Genomic Epidemiology of NDM-1-Encoding Plasmids in Latin American Clinical Isolates Reveals Insights into the Evolution of Multidrug Resistance

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Data deposition: The annotated, complete NDM-1-encoding plasmids sequenced in this study are available in the DDBJ/EMBL/GenBank public databases under the GenBank accession numbers KX832927 (p16Pre36-NDM), KX832926 (p16Pre36-2), CP017672 (pRB151-NDM), KX832928 (p06-1619-NDM), and KX832929 (p06-1619-2). The trimmed and filtered MiSeq sequencing reads for all genomes sequenced in this study have been deposited in the Sequence Read Archive under Bioproject accession number PRJNA342046 with sample accession numbers for each strain listed in supplementary data set 1, Supplementary Material online.

Abstract

Bacteria that produce the broad-spectrum Carbapenem antibiotic New Delhi Metallo-β-lactamase (NDM) place a burden on health care systems worldwide, due to the limited treatment options for infections caused by them and the rapid global spread of this antibiotic resistance mechanism. Although it is believed that the associated resistance gene bla_{NDM-1} originated in *Acinetobacter* spp., the role of *Enterobacteriaceae* in its dissemination remains unclear. In this study, we used whole genome sequencing to investigate the dissemination dynamics of bla_{NDM-1}-positive plasmids in a set of 21 clinical NDM-1-positive isolates from Colombia and Mexico (*Providencia rettgeri*, *Klebsiella pneumoniae*, and *Acinetobacter baumannii*) as well as six representative NDM-1-positive *Escherichia coli* transconjugants. Additionally, the plasmids from three representative *P. rettgeri* isolates were sequenced by PacBio sequencing and finished. Our results demonstrate the presence of previously reported plasmids from *K. pneumoniae* and *A. baumannii* in different genetic backgrounds and geographically distant locations in Colombia. Three new previously unclassified plasmids were also identified in *P. rettgeri* from Colombia and Mexico, plus an interesting genetic link between NDM-1-positive *P. rettgeri* from distant geographic locations (Canada, Mexico, Colombia, and Israel) without any reported epidemiological links was discovered. Finally, we detected a relationship between plasmids present in *P. rettgeri* and plasmids from *A. baumannii* and *K. pneumoniae*. Overall, our findings suggest a Russian doll model for the dissemination of bla_{NDM-1} in Latin America, with *P. rettgeri* playing a central role in this process, and reveal new insights into the evolution and dissemination of plasmids carrying such antibiotic resistance genes.

Key words: metallo-beta-lactamase, genomics, antibiotic resistance, *Providencia rettgeri*, mobile genetic elements, bacterial evolution.

Introduction

In the first global report on antimicrobial resistance issued by the World Health Organization very high resistance rates were found in the bacteria that are the main causes of community and health care associated infections, including *Enterobacteriacea* and *Acinetobacter baumannii* (World Health Organization 2014). For instance, the prevalence of *Enterobacteriaceae* resistant to broad-spectrum β-lactam type antibiotics often used as "last-line-of-defence" agents, such as carbapenems, are persistently increasing across the world (Rhomberg and Jones 2009; Prabaker and Weinstein 2011; van Duijn et al. 2011; CDC 2013). Infections caused by carbapenem-resistant bacteria increase health care costs by requiring hospitalization of patients and increase the risk of mortality (Lemos et al. 2014; World Health Organization 2014). It is therefore important to gain greater insight into how resistance spreads, recognizing in doing so that resistance can spread either vertically, through distribution of clones of established "successful" resistant bacterial species, or horizontally, through dispersal of mobile genetic elements (e.g., transposons, plasmids, and prophages) carrying genes for antimicrobial resistance (Woodford et al. 2011). The horizontal transfer of antibiotic resistance genes between bacteria can contribute rapid expansion in the suite of resistance mechanisms present in a bacterial strain.

One mechanism for resistance that can be acquired from the mobile gene pool is the capability for drug modification, an example of which is the group of metallo-β-lactamases. These enzymes have great impact on public health due to their broad substrate range, and are increasing in frequency in clinically important Gram-negative bacteria (Palzkill 2013). A new member of this group of enzymes was identified in 2008 in a patient treated in New Delhi, India (Yong et al. 2009), and named New Delhi Metallo-β-lactamase (NDM). Initially, NDM dissemination was epidemiologically linked to the Indian subcontinent, though the complexity of transmission of this antibiotic resistance determinant became apparent rapidly, due to the presence of NDM-encoding genes (bla_{NDM}) in diverse Gram-negative bacteria, both fermenters (*Enterobacteriaceae*) and nonfermenters (*Acinetobacter baumannii* and *Pseudomonas aeruginosa*) (Johnson and Woodford 2013). Subsequently, NDM-positive strains were isolated in multiple countries on all continents in a great variety of bacterial genera without any epidemiological or molecular links to the strains circulating in the Indian subcontinent (Johnson and Woodford 2013).

