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Characterization of Tn*3000*, a Transposon Responsible for bla_{NDM-1} Dissemination among *Enterobacteriaceae* in Brazil, Nepal, Morocco, and India

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In *Enterobacteriaceae*, the bla_{NDM} genes have been found in many different genetic contexts, and a wide diversity of plasmid scaffolds bearing those genes has been found. In August 2013, we identified NDM-1-producing *Escherichia coli* and *Enterobacter hormaechei* strains from a single rectal swab sample from a patient hospitalized in Rio de Janeiro, Brazil, who had no history of travel abroad. Complete DNA sequencing using the Illumina platform and annotation of the two plasmids harboring the bla_{NDM-1} gene, one from each strain, showed that they belonged to incompatibility groups $IncFII_K$ and IncX3 and harbored a novel transposon named Tn*3000*. Similar genetic structures have been identified among other isolates in Brazil but also on plasmids from other continents. Our findings suggest that the bla_{NDM-1} gene may be transmitted by Tn*3000* in different parts of the world.

Since the original description of NDM-1 carbapenemase in *Escherichia coli* and *Klebsiella pneumoniae* (1), 11 variants of this enzyme have been reported, with NDM-1 being the most prevalent (2). These enzymes have now been detected worldwide in *Enterobacteriaceae* (3), in *Pseudomonas aeruginosa* (4), and in many different *Acinetobacter* species (5). It has been proposed that the dissemination of the bla_{NDM-1} gene among *Acinetobacter* strains is mediated by a composite transposon designated Tn*125*, with two IS*Aba125* copies bracketing the resistance gene module (6). Although in *Acinetobacter* the bla_{NDM-1} has most frequently been found chromosomally located, some reports have described this gene located on plasmids (7, 8).

In *Enterobacteriaceae*, the bla_{NDM} genes have been found mainly on plasmids (9). In contrast to the more conserved genetic environment observed in *Acinetobacter* spp., many different genetic contexts have been described in *Enterobacteriaceae*, with a wide diversity of plasmids harboring bla_{NDM} genes (10–13). Among NDM variants described to date, all but NDM-2 and NDM-14 were detected in *Enterobacteriaceae* (14). Most of the sequences available in GenBank have a complete or truncated IS*Aba125* upstream and the ble_{MBL} gene downstream from the bla_{NDM} gene. Many different mobile elements have been found bracketing these genes and can potentially mobilize them (15). Three examples of genetic elements bearing the bla_{NDM-1} gene are (i) the Tn*125* transposon (6), originally described in *Acinetobacter* but now detected in *Enterobacteriaceae* (16), (ii) the one detailed under GenBank accession no. KP900016 (17), in which an IS*5* family transposase is located upstream from a truncated IS*Aba125* and the bla_{NDM-1} gene and is also found 6.064 kb downstream from the bla_{NDM-1} gene, bracketing a 9.476 kb genetic element, and (iii) the one detailed under GenBank accession no. KR059865 (18), in which IS*3000* (IS*3* family) is found 2.479 kb upstream from the bla_{NDM-1} gene and a Tn*Asn3*-like *tnpA*, also from IS*3* family, is found 4.757 kb downstream from the bla_{NDM-1} gene, bracketing a 12.802-kb genetic element.

There are few reports on genes other than bla_{NDM-1} which include complete mobile elements both upstream and downstream from the bla_{NDM} gene. In GenBank deposit AB898038 (19), an IS*6* family transposase truncates the IS*Aba125* and an unknown transposase is present 2.367 kb downstream from the bla_{NDM-3} gene. In *K. pneumoniae* plasmid pJEG027 (20), an IS*5* family transposase truncates the IS*Aba125* and IS*26* is found 2.189 kb downstream from the bla_{NDM-4} gene. A similar genetic structure is present in GenBank deposits KP826705 (unpublished) and KP178355 (21), containing, respectively, the bla_{NDM-7} and bla_{NDM-5} genes.

In Brazil, the first NDM-positive strain was reported in 2013, bearing a chromosomally located bla_{NDM-1} gene in *Providencia rettgeri* (22). Subsequently, plasmid-borne bla_{NDM-1} genes were identified in *Enterobacter hormaechei* (23), *Enterobacter cloacae*, *P.*

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rettgeri, *K. pneumoniae* (24), and *Acinetobacter baumannii* (25), but the sequences of these plasmids remain unknown. In *E. hormaechei*, the plasmid was reported be ~420 to 490 kb (23), while in *E. cloacae*, *P. rettgeri*, and *K. pneumoniae*, the plasmid was reported to be ~230 kb (24) and in *A. baumannii* the estimated plasmid size was 100 kb (25).

In this study, we aimed to characterize the genetic environment surrounding the bla_{NDM-1} gene in two *Enterobacteriaceae* species, *E. coli* and *E. hormaechei*, which were simultaneously recovered from a rectal swab from a hospitalized patient who had never traveled outside Brazil. Our investigation revealed that in both isolates, the bla_{NDM-1} gene was carried in an original transposon structure.

MATERIALS AND METHODS

Bacterial strains. Two NDM-producing strains, *E. hormaechei* E0083033-1 and *E. coli* E0083033-2, recovered from the same rectal swab sample from a pediatric patient on August 2013 in Rio de Janeiro, Brazil, were used in this study. The patient was under treatment for acute lymphoblastic leukemia and was admitted at Children's Hospital for 2 days for skin-tunneled central venous catheter placement. She had no history of previous infections or colonization by carbapenem-resistant *Enterobacteriaceae* (CRE), but since she had been previously hospitalized in another institution, according to institutional infection control recommendations, a rectal swab sample was collected for CRE surveillance.

Species identification. Identification of species was done by mass spectrometry (MS) using the Vitek MS system (bioMérieux), as recommended by the manufacturer.

Molecular identification was performed by partial sequencing of the *gyrB* gene, as previously described (26). The identification of the *Enterobacter* strains at the species level was confirmed by partial sequencing of the *hsp60* gene, as previously described (27, 28), except that Platinum *Taq* DNA polymerase was used in PCRs and DNA sequences were obtained using BigDye Terminator version 3.1 and a 3130xl genetic analyzer (Applied Biosystems), according to the manufacturer's instructions. Contigs were assembled using DNABaser program version 3.4.5 (Heracle Biosoft) and subsequently compared to the sequences from the type strains available at GenBank, using the BLAST program.

Detection of carbapenemase-encoding genes by PCR and sequencing. Multiplex PCRs for the bla_{NDM}, bla_{OXA-48}, bla_{KPC}, bla_{IMP}, bla_{VIM}, and bla_{SPM} genes were performed as previously described (29), except that primers 27F (AGAGTTTGATYMTGGCTCAG) and 1492R (GGTTACC TTGTTACGACTT) were included in order to amplify the 16S rRNA gene as an internal control (30). For full-length amplification of the bla_{NDM-1} gene, primers NDM-L-bleo-FW (5′-TGGGTCGAGGTCAGGATAGG) and NDM-R-Aba-125-RV (5′-GCTTTTGAAACTGTCGCACCT) were designed using Primer-BLAST. Amplicons were sequenced and assembled as described above.

Plasmid extraction, transformation, and conjugation assays. Plasmid DNA was obtained from the wild-type (WT) strains by alkaline extraction (31) and subsequently used to transform *E. coli* TOP10 (Invitrogen) by electroporation. Transformants were selected on LB agar containing ceftazidime (4 mg/liter). Conjugation experiments were performed using WT strains as donors and *E. coli* J53 as the recipient, as described previously (32). Transconjugants were selected on LB agar containing ceftazidime (4 mg/liter) plus sodium azide (125 mg/liter). The presence of the bla_{NDM-1} gene in transformants and transconjugants was confirmed by PCR (29).

Estimation of plasmid size was performed after 0.7% agarose gel electrophoresis, using a curve obtained by plotting the distance (millimeters) from the origin against the decimal logarithm of the plasmid size (154 kb, 66.2 kb, 37.6 kb, and 7.4 kb) from the reference strain *E. coli* 39R861 (33).

Antimicrobial susceptibility profile of WT strains and their transformants. Antimicrobial susceptibility profiles were determined by broth microdilution (34) using cation-adjusted Mueller-Hinton broth (Becton-Dickinson) and Etest strips for fosfomycin and aztreonam. *E. coli* ATCC 25922 was used as a control. Results were interpreted according to the M100-S25 document from CLSI (35), except for tigecycline and fosfomycin, for which results were interpreted according to the EUCAST breakpoints (36). For polymyxin B, the colistin criteria from EUCAST were applied. The disk diffusion method (35, 37) was used to test for ampicillin susceptibility, with and without the addition of 10 μl of a 0.1 M EDTA solution to the disks in order to inhibit the NDM-1 activity. A blank disk containing only 0.1 M EDTA was also included as control.

Complete plasmid sequencing, assembly, annotation, and analysis. Plasmid DNA was extracted (31) from transformants grown overnight at 37°C in an orbital shaker in LB broth containing imipenem (1 mg/liter). DNA samples were tagmented using the Nextera DNA sample preparation kit before fragments of ~2,000 bp were captured, purified, and sequenced using a MiSeq Reagent Nano kit, v2 (500 cycles), in MiSeq equipment from Illumina. Sequences were assembled *de novo* in contigs using the SeqMan NGen program version 4.0 (DNAStar) and subsequently aligned using SeqMan Pro version 10.1.1 (DNAStar). Open reading frames (ORFs) were predicted and annotated using RAST (http://rast.nmpdr.org/) (38). Manual curation and sequence similarity searches directed against the GenBank database were carried out using the ARTEMIS genome browser and annotation tool (39). Insertion sequences were manually reviewed, directing searches against the IS Finder database (https://www-is.biotoul.fr/) (40). The full plasmid sequences were compared to those available at GenBank using BLAST.

Nucleotide sequence accession numbers. The complete nucleotide sequences of the pEh1A and pEc2A plasmids were deposited in GenBank under accession numbers KR822246 and KR822247, respectively.

RESULTS

Species identification and screening for carbapenemase-encoding genes. Identification using the Vitek MS system identified the E0083033-1 strain as *E. cloacae* complex with 99% confidence. When the *gyrB* partial sequence (1,138 bp) was compared to those pertaining to the reference strains published by Brady et al. (41), the highest similarity (96%) was obtained with *E. hormaechei* strain CCUG 27126. The partial sequence of the *hsp60* gene (341 bp) was identical to that from the type strain of "*E. hormaechei* subsp. *steigerwaltii*" DSMZ16691. The Vitek MS system (bioMérieux) identified strain E0083033-2 as *E. coli* with 99% confidence, which was further confirmed by sequencing of the partial *gyrB* sequence (1,138 bp). When the WT strains were tested by multiplex PCR for detection of carbapenemase-encoding genes, both were positive for bla_{NDM} and negative for the other genes evaluated. Full sequencing of amplicons identified the bla_{NDM-1} gene in both strains.

Plasmid profile, transformation, and conjugation assays. *E. hormaechei* strain E00383033-1 possessed five plasmid bands (ca. 130 kb, ca. 90 kb, ca. 70 kb, ca. 7 kb, and ca. 6 kb), while the *E. coli* transconjugant and transformant strains showed only a single plasmid band of approximately 90 kb (data not shown). *E. coli* strain E0083033-2 exhibited two plasmid bands (160 kb and ca. 70 kb), while the transformant and the transconjugant possessed a single plasmid band of approximately 70 kb (data not shown). The plasmids carrying the bla_{NDM-1} gene were successfully transferred by conjugation, at a frequency of 5.3×10^{-1} with *E. hormaechei* strain E0083033-1 as the donor and at a frequency of 6.0×10^{-1} with *E. coli* strain E0083033-2 as the donor.

Antimicrobial susceptibility profiles. The transformant obtained with plasmid DNA extracted from *E. hormaechei*

TABLE 1 MICs for wild-type strains and their transformants

Antimicrobial	MIC (μg/ml) for strain[a]:				
	E0083033-1	TF1A	E0083033-2	TF2A	TOP10
Ampicillin	≥2,056	≥2,056	≥2,056	≥2,056	8
Aztreonam	64	0.094	0.064	0.125	0.094
Cefepime	≥64	32	≥64	≥64	0.06
Cefoxitin	≥1,024	512	≥1,024	≥1,024	8
Ceftazidime	≥64	≥64	≥64	≥64	0.25
Ceftriaxone	≥64	≥64	≥64	≥64	0.06
Ertapenem	64	16	64	16	0.015
Imipenem	64	32	32	32	0.25
Meropenem	32	16	32	16	0.015
Amikacin	8	4	16	4	2
Gentamicin	2	0.5	0.5	0.25	0.5
Kanamycin	32	4	32	32	2
Tobramycin	16	0.5	16	8	0.25
Ciprofloxacin	1	0.004	0.5	0.016	0.004
Levofloxacin	0.5	≤0.008	0.25	≤0.008	0.015
Chloramphenicol	4	2	4	2	2
Fosfomycin	0.75	0.38	0.5	0.38	0.38
Tigecycline	0.25	0.03	0.25	0.125	0.5
Polymyxin B	1	0.5	1	0.25	0.5
Rifampin	512	8	512	512	8

[a] E0083033-1, WT *E. hormaechei* strain; TF1A, transformant derived from *E. hormaechei* E0083033-1; E0083033-2, WT *E. coli* strain; TF2A, transformant derived from *E. coli* E0083033-2.

E0083033-1 as the donor showed resistance to all β-lactams tested except aztreonam. It remained susceptible to aminoglycosides, fluoroquinolones, rifampin, and chloramphenicol (Table 1).

E. coli strain E0083033-2 and its transformant were resistant to all β-lactams tested except aztreonam. MICs of tobramycin, amikacin, kanamycin, ciprofloxacin, and rifampin for the corresponding transformants were 2- to 64-fold increased, while those of chloramphenicol and gentamicin were unchanged (Table 1).

No inhibition zones were observed with blank disks containing 0.1 M EDTA or ampicillin disks when testing the transformant harboring pEc2A. Of note, an inhibition zone of 19 mm in diameter was observed with the ampicillin disk with addition of 0.1 M EDTA when testing the transformant containing pEh1A, in which the only antimicrobial resistance gene is bla_{NDM-1}.

Plasmid pEh1A sequence analysis. The complete DNA sequence of plasmid pEh1A from *E. hormaechei* E0083033-1 was obtained, with an average depth of coverage of 470. It is a circular 96,124-bp plasmid with a G+C content of 53.1% and carries a total of 100 open reading frames (Fig. 1). DNA sequence comparison with sequences available in GenBank revealed a similarity index of 99% with two IncF plasmids, one from "*E. hormaechei* subsp. *oharae*" recovered in Brazil (GenBank accession no. NG_041719.1) (23) and plasmid pKPX-1 from *K. pneumoniae* recovered in Taiwan from a patient with a history of hospitalization in India (GenBank accession no. AP012055.1) (42). The pEh1A DNA sequence differed from that of *E. hormaechei* by the presence of a 40-bp repeat region at position 70886 (GenBank accession no. NG_041719.1) (23) downstream of the *parA* gene and the lack of a 1,370-bp fragment (partial sequence of the second copy of IS*3000*).

Comparison of the 250,444-bp plasmid pKPX-1 (42) showed that it contains all gene clusters and operons found in plasmid pEh1A (96,124 bp). These two plasmids differed in the ordering of operons, as the arsenic resistance operon is inverted with respect to the bla_{NDM-1} gene in pEh1A. They also differed by the presence of a gene coding for a hypothetical protein and a truncated *tnpA* gene, both occurring downstream of the arsenic operon in pEh1A, and by the presence of a *tnpR* gene truncating IS*3000* downstream of the *groEL* gene. The nucleotide sequences from the two plasmids share 93.8% similarity (90,184 bp identical over the 96,124 bp of pEh1A).

The sequences of the *oriV* and *repA* genes (nucleotide positions 1 to 1276) from plasmid pEh1A were compared to those previously studied by Villa et al. (43). The highest similarity index (99%; 1,273/1,276) was observed with plasmid pKF3-94 (GenBank accession no. FJ876826.1) (44), belonging to the $IncFII_K$ group. The *oriV* region from pEh1A possessed two DnaA boxes upstream from the *repA* gene, with an AT-rich region of 63.3% (nucleotide positions 146 to 224 bp) and five iterons characterized by GGTG(T/G)(G/T) nucleotide sequences distant from each other by 15 or 16 bases (nucleotide positions 245 to 335).

Looking at the features related to plasmid transfer and stability, plasmid pEh1A carries *tra* and *trb* operons, which enable conjugal transfer. A *ccdAB* operon encoding a toxin/antitoxin system involved in postsegregation killing of plasmid-free cells was also identified. A complete arsenic resistance operon was identified at nucleotide positions 21296 to 25604.

The plasmid has a single copy of the bla_{NDM-1} gene flanked upstream by a truncated IS*Aba125* and downstream by the ble_{MBL} gene, encoding resistance to bleomycin. That overall structure containing the bla_{NDM-1} gene was designated transposon Tn*3000*.

The Tn*3000* transposon is conserved among plasmids from different continents. Transposon Tn*3000* is 11,823 bp long and is bracketed by two copies of IS*3000*. The first copy truncates the 5′ portion of the IS*Aba125* upstream of the bla_{NDM-1} gene. Downstream of the bla_{NDM-1} gene, the ble_{MBL} gene was present, followed by genes encoding a phosphoribosylanthranilate isomerase (*trpF*), a twin-arginine translocation pathway signal protein (*tat*), and a

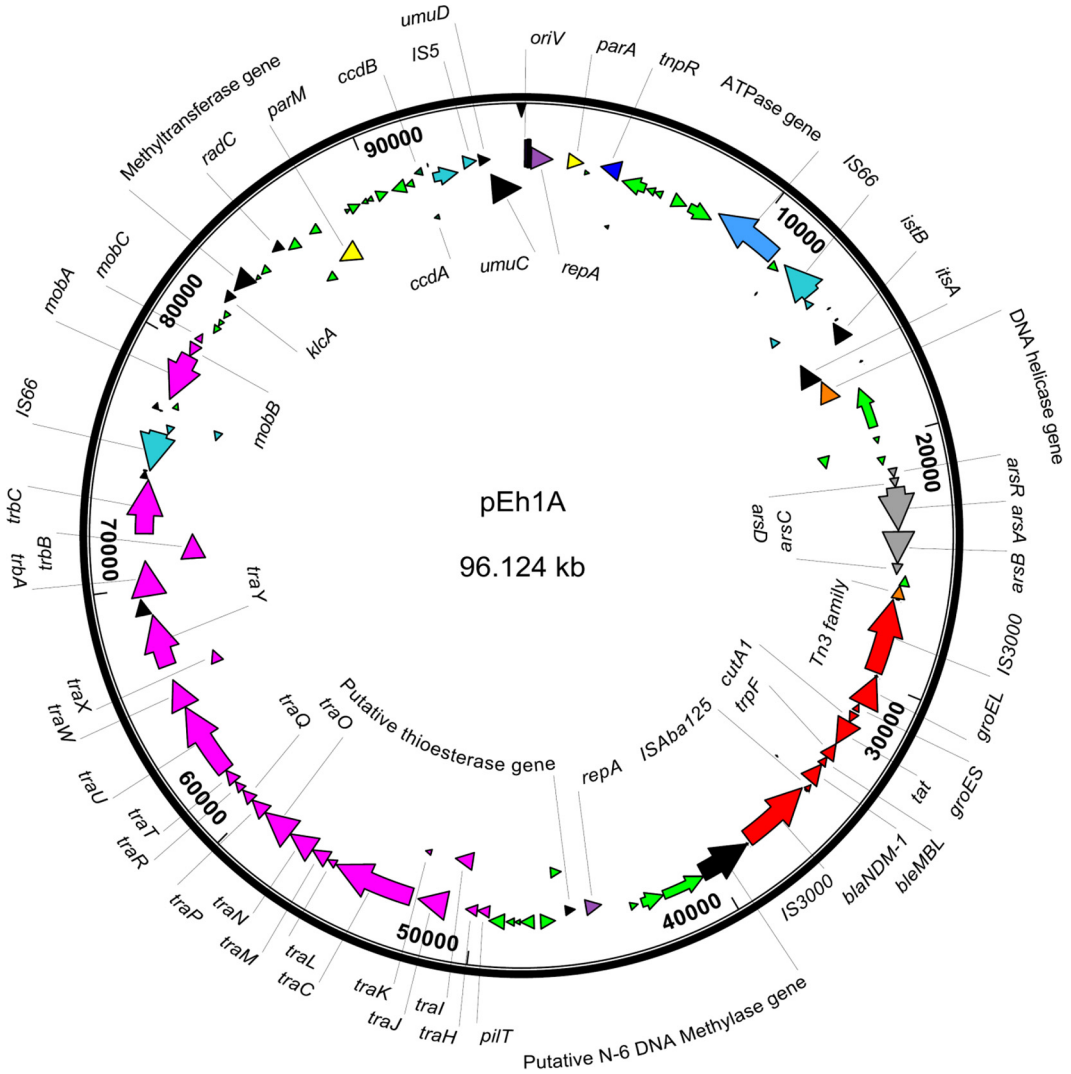

FIG 1 Circular map of IncFII$_K$ plasmid pEh1A from *E. hormaechei*. Genes encoding hypothetical proteins are in green, genes encoding the conjugation apparatus are in pink, and genes from Tn*3000* are in red.

divalent ion tolerance protein (*cutA1*). The *groEL* and *groES* genes were also part of Tn*3000*, but the *groEL* gene was truncated at its 3′ extremity by insertion of a second copy of IS*3000*. The Tn*3000* nucleotide sequences identified on plasmids pEh1A and pEc2A were 100% identical. *In silico* analysis revealed sequences showing high similarities with Tn*3000* in five plasmid sequences (Fig. 2) originating from isolates distributed over different continents. *In silico* analysis revealed that transposon Tn*3000* was 99.9% identical to sequences identified on plasmids from incompatibility groups IncF and IncH originating from *K. pneumoniae* from Nepal (GenBank accession no. CP008933.1) (45) and Morocco (GenBank accession no. JN420336.1) (13). Therefore, those two plasmid sequences also harbored transposon Tn*3000* (Fig. 2).

Two other plasmids, one from Porto Alegre, Brazil (GenBank accession no. NG_041719.1) (23), and one from New Delhi, India (pKPX-1; GenBank accession no. AP012055.1), also harbored transposon Tn*3000*, but the right-hand copy of IS*3000* was truncated in those two cases. In the plasmid from Brazil, two inverted repeats (IRs) from the second copy of IS*3000* were identified, but the *tnpA* gene lacked a fragment of 1,370 bp (Fig. 2).

In another plasmid (pNDM-BTR) from China (unpublished; GenBank accession no. KF534788.1), the left-hand extremity of transposon Tn*3000* was conserved but the second copy of IS*3000* located at the right extremity was aborted, truncated by IS*Kpn19* (Fig. 2).

Transposon Tn*3000* identified on plasmids from Brazil recovered in 2013 and described in this study was closely related to that identified from isolates from Nepal and Morocco, differing by 3 and 5 bp, respectively (Fig. 3).

In none of the plasmid sequences analyzed were direct repeats flanking the Tn*3000* transposon observed, suggesting that this structure may have been acquired by homologous recombination rather than by transposition.

Plasmid pEc2A sequence analysis. The complete DNA sequence from pEc2A from *E. coli* E0083033-2 was obtained, with an average depth of coverage of 2,771. It is a circular 74,852-bp plasmid with a 50.2% G+C content and 85 ORFs (Fig. 4).

The 29.5-kb backbone structure of plasmid pEc2A is typical of IncX plasmids, with genes encoding replication-associated proteins (*pir*, *bis*, *parA*, *hns*, and *topB*). It has a complete *pilX* operon,

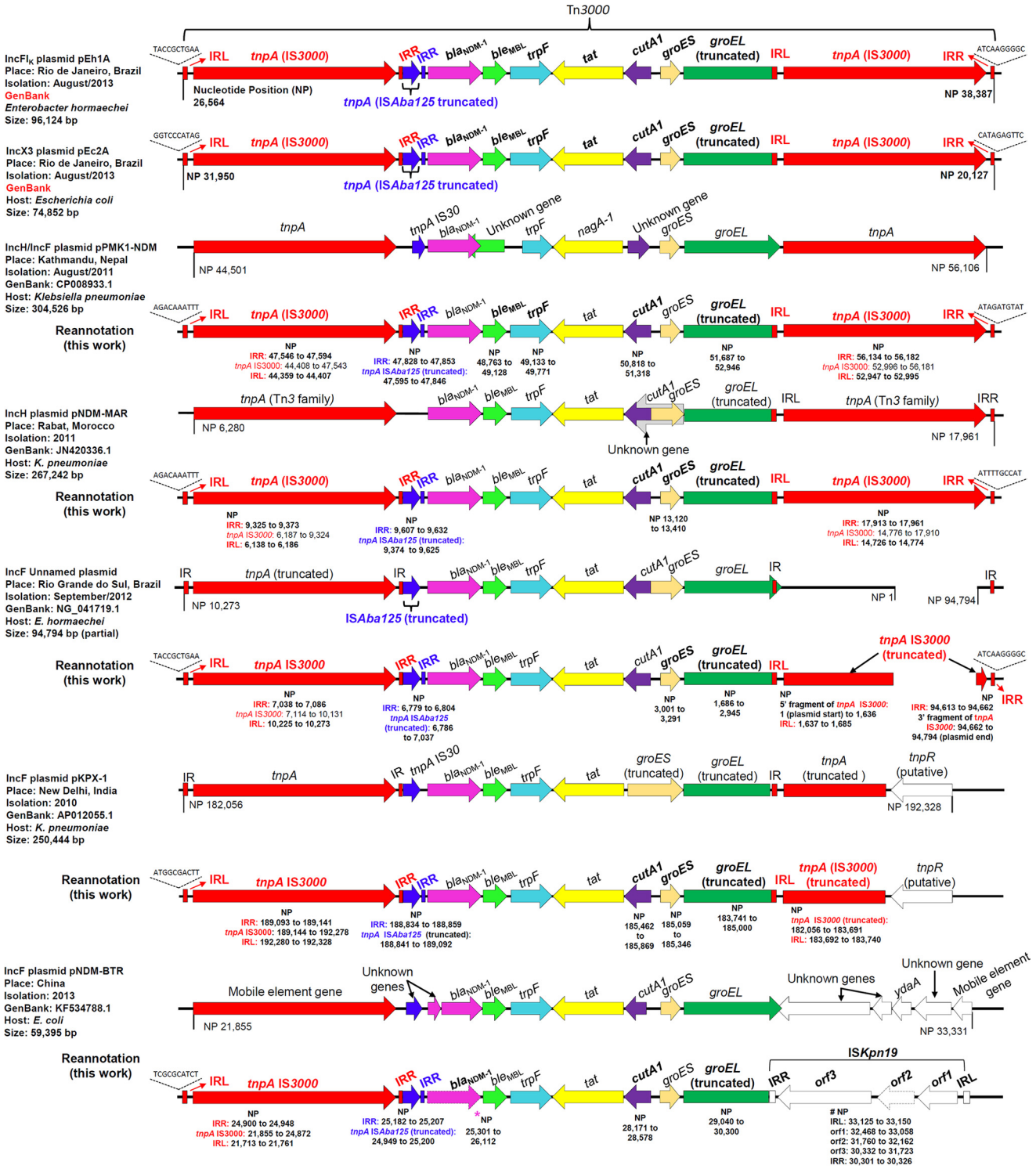

FIG 2 Comparison of Tn*3000* transposons of plasmids detected in different continents. *, a single-base-pair deletion in the bla_{NDM-1} gene at position 25509 created a stop codon at positions 25532 to 25534. #, a single-base-pair deletion in the *orf2* gene from IS*Kpn19* at position 32162 altered the reading frame originally described. Gene names in bold indicate revision of the original annotation.

encoding a conjugation apparatus, and also *taxA*, *taxB*, and *taxC* genes, implicated in plasmid transfer. The *taxC* gene sequence was compared to IncX plasmids recently reviewed (46), and the highest similarity was observed with IncX3 plasmids pEC14_35 (GenBank accession no. JN935899) (95.4%) (47) and pIncX-SHV (GenBank accession no. JN247852) (95.3%) (48).

Plasmid pEc2A has a single copy of the bla_{NDM-1} gene flanked upstream by a truncated IS*Aba125* and downstream by the ble_{MBL}

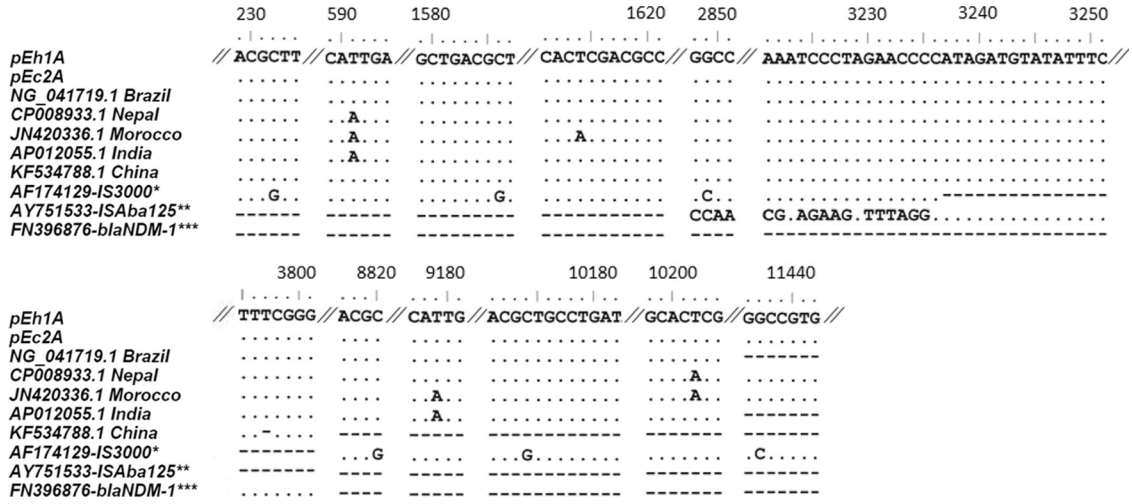

FIG 3 Polymorphisms in the Tn*3000* transposon in unique plasmids detected in different continents. A dot indicates a nucleotide identical to that from the Tn*3000* of the pEh1A plasmid in a given position. A dash indicates the absence of a nucleotide in a given position compared to Tn*3000* of the pEh1A plasmid in a given position. Nucleotide numbering refers to the Tn*3000* sequence. *, original IS*3000* GenBank deposit; **, original IS*Aba125* GenBank deposit; ***, original bla_{NDM-1} gene GenBank deposit.

gene. As observed for the IncF plasmid pEh1A, the bla_{NDM-1} gene occurred within Tn*3000*.

Plasmid pEc2A has a class 1 integron that is 99% similar to In*37* (GenBank accession no. AY259086) (49). It possesses a variable region encompassing four gene cassettes, namely, *aac*(*6′*)*-lb-cr*, bla_{OXA-30}, *catB3*, and *arr3*. MICs of tobramycin, amikacin, kanamycin, ciprofloxacin, and rifampin in the transformants harboring plasmid pEc2A were 2- to 64-fold increased compared to those for *E. coli* TOP10, while no elevation in chloramphenicol and gentamicin MICs was observed (Table 1).

DISCUSSION

The present study describes a new genetic element harboring bla_{NDM-1}, Tn*3000*, which was found on plasmids of distinct incompatibility groups detected in different continents. Upon isolation of NDM-1-producing *E. coli* and *E. hormaechei* from a single rectal swab, our first hypothesis was that plasmid transfer occurred between these enterobacterial species, but plasmid analysis showed sizes that were significantly different. We subsequently introduced both plasmids into a single *E. coli* TOP10 strain and observed that they replicated and coexisted stably, which suggested different incompatibility groups. DNA sequence analysis confirmed that they belonged to different incompatibility groups: $IncFII_K$ and IncX3. These are the first complete sequences of bla_{NDM-1}-carrying plasmids from Brazil. bla_{NDM-1} has so far been found on plasmids of incompatibility groups IncF, IncH, IncL, IncM, and IncX (7, 50), as well as untypeable ones. Plasmid pEh1A, belongs to the $IncFII_K$ incompatibility group and was found from "*E. hormaechei* subsp. *steigerwaltii*" in 2013; it is highly similar to the partial sequence of a plasmid isolated from "*E. hormaechei* subsp. *oharae*" in 2012 (23) in Porto Alegre, 1,571 km away from Rio de Janeiro.

The pEh1A $IncFII_K$ plasmid has genes commonly found in IncF plasmid backbones, such as *repA*, *parA*, *resD*, and *ccdAB*, but is unusual in having an arsenic resistance operon (*arsR*, *arsD*, *arsA*, *arsB*, and *arsC*) instead of a mercury resistance operon (51).

The genetic structure observed in the plasmid extracted from *E. coli* (pEc2A) is as described by Norman and colleagues (52): *pir-bis-par-hns-topB-pilX-actX-taxCA*. The antimicrobial resistance genetic determinants located on the plasmid were embedded into two distinct genetic structures, namely, In*37* and Tn*3000*. Concerning the In*37* integron, the increased MICs of tobramycin, amikacin, kanamycin, ciprofloxacin, ampicillin, and rifampin observed for the transformant harboring plasmid pEc2A were consistent with the expression of gene cassettes driven by the Pc promoter. Of note, there was likely a lack of expression of the third gene cassette in In*37* (*catB3*), as indicated by the low MICs observed for chloramphenicol in both the wild type and the transformant. If we consider that the genes upstream (bla_{OXA-30}) and downstream (*arr3*) of the *catB3* gene are expressed, the lack of chloramphenicol MIC elevation is most probably due to a post-transcriptional attenuation, as previously reported by Stokes and Hall (53).

The pEc2A plasmid isolated from *E. coli* belongs to the IncX3 incompatibility group. This suggests considerable potential for dissemination of bla_{NDM-1} in Brazil, as recently reported from China (54) and the United Arab Emirates (55).

We have found that the same genetic structure Tn*3000* is present in plasmids of different sizes and incompatibility groups detected during the period from 2010 to 2013 in different countries and continents. IS*3000* was originally described by Sabaté et al. (56). It was found in the In*60* integron but oriented in the opposite direction of gene cassettes. These authors detected the presence of In*60* containing IS*3000* in a total of 30 *E. coli* and *Salmonella* species strains isolated from unrelated sources, but they were not able to demonstrate the occurrence of transposition events using a positive-selection vector strategy (57). One possibility to explain the presence of this element in different plasmids would be homologous recombination, but in this case the regions flanking IS*3000* would be identical in different plasmids. This is not the case in the plasmids we have described or cited. If IS*3000* and Tn*3000* are not mobile elements, it would be hard to explain how they could be found flanked by different structures.

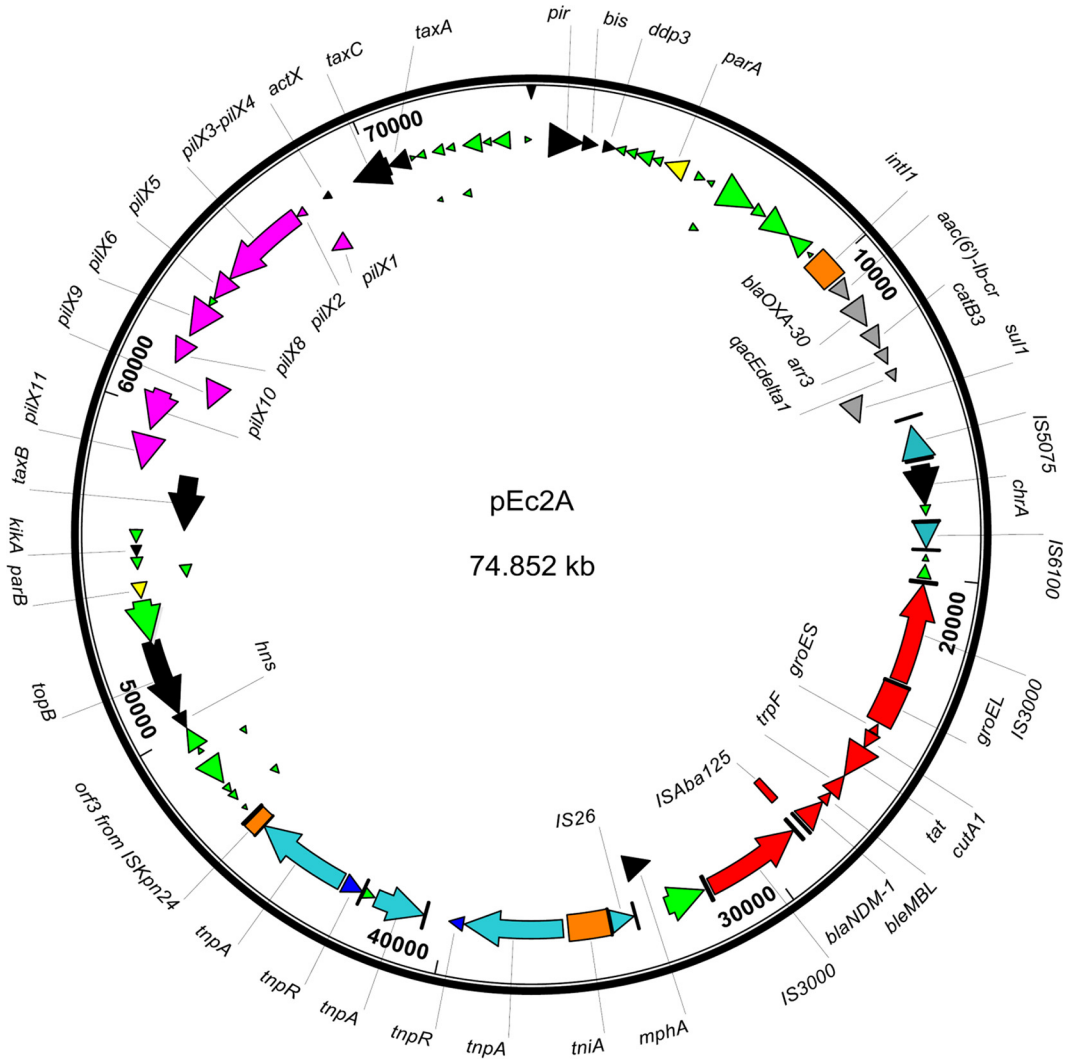

FIG 4 Circular map of IncX3 plasmid pEc2A plasmid from *E. coli*. Genes encoding hypothetical proteins are in green, genes encoding the conjugation apparatus are in pink, and genes from Tn*3000* are in red.

The presence of a truncated IS*3000* in the 3′ portion of Tn*3000* in plasmid pKPX-1 from India indicates that Tn*3000* is the ancestral structure. Its insertion into this plasmid preceded a second transposition event that resulted in truncation of the IS*3000*. The full Tn*3000* transposon sequence was found in two others plasmids, pPMK1-NDM (GenBank accession no. CP008933.1) (45) and pNDM-MAR (GenBank accession no. JN420336.1) (13), from Nepal and Morocco, respectively. If we consider that the Tn*3000* sequence from the plasmids we described in this work, which were isolated in Rio de Janeiro, Brazil, in August 2013, is identical to that from the plasmid isolated in Porto Alegre, Brazil, in September 2012, the frequency of mutations in Tn*3000* is less than one per 11 months. Zhao et al. (44) analyzed 110 strains harboring three plasmids with lengths of 70,057 to 147,416 bp by Illumina sequence analysis. When they compared the full plasmid sequences obtained in different years from different strains, they found 331 to 1,256 SNPs, depending on the plasmid studied (44). If we extrapolate this number to a pair of strains and a 11.8-kb structure as in the case of Tn*3000*, this range would be from 1 to 2.8 SNPs in 4 years in a 11.8-kb fragment. Consequently our finding of no SNPs when comparing the DNA sequences from Tn*3000* in the plasmids isolated in Brazil 11 months apart is consistent with the findings of Zhao et al. (44). If we use these mutation rates to calculate the evolutionary distance in years between Tn*3000* detected in Brazil and those detected in different continents, the smallest distance would be with the element from Nepal, with the time required to accumulate the three observed SNPs being 4.3 to 12 years. The plasmid isolated in Nepal was detected in August 2011. If we compare the Tn*3000* DNA sequence from Brazil to that from Morocco, also detected in 2011, there are five SNPs, and their evolutive distance would be 7.1 to 20 years.

Conclusions. In summary, we have described the first two complete plasmid sequences harboring $bla_{\text{NDM-1}}$ from Brazil and have described a new transposon, designated Tn*3000*, which appears to mediate the transfer of $bla_{\text{NDM-1}}$ among plasmids from different incompatibility groups in Brazil, Nepal, Morocco, and India.

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We declare no conflicts of interest.

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