance) gene and IS*Cfr1* (33). The adjacent fragment of the Tn*402 tni* region
162 has the same boundary with IS*26* as in ARI-A of pRMH760, but only 217 bp of IS*26* is
163 present. This is followed by an 8,913 bp region matching *Acinetobacter* plasmids such as
164 pNDM-BJ01, which includes 662 bp of the right end of IS*Aba14*, *aphA6*, one copy of
165 IS*Aba125*, *bla*$_{NDM-1}$ and a fragment of IS*CR27*.

166 pNDM10505, pNDM-PstGN576 and pNDM-EcoGN568 (Table 1) have a variant of the
167 pNDM102337 ARI-A with a second IS*Kpn14* inserted 130 bp upstream of the left end of
168 IS*Aba125* (Fig. 1B). IS*Kpn14*-mediated deletion may have been responsible for creating the
169 ARI-A variant present in the other closely-related type 1 IncA/C_2 plasmids pNDM-US,
170 pNDM-US-2, pNDM-KN and pNDM10469, which lack the *aac(3)-IId* region (Table 1; Fig.
171 1B) (3). pKP1-NDM-1 sequenced here has an almost identical ARI-A except that only 89 bp

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172 of IS*Aba125* are present adjacent to IS*Kpn14* upstream of bla_{NDM}. This difference was
173 confirmed by re-examining raw reads, has been seen in other partial sequences (17, 34) and
174 IS*Kpn14* is ~89% identical to IS*1*, which is known to cause adjacent deletions (33). All of
175 these type 1 IncA/C_2 plasmids except pNDM-KN have the same cassette array, consisting of
176 single fused cassette comprised of the first 87 bp of the bla_{OXA-30} cassette and position 17 to
177 the end of the *aacA4* cassette, overlapping by a single A (35). The mechanism(s) responsible
178 for insertion of the bla_{NDM} region into the proposed pNDM102337-like progenitor plasmid
179 are unclear, but it is possible that they involved IS*CR27* and/or IS*26* and subsequent
180 deletion(s).

181 The backbone of pEC2-NDM-3 is almost identical to the pNDM102337-like plasmids
182 described above (Table S1) but IS*Ec23* is inserted 222 bp upstream of ARI-A, flanked by 8
183 bp DR characteristic of this element. ARI-A of pEC2-NDM-3 includes the same *rmtC* region
184 as described above except that IS*3000* is inserted upstream of *rmtC*, flanked by characteristic
185 5 bp DR. The region containing bla_{NDM}, however, is different from the one in the other
186 IncA/C_2 plasmids and is inserted between IS*Kpn14* and the *aac(3)-IId*/IS*Cfr1*/tni_{402} region.
187 The region matching pNDM-BJ01 encompasses 198 bp of IS*Aba14*, *aphA6*, one copy of
188 IS*Aba125*, bla_{NDM}, ble_{MBL} and *trpF*. IS*Kpn25*, carrying a restriction-modification system, is
189 inserted in IS*Aba125* upstream of the -35 promoter region, flanked by characteristic 8 bp DR
190 (Fig. 1B). The bla_{NDM} gene has the single nucleotide change giving bla_{NDM-3} rather than
191 bla_{NDM-1} and *trpF* is followed by a truncated bla_{DHA} gene and the associated *ampR* gene,
192 nucleotides 180-1,313 of the 3'-CS and IS*CR1*. This region is separated from a complete
193 IS*Aba14* by 934 bp matching the region upstream of IS*Aba14* in pNDM-BJ01. ARI-A in
194 pEC2-NDM-3 ends with the *aac(3)-IId*/IS*Cfr1*/tni_{402} region but a complete copy of IS*26*
195 truncates the *rhs* gene in the IncA/C_2 backbone. The only other known location of the bla_{NDM-}

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196 $_3$ variant is on an IncFII plasmid (36) associated with IS*CR1* but not with the truncated
197 *bla*$_{DHA}$/*ampR* region present in pEC2-NDM-3.