The bla_{NDM} gene is usually carried by conjugative plasmids, although the host plasmid characteristics can vary greatly in attributes such as size, incompatibility group, gene content, and organization. As conjugative plasmids are self-transmitting, this underscores the point that these resistance genes can spread independently of clones of the original bacterial host. In *Acinetobacter* spp., although the bla_{NDM} gene has been reported to be located in the chromosome (Espinal et al. 2011), it mainly resides in a family of plasmids known as pNDM-BJ01-like, named after the first completely sequenced *Acinetobacter* spp. NDM-plasmid, reported in 2012 (Hu et al. 2012). The pNDM-BJ01-like plasmids are highly conserved, with >99% nucleotide identity extending over at least 85% of the 47-kb pNDM-BJ01 length; they do not belong to any reported incompatibility (Inc) group (Hu et al. 2012); they have a Type IV Secretion System (T4SS); and they have a region of replication and transfer genes separated by a variable region containing a Tn*125* composite transposon (Hu et al. 2012). It is within Tn*125* that bla_{NDM} is located, along with other genes conserved in the order 5′-bla_{NDM}-ble_{MBL}-*trpF*-*tat*-*dct*-*groES*-*groEL*-IS*CR21*-Δ*pac*-3′, flanked upstream and downstream by IS*Aba125*, though the Tn*125* structure has been found to be truncated in some strains. Among non-*Acinetobacter* bacteria—with the exception of *Enterobacter aerogenes* (Chen et al. 2015) where bla_{NDM-1} was found in a pNDM-BJ01-like plasmid—bla_{NDM} is carried in a great variety of plasmids belonging to diverse Inc groups (FII, FIB, A/C2, HI1A, HI1B, L/M, N, N2, X3, R, T as well as unclassified plasmids) (Johnson and Woodford 2013; Khong et al. 2016). Despite the diversity of NDM-encoding plasmids in non-*Acinetobacter*, the immediate genetic context of the bla_{NDM} gene remains the same in all known cases to date, in that it is always found within Tn*125* or its remnants (Poirel et al. 2011; Partridge and Iredell 2012; Wailan, Paterson, et al. 2016). However, Tn*125* is often surrounded by other transposons (Tn) or insertion sequence (IS) elements, including IS*Kpn14*, IS*26*, IS*5*, IS*CR1* or Tn*3*-like elements, which are frequently found in *Enterobacteriaceae* and may be involved in the further dissemination of bla_{NDM} through a combination of transposition and homologous recombination (Toleman et al. 2012; Khong et al. 2016; Wailan, Sidjabat, et al. 2016). Although the bla_{NDM} gene is believed to have originated in an *Acinetobacter* spp. as the result of the fusion of an aminoglycoside resistance gene with a pre-existing metallo-β-lactamase (Toleman et al. 2012) and later transferred to *Enterobacteriaceae*, aside from the Tn*125* remnants and the case of the *Enterobacter aerogenes* harboring a pNDM-BJ01-like plasmid, little is known about this transmission from *Acinetobacter* spp. to *Enterobacteriaceae*, nor about how the diverse bla_{NDM} positive plasmids in *Enterobacteriaceae* evolved.

NDM-positive *Enterobacteriaceae*, particularly *Providencia* spp. play an increasingly important role in multidrug resistant infections and dissemination of bla_{NDM} around the world, as evidenced by the rapidly accumulating reports of isolation of this bacteria harboring this gene (Carvalho-Assef et al. 2013; Mataseje et al. 2014; Pollett et al. 2014; Tada et al. 2014; Carmo Junior et al. 2015; Manageiro et al. 2015; Nachimuthu et al. 2015; Wailan, Paterson, et al. 2016). Previously, we reported the first South American bla_{NDM-1} outbreak, which occurred in Colombia in *Klebsiella pneumonia*, as well as an NDM-1-positive *Providencia rettgeri* outbreak in Mexico, both

of which occurred in 2011–2012, without any link to the Indian subcontinent (Barrios et al. 2013; Escobar Perez et al. 2013). Shortly thereafter, we commenced a surveillance study across Colombia, and found the majority of NDM-positive bacteria isolated were *P. rettgeri*. Here we describe the use of whole genome sequencing (WGS) to investigate the dissemination dynamics of bla_{NDM-1}-positive plasmids among *Enterobacteriaceae* and *A. baumannii* clinical isolates from this surveillance study, as well as from the previous outbreaks in Mexico and Colombia. Our results demonstrate interesting genetic links between NDM-1-positive *P. rettgeri* from distant geographic locations, and between their plasmids and those present in *K. pneumoniae* and *Acinetobacter* spp. isolates, providing insights into the central role of *P. rettgeri* in antibiotic resistance dissemination in Latin America.

Materials and Methods

Isolate Collection and Culture Conditions

Twenty-one NDM-1-positive clinical isolates were included in this study (supplementary data set 1, Supplementary Material online, strains used in this study and statistics of assemblies): *P. rettgeri* (14), *K. pneumoniae* (6), and *A. baumannii* (1). Of these, 12 (11 *P. rettgeri* and one *A. baumannii*) were isolated from samples obtained in a surveillance study for carbapenