Evolution of IncA/C *bla*_{CMY-2}-Carrying Plasmids by Acquisition of the *bla*_{NDM-1} Carbapenemase Gene

Alessandra Carattoli,^a Laura Villa,^a Laurent Poirel,^b Rémy A. Bonnin,^b and Patrice Nordmann^b

Department of Infectious, Parasitic, and Immuno-Mediated Diseases, Istituto Superiore di Sanità, Rome, Italy^a; and Service de Bactériologie-Virologie, INSERM U914 Emerging Resistance to Antibiotics, Hôpital de Bicêtre, Faculté de Médecine et Université Paris-Sud, K-Bicêtre, France^b

The *bla*_{NDM-1} gene has been reported to be often located on broad-host-range plasmids of the IncA/C type in clinical but also environmental bacteria recovered from the New Delhi, India, area. IncA/C-type plasmids are the main vehicles for the spread of the cephalosporinase gene *bla*_{CMY-2}, frequently identified in the United States, Canada, and Europe. In this study, we completed the sequence of IncA/C plasmid pNDM-KN carrying the *bla*_{NDM-1} gene, recovered from a *Klebsiella pneumoniae* isolate from Kenya. This sequence was compared with those of three IncA/C-type reference plasmids from *Escherichia coli*, *Yersinia ruckeri*, and *Photobacterium damsela*. Comparative analysis showed that the *bla*_{NDM-1} gene was located on a widely diffused plasmid scaffold known to be responsible for the spread of *bla*_{CMY-2}-like genes and consequently for resistance to broad-spectrum cephalosporins. Considering that IncA/C plasmids possess a broad host range, this scaffold might support a large-scale diffusion of the *bla*_{NDM-1} gene among Gram-negative rods.

The *bla*_{NDM-1} gene was initially identified in *Klebsiella pneumoniae* and *Escherichia coli* isolates but has since been reported for many different enterobacterial species and also *Acinetobacter baumannii* (7, 14–16, 19, 29, 32). Noteworthy, the *bla*_{NDM-1} gene was most often reported to be carried on plasmids in enterobacterial species, whereas it was chromosomally carried in *A. baumannii* (14). In particular, it has been often reported to be carried on IncA/C-type plasmids, as found for *bla*_{NDM-1}-positive *K. pneumoniae* isolates from Switzerland (25) and Kenya (24), *Proteus mirabilis* isolates from Switzerland (24), and *E. coli* isolates from France (23). A recent study focusing on environmental water samples recovered from the New Delhi, India, area reported IncA/C-type plasmids carrying the *bla*_{NDM-1} gene in *E. coli*, *Citrobacter freundii*, and also *Vibrio cholerae* (32), thus emphasizing the potential of this type of plasmid to act as a vehicle for the spread of the *bla*_{NDM-1} gene.

IncA/C-type plasmids have been extensively investigated in the last 10 years, since they have been shown to be the main vehicles for the spread of *bla*_{CMY-2} cephalosporinase genes frequently identified in *E. coli* and *Salmonella* sp. isolates in the United States, Canada, and Europe (1, 2, 4, 9–12, 17, 18). IncA/C-type plasmids have been identified from isolates of human but also of animal origins, for example, from beef, chicken, turkey, and pork samples (34). Those plasmids possess a broad host range of replication, being identified in all enterobacterial species but also in *Photobacterium damsela* and *Aeromonas salmonicida* (33).

In this study, we completed the sequence of IncA/C plasmid pNDM-KN (formerly referred to as pKp7 [24]) carrying the *bla*_{NDM-1} gene, recovered from a *K. pneumoniae* isolate. That clinical isolate was recovered in November 2009 at a hospital in Nairobi, Kenya. In addition to the *bla*_{NDM-1} gene, this isolate carried supplementary resistance genes, including the extended-spectrum- β -lactamase (ESBL) genes *bla*_{SHV-28} and *bla*_{CTX-M-15} together with the AmpC gene *bla*_{CMY-6} and the class D narrow-spectrum β -lactamase genes *bla*_{OXA-1} and *bla*_{OXA-9}. It also carried the *rmtC* 16S RNA methylase gene conferring high-level resistance to all aminoglycosides.

A comparative analysis of the sequence of plasmid pNDM-KN with the sequences of other plasmids belonging to the IncA/C

family demonstrated the evolutionary relationship between this *bla*_{NDM-1}-bearing plasmid and the IncA/C plasmids carrying *bla*_{CMY-2}, suggesting a recent acquisition of the *bla*_{NDM-1} gene by this successful plasmid type.

MATERIALS AND METHODS

Whole-plasmid sequencing. Plasmid pNDM-KN was characterized previously (24). To obtain the DNA sequence, plasmid DNA was purified by using the Qiaquick kit (Qiagen, Courtaboeuf, France) according to the manufacturer's protocol. The complete sequencing work flow of the Illumina Genome Analyzer IIx system was performed by the DNA Vision Company (Gosselies, Belgium). A single contiguous sequence with at least 15-fold coverage was obtained for plasmid pNDM-KN using draft assembly and PCR-based gap closure.

Bioinformatics. Open reading frames were predicted and annotated by using DNAMAN 5.2.10 software (Lynnon BioSoft; Lynnon Corporation). Each predicted protein was compared against an all-protein database by using BlastP (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), with a minimum cutoff of 30% identity and over 80% length coverage. Gene sequences were further compared and aligned with GenBank data by using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and CLUSTAL W (<http://www.ebi.ac.uk/clustalw>). IncA/C plasmids pAR060302 from *E. coli* (GenBank accession no. FJ621588), pYR1 from *Y. ruckeri* (GenBank accession no. CP000602), and pP91278 from *P. damsela* (GenBank accession no. AB277724) were used as references for annotating pNDM-KN.

Nucleotide sequence accession number. The GenBank accession number for plasmid pNDM-KN is JN157804.

RESULTS AND DISCUSSION

Comparison of plasmid pNDM-KN and other IncA/C plasmids. A BLAST analysis of the completed nucleotide sequence of plas-

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Address correspondence to Patrice Nordmann, nordmann.patrice@bct.aphp.fr.

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IncA/C pNDM-KN, *Klebsiella pneumoniae*, Kenya, 162746 bp, JN157804

FIG 1 Major structural features of pNDM-KN in comparison with IncA/C plasmids pAR060302 (GenBank accession no. [FJ621588](#)), pYR1 (GenBank accession no. [CP000602](#)), and pP91278 (GenBank accession no. [AB277724](#)). White boxes indicated plasmid scaffold regions that are in common among plasmids. The *tra* locus is indicated within the boxes. Resistance genes are indicated by orange boxes, except for the *bla*_{NDM-1} gene, which is indicated by a blue box. Transposon-related genes (*tnpA*, *tnpR*, and *tnpM*) and insertion sequences are indicated by red boxes. Other genes are indicated by boxes colored as follows: violet, the replicase gene *repA*; gray, restriction enzyme and DNA methylase genes; green, the *groEL*-*groES* cluster; pale blue, the *sugE* and *bcl* genes; brown, the *bla*_{CMY} genes. *hyp*, hypothetical.

mid pNDM-KN performed in comparison with the reference IncA/C-type plasmids pAR060302 from *E. coli* (4), pYR1 from *Y. ruckeri*, and pP91278 from *P. damsela* showed that pNDM-KN is an IncA/C plasmid with a backbone similar to those of other IncA/C plasmids (>99% nucleotide sequence identity), with the exception of four major regions of discontinuity (Fig. 1).

(i) The phage integrase-*rhs* regions as hot spot for integration. The sequence of plasmid pNDM-KN shows the acquisition of a novel type I site-specific restriction-modification system of the HsdR type that includes genes encoding a DNase, a restriction enzyme, and a related ATP-dependent DNA helicase (Fig. 1). The best BLAST hit for this novel type I restriction system was with that identified in the genome of *Xanthomonas campestris* pv. *vesicatoria* (>84% nucleotide sequence identity; GenBank accession no. [AM039952](#)). This HsdR operon was inserted into the gene encoding a site-specific recombinase of phage origin, being a common feature of all IncA/C plasmid scaffolds (Fig. 1), and was flanked by a Tn7-like transposon. The Tn7-like transposition occurred within the *rhs* gene that is also a common feature of IncA/C plasmids. The region encompassing the phage-integrase and the *rhs* genes is likely a hot spot for the integration of accessory genes within IncA/C plasmids.

(ii) Evolution of the *bla*_{NDM-1} locus. The *mer* operon of pAR060302 is part of a complex hybrid transposon inserted into the IncA/C backbone flanked by direct repeats, and related structures are found in other IncA/C plasmids. The *strA*-*strB* genes in pYR1 are part of a Tn5393 that was recently described (3, 4). The synteny among the compared plasmids restarted at the DNA primase gene (Fig. 1). IncA/C plasmid pP91278 from *P. damsela* likely represents the IncA/C scaffold before the acquisition of the accessory genes, since it does not show any insertion between the phage-integrase, *rhs*, and DNA primase genes (Fig. 1).

An analysis of the pNDM-KN sequence revealed that the *rhs* gene was deleted at its 3' and 5' extremities and was adjacent to the heat shock chaperone *groEL*-*groES* cluster (28). In pAR060302, this cluster flanked a class 1 integron that contained the *aac*(3)-*Iva* and *aadA1* gene cassettes, but it was absent in plasmids pYR1 and pP91278 (Fig. 1). Analysis of the sequences located at the right extremity of the *bla*_{NDM-1} locus on plasmid pNDM-KN revealed the presence of a class 1 integron structure bearing the *cmlA7* (chloramphenicol resistance), *aadA1* (streptomycin resistance), *ereC* (erythromycin resistance), and *arr2* (rifampin resistance) gene cassettes. This integron was previously described for an NDM-1-producing *K. pneumoniae* strain from a Swedish patient

of Indian origin (35). The similarity between these two plasmids continues through the *intI1* integrase gene and the Tn1696 resolvase and transposase genes. However, the integron identified in plasmid pAR060302 was associated with an ISCR16 element, which was not detected in plasmid pNDM-KN (30).

A detailed analysis of the immediate genetic environment surrounding the *bla*_{NDM-1} gene (considering those present on pNDM-KN and absent on pAR060302) revealed novel features. As previously described for other *bla*_{NDM-1}-carrying plasmids, upstream of the *bla*_{NDM-1} gene, part of IS*Aba125* was identified (our unpublished data). This insertion sequence, which provides the –35 promoter region for *bla*_{NDM-1} expression, was truncated by the insertion of IS*Kpn14* (previously identified in *K. pneumoniae*). However, no remnant of IS*Aba125* was identified on the other extremity of IS*Kpn14*. Instead, a truncated fragment of IS*Ecp1* was identified, followed by the 16S rRNA methylase *rmtC* gene. This association between IS*Ecp1* and *rmtC* was previously described (31). Genes encoding a type III endonuclease and a methyltransferase were then identified, with both sharing sequence similarities with genes from the *X. campestris* genome, which, however, are not colinear, as they appear on plasmid pNDM-KN (>84% nucleotide sequence identity; GenBank accession no. AM039952). Downstream of the *bla*_{NDM-1} gene, the *bleMBL* (bleomycin resistance associated with metallo- β -lactamase [MBL]) gene, encoding a putative protein conferring resistance to bleomycin, was identified, as found previously for other *bla*_{NDM-1}-bearing plasmids (21, 23).

According to this *in silico* analysis, it was not possible to determine the genetic events that led to the formation of this heterogeneous genetic structure. However, it is likely that independent and multiple genetic events contributed to the acquisition of the *bla*_{NDM-1}⁺ and *rmtC*-containing locus.

(iii) The IS*Ecp1*-*bla*_{CMY} module region. In IncA/C plasmids, one or more copies of the IS*Ecp1*-*bla*_{CMY} transposition unit gene are inserted within the Tra region in a module that also contains the *btc* and *sugE* genes (4, 22, 27). The IS*Ecp1*-*bla*_{CMY-6} (CMY-6 differs just in the W661→L amino acid substitution from CMY-2) module was found to be inserted in the same position into the Tra region of plasmid pNDM-KN but with an IS*Ec23* inserted into the *sugE* gene. The insertion of the IS*Ecp1*-*bla*_{CMY} module within the Tra locus was demonstrated previously to significantly reduce the conjugation frequency of IncA/C plasmids compared to those that do not carry this module (27). The presence of this very well conserved region showed plasmid pNDM-KN was an IncA/C *bla*_{CMY}-carrying plasmid that has acquired the *bla*_{NDM-1} gene within its scaffold as a secondary event.

(iv) The *parA*-*parB* gene region. The remaining part of plasmid pNDM-KN was similar to the corresponding region of plasmid pYR1 from *Y. ruckeri*, including the *korB*, *parB*, and *parA* and genes, encoding the putative restriction methyltransferase and DNA methylases. In this region, plasmids pAR060302 and pP91278 carried other resistance genes integrated between the *parA* and *parB* genes (*sul2*, *strA*, *strB*, *dhfr*, and *tetA*-*tetR* of the A and D types, respectively) (Fig. 1).

Conclusion. Epidemiological studies focusing on the *bla*_{CMY}-carrying IncA/C plasmids showed that these plasmids possess a high capacity for diffusion and spread, as they have been detected worldwide. They have been reported extensively for *Salmonella* strains of animal origins (6, 34). According to the most recent studies that have been conducted, these plasmids likely play an

important role in the rapid and efficient dissemination of the *bla*_{NDM-1} gene in several bacterial species from both human cases and the environment. In particular, these plasmids, which are known to be of broad host range, have been detected in numerous multidrug-resistant Gram-negative species isolated from India and the United Kingdom and from water samples from New Delhi (32).

Interestingly, a study performed on commensal and pathogenic *E. coli* isolates that did not consider antimicrobial resistance as a criterion of selection revealed that the RepA/C replicon occurred in only 1% of *E. coli* strains obtained from healthy humans not exposed to antimicrobials from United States and was absent from the fecal flora of healthy animals (13). Therefore, the rate of occurrence of IncA/C plasmids in nonresistant bacterial populations is not high, and these plasmids are probably advantaged in populations that are under antimicrobial selective pressure (34).

The *bla*_{NDM-1} gene is not the first carbapenemase gene that has been identified on an IncA/C scaffold, since IncA/C-type plasmids carrying the *bla*_{VIM-4} gene have been identified in *K. pneumoniae* and *Enterobacter cloacae* clinical isolates in Italy (5, 8, 12). IncA/C-IncP multireplicon plasmids carrying the *bla*_{IMP-13} gene were also identified in *Salmonella* isolates from food sources in Colombia (20). Apart from carbapenemase genes, it was also demonstrated previously that IncA/C-type plasmids were at the origin of the worldwide spread of the ESBL gene *bla*_{VEB-1} (often associated to the quinolone resistance gene *qnrA1*) in members of the *Enterobacteriaceae* (26).

We report here the whole sequence of an IncA/C-type plasmid carrying the *bla*_{NDM-1} gene identified in a *K. pneumoniae* clinical isolate from Kenya. The acquisition of this emerging MBL resistance gene occurred on a widely diffused plasmid scaffold known to be responsible for the spread of *bla*_{CMY-2}-like genes. Considering that IncA/C plasmids possess a broad host range, this scaffold might enhance the large-scale diffusion of the *bla*_{NDM-1} gene among Gram-negative organisms.

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