198 This context in pEC2-NDM-3 suggests insertion of *bla*$_{NDM}$ from a circular molecule mediated
199 by IS*CR1*. IS*CR1* is proposed to transpose by a rolling-circle mechanism, similar to the
200 related IS*91* family elements (37), in which replication proceeds from the *ori*IS end, located
201 downstream of *rcr* (rolling circle replicase gene), towards the *ter*IS upstream and can
202 continue into and capture an adjacent region. IS*CR1* has generally been found associated with
203 class 1 integrons, after position 1,313 of the 3′-CS, suggesting integration of circular
204 molecules by recombination in either the 3′-CS or an existing IS*CR1* (37). IS*CR1* has
205 previously been suggested to be associated with movement of *bla*$_{NDM}$ (38) and was recently
206 shown to be responsible for mobilising a region containing *bla*$_{NDM}$ and part of the 3′-CS, but
207 without the *bla*$_{DHA}$Δ/*ampR* region, between plasmids (20).

208 IS*CR1* appears to have been responsible for capturing the *bla*$_{DHA}$Δ/*ampR* region from the
209 *Morganella morganii* chromosome and inserting it into a class 1 integron (39) (Fig. 1C).
210 Generation of a circular molecule by recombination between the two flanking 3′-CS and
211 reintegration at IS*CR1* could create the arrangement seen in e.g. pKP048 (GenBank accession
212 no. NC_014312), with IS*CR1* downstream of the *bla*$_{DHA}$Δ/*ampR* region and the 3'-CS, and
213 the usual 3'-CS/IS*CR1* boundary (Fig. 1C). IS*CR1* may then have mobilised this 3'-CS
214 segment and the *bla*$_{DHA}$Δ/*ampR* region and inserted them downstream of *bla*$_{NDM}$, before
215 picking up the *bla*$_{NDM}$ region as part of a circular molecule (Fig. 1C).

216 The complete IS*Aba14* in pEC2-NDM-3 has the same boundary with the *aac(3)-IId* region as
217 the IS*Aba14* fragment in pNDM102337, suggesting that homologous recombination between
218 the complete and partial copies of IS*Aba14* could have been responsible for the insertion of
219 this circular molecule into pEC2-NDM-3 (Fig. 1D). The same circular molecule carrying
220 *bla*$_{NDM}$ also appears to have inserted in a *P. mirabilis* genomic island to create PGI-*Pm*PEL

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221 (38) but in this case by recombination in IS*CR1* (Fig. 1D), supporting the proposed
222 mechanism of IS*CR1*-mediated capture of *bla*$_{NDM}$. Regions containing the same IS*CR1*, 3'-
223 CS, *bla*$_{DHA}$Δ/*ampR* region, but adjacent to shorter fragments of the *bla*$_{NDM}$ region, are found
224 in the original *bla*$_{NDM-1}$ plasmid pKpANDM-1 (FN396876.1) (1) and in plasmids of other Inc
225 types (3) (e.g. the IncL/M plasmid pNDM-HK) (21)), suggesting capture of shorter *bla*$_{NDM}$
226 regions and/or subsequent deletions.

227 **IncFII$_Y$ plasmids carry *bla*$_{NDM}$ flanked by TIMEs.** Several very closely related IncFII$_Y$
228 plasmids carrying a *bla*$_{NDM}$ gene have now been identified (Table 1). They all have almost
229 identical backbones with the same insertions of multiple IS elements in the same places,
230 mostly between the replication (*repA*) and plasmid stability (*parA*) regions (Fig. 2) and minor
231 sequence differences (Table S2). pKP351 (previously named pYDC644) alone appears to
232 have a deletion adjacent to one copy of IS*1* (40). In all of these plasmids *bla*$_{NDM}$ lies within a
233 5,945 bp region matching Tn*125* that includes 101 bp of IS*Aba125* and a fragment of
234 IS*CR27*. This region is flanked by two copies of a 256 bp Tn*3*-derived Inverted-repeat
235 Transposable Element (TIME), each bounded by 38 bp IRs (41). These TIMEs, previously
236 described as MITEs (Miniature Inverted-repeat Transposable Element), may have been
237 responsible for capturing the *bla*$_{NDM}$ region from a pNDM-BJ01-like plasmid (18, 19, 42).
238 pEC4-NDM-6 is very closely related to these plasmids (Table S2) but has the single
239 nucleotide change giving *bla*$_{NDM-6}$ (43) rather than *bla*$_{NDM-1}$, suggesting mutation in this
240 context.

241 In most of these IncFII$_Y$ plasmids carrying *bla*$_{NDM}$, an 11,029 bp region that includes the
242 *rmtC* gene and an IS*CR6*-like element separates the TIME upstream of *bla*$_{NDM-1}$ from a third
243 copy of this TIME. TIME create 5-6 bp DR on transposition like the Tn*3* transposons from
244 which they appear to be derived (41). In these plasmids the 5 bp sequences adjacent to the
245 "inside" of each TIME flanking the *rmtC* region are identical (TATAA). This configuration

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246 could be explained by insertion of a circular molecule, consisting of this region plus one copy
247 of the TIME (flanked by these 5 bp sequences as DR), into the TIME upstream of *bla*$_{NDM-1}$
248 (Fig. 2B). Gain and loss of the *rmtC* region in this way is supported by the sequences of the
249 IncFII$_Y$ plasmids pP10164-NDM and pNDM-EC14653 (Table 1; Fig. 2B), which lack the
250 *rmtC* region. Removing the TIME and one DR of this circular molecule also gives a region
251 that matches the *rmtC* region found in ARI-A of the IncA/C$_2$ plasmids, also supporting this
252 hypothesis. *rmtC* was originally identified in a transposition unit flanked by DR with a
253 complete copy of IS*Ecp1* that also matches part of this structure (Fig. 2C) (44). The same 30
254 bp separate *rmtC* from this complete IS*Ecp1* and the IS*Ecp1* fragment in IncA/C$_2$ plasmids,
255 while an additional 10 bp are present between IS*CR6* and *rmtC*. While these contexts are
256 clearly related, without additional examples of *rmtC* contexts it is difficult to say exactly how
257 each arose.

258 pECL3-NDM-1 carries the same *rmtC* region as the other IncFII$_Y$ plasmids but its backbone
259 has a number of confirmed nucleotide differences (Table S2) and a different region carrying
260 *bla*$_{NDM-1}$ has been inserted in a different location (Fig. 2B). This region matches pNDM-
261 BJ02, which lacks the copy of IS*Aba125* downstream of *bla*$_{NDM}$ (3), rather than pNDM-BJ01,
262 and also includes 1,369 bp of pNDM-BJ02 backbone. An IS*903*-like element truncates
263 IS*Aba125*, leaving 83 bp upstream of *bla*$_{NDM-1.}$ This 10,411 bp region replaces a 15,560 bp
264 region present in the other IncFII$_Y$ plasmids and it is possible that the IS*903*-like element was
265 involved in the insertion of this *bla*$_{NDM}$ region into pECL3-NDM-1.

266 **Conclusions.** In summary, the analysis presented in this study supplements and complements
267 the catalogue of previously characterised IncA/C$_2$ and IncFII$_Y$ plasmids carrying *bla*$_{NDM}$. All
268 four plasmids studied here carry segments that align to different parts of the *bla*$_{NDM}$ regions
269 found on *Acinetobacter* plasmids. Different mechanisms appear to have been responsible for
270 independently transferring different segments of Tn*125* into ARI-A in the same IncA/C$_2$

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271 plasmid backbone (giving pKP1-NDM-1-type plasmids or pEC2-NDM-3). Other less
272 closely-related type 1 IncA/C_2 plasmids e.g. pNDM-1_Dok01 from *E. coli* (45) and
273 pMR0211 from *Providencia stuartii* (46), also carry segments matching different parts of
274 Tn*125* and adjacent *Acinetobacter* plasmid backbone in ARI-A, illustrating further variation
275 in the ways in which *bla*$_{NDM}$ genes appear to have been acquired by similar plasmids.
276 Different mechanisms also appear to have transferred different segments matching *bla*$_{NDM}$
277 contexts found in *A. baumannii* to slightly different IncFII$_Y$ backbones (giving pEC4-NDM-
278 1-type plasmids or pECL3-NDM-1).

279 At least theoretically, transfer of *bla*$_{NDM}$ segments between *Acinetobacter* and
280 *Enterobacteriaceae* plasmids could have occurred in either *Acinetobacter* or in one or more
281 of the *Enterobacteriaceae*. Transfer of *Acinetobacter* plasmids carrying *bla*$_{NDM}$ into *E. coli*
282 J53 by conjugation has been demonstrated (12, 13) and recently a pNDM-BJ01-like plasmid
283 (p3SP-NDM) was found in an *Enterobacter aerogenes* isolate (47). IncA/C plasmids have
284 also been reported in a few *A. baumannii* clinical isolates on the basis of PCR (48). While
285 independent transfer from *Acinetobacter* plasmids to different types of plasmids found in the
286 *Enterobacteriaceae* is possible, it may be more likely that *bla*$_{NDM}$ regions have subsequently
287 moved between these plasmids in the *Enterobacteriaceae.*

288 The four plasmids in this study were carried by clinical isolates from Australia or New
289 Zealand, from different patients recently returning from India. We have also recently reported
290 partial sequences of *bla*$_{NDM}$ contexts matching pKP1-NDM-1 (with the 89 bp IS*Aba125*
291 fragment) in IncA/C plasmids harboured by isolates from a hospital in Pakistan (17) and
292 those matching pECL3-NDM-1 or pEC4-NDM-6 in IncFII$_Y$ plasmids in isolates from
293 multiple Australian healthcare facilities (16). The other related IncA/C_2 and IncFII$_Y$ plasmids
294 harbouring *bla*$_{NDM}$ genes discussed here were also isolated in several different countries

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295 (Table 1). This distribution illustrates the geographical spread of *bla*$_{NDM}$ genes on these
296 particular plasmid types.
297 There appears to be an underlying complex network of interactions between *bla*$_{NDM}$, different
298 mobile elements and different plasmids, but without access to the sequences of additional
299 intermediate and progenitor plasmids it is difficult to fully understand the contributions that
300 different factors have to the transmission of *bla*$_{NDM}$ genes. The different mechanisms
301 observed here to capture relevant genes onto different plasmid types emphasize the capability
302 of *Enterobacteriaceae* to adapt to their environment, especially where antimicrobial pressure
303 is present.
304

305 **ACKNOWLEDGEMENTS**
306 We thank George Jacoby for providing the *E. coli* J53Az^{r} strain. Sequencing was funded by
307 the Australian Infectious Diseases Research Centre. Part of this work was funded by Project
308 grant G1033799 from the National Health and Medical Research Council of Australia
309 (NHMRC). MAS is supported by a NHMRC Senior Research Fellowship (G1106930). SAB
310 is supported by a NHMRC Career Development Fellowship (G1090456).

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500 **Figure legends**

501 **FIG 1** ARI-A of type 1 IncA/C_2 plasmids carrying *bla*$_{NDM}$, and potential routes for *bla*$_{NDM}$
502 insertion. IS are shown as block arrows labelled with their name or number. DR are
503 represented by flags of the same colour. Triangles indicate the insertion sites of IS elements
504 flanked by DR. Vertical black bars represent the transposon IR of ARI-A and IRi of class 1
505 In/Tn. Horizontal green and black lines represent *Acinetobacter* and IncA/C_2 plasmid
506 backbones, respectively. Vertical dotted lines indicate boundaries of closely related
507 sequences. Vertical black arrows and dotted diagonal lines indicate possible deletion and
508 insertion events. (A) Tn*125* in *Acinetobacter lwoffii* plasmid pNDM-BJ01. (B) ARI-A of type
509 1 IncA/C_2 plasmids closely related to pKP1-NDM-1 and pEC2-NDM-3. (C) Possible
510 derivation of the circular molecule inserted in pEC2-NDM-3. (D) Insertion of circular
511 molecular carrying *bla*$_{NDM}$ into pEC2-NDM-3 and a *P. mirabilis* genomic island. The
512 sequences used to draw these diagrams are from GenBank accession numbers listed in Table
513 1, plus: pNDM-BJ01, NC_019268; pSAL-1, AJ237702; pKP048, NC_014312; SGI1-V,
514 HQ888851; PGI1-*Pm*PEL, KF856624.

515

516 **FIG 2** Contexts of *bla*$_{NDM}$ on IncFII$_Y$ plasmids. Features are generally shown as in Fig. 1.
517 Solid black lines represent IncFII$_Y$ plasmid backbone. Grey shaded areas indicate matching
518 plasmid backbone regions, with their sizes given. (A) Tn*125* in *Acinetobacter lwoffii* plasmid
519 pNDM-BJ01. (B) Comparison of IncFII$_Y$ plasmids. (C) Comparison of *rmtC* contexts in
520 IncFII$_Y$, plasmids, IncA/C_2 ARI-A and *Proteus mirabilis*. The sequence shown is the spacer
521 between *rmtC* and the associated transposable element. The pink triangle indicates the
522 insertion site of the TIME. The sequences used to draw these diagrams are from GenBank
523 accession numbers listed in Table 1 plus: pNDM-BJ01, NC_019268; pNDM-BJ02,
524 NC_019281.1; IS*Ecp1* transposition unit in *P. mirabilis*, AB194779.

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525 **TABLE 1** General features of IncA/C_2 and IncFII_Y plasmids studied here and close relatives.

Plasmid[a]	NDM	Size (bp)	Species	ST[b]	Country[c]	Year	Source	GenBank accession no.	Reference
A/C_2									
pKP1-NDM-1	1	137,552	*K. pneumoniae*	147	India/Australia	2010	Human	KF992018.2	This study
pEC2-NDM-3	3	160,989	*E. coli*	443	India/Australia	2010	Human	KC999035.2	This study
pNDM-EcoGN568	1	166,750	*E. coli*	1289	India/Canada	na	Human	KJ802404.1	(49)
pNDM-PstGN576	1	147,886	*P. stuartii*	N/A	India/Canada	na	Human	KJ802405.1	(49)
pNDM102337	1	165,974	*E. coli*	na	Canada	na	na	NC_019045.2	-
pNDM10505	1	166,744	*E. coli*	na	Canada	na	na	NC_019069.1	-
pNDM10469	1	137,813	*K. pneumoniae*	na	Canada	na	na	NC_019158.1	-
pNDM-KN	1	162,746	*K. pneumoniae*	14	Kenya	2009	Human	JN157804.1	(50, 51)
pNDM-US	1	140,825	*K. pneumoniae*	11	India/USA	2010	Human	CP006661.1	(52)
pNDM-US-2	1	140,821	*K. pneumoniae*	na	-[d]	-	-	KJ588779.1	-
FII_Y									
pECL3-NDM-1	1	99,435	*E. cloacae*	265	India/Australia	2011	Human	KC887917.2	This study
pEC4-NDM-6	6	110,786	*E. coli*	101	India/New Zealand	2010	Human	KC887916.2	This study
pKOX_NDM1	1	110,781	*K. oxytoca*	na	China/Taiwan	2010	Human	NC_021501.1	(18)
pNDM1_EC14653	1	109,353	*E. cloacae*	177	China	2014	Human	KP868647.1	(42)
pNDM-EclGN574	1	110,786	*E. cloacae*	na	India/Canada	na	Human	KJ812998.1	(49)
pP10164-NDM	1	99,276	*L. adecarboxylata*	N/A	China	2012	Human	KP900016.1	(19)
pRJF866	1	110,786	*K. pneumoniae*	11	China	2011	Human	NC_025184.1	(53)
pK351[e]	1	106,844	*K. pneumoniae*	147	Iran/USA	2014	Human	KR351290.1	(40)

526 [a] Plasmids with names in bold typeface were sequenced in this study.
527 [b] na, not available; N/A, not applicable (no multi-locus typing schemes for these species).
528 [c] Travel history is given if available e.g. India/Australia indicates isolation in Australia from a patient recently returned from India.
529 [d] GenBank accession no. KJ588779 implies that pNDM-US-2 was extracted in China from the same strain, (ATCC BAA-2146) as pNDM-US.
530 [e] pK351 was previously named pYDC644.
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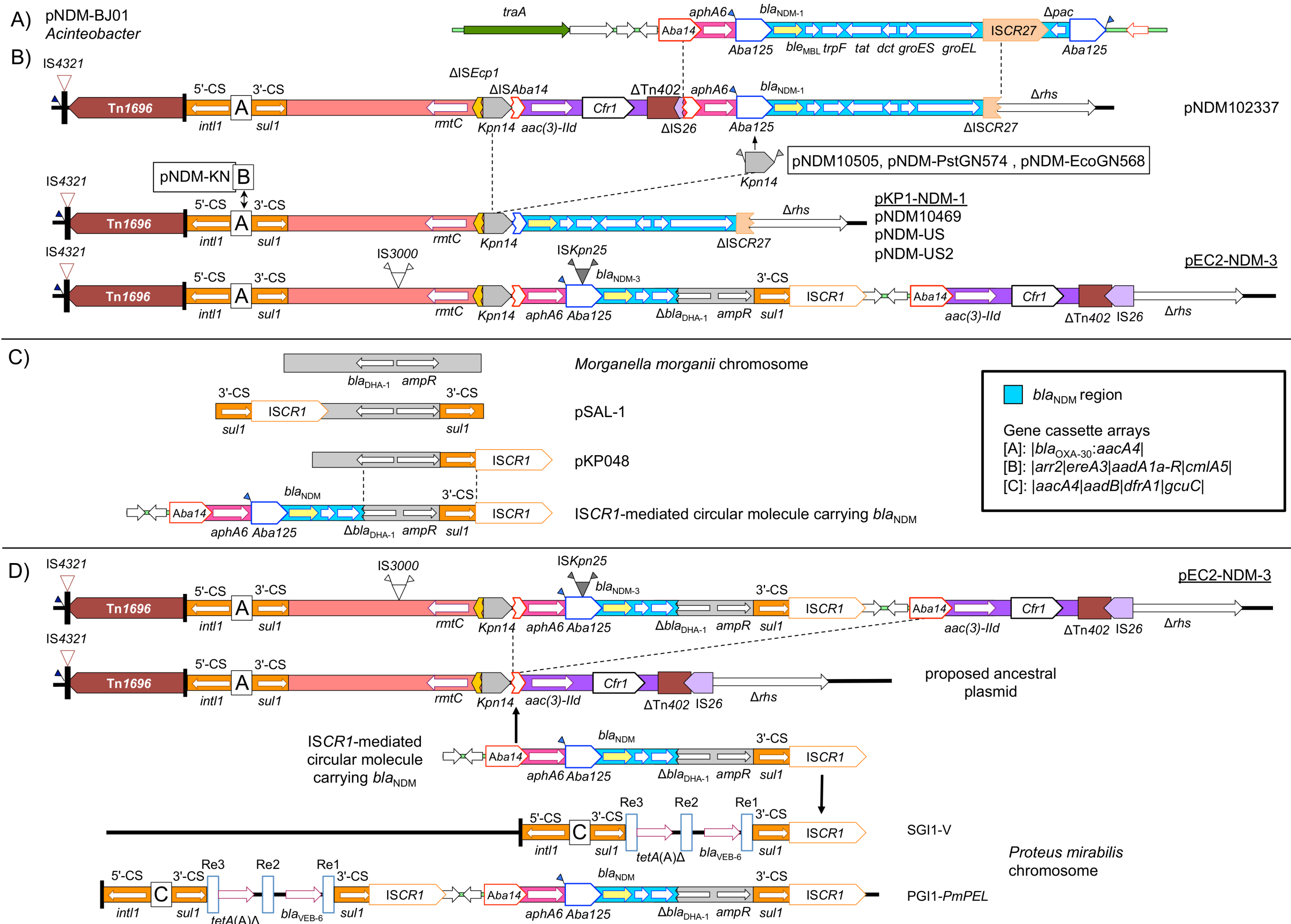

Accepted Manuscript Posted Online
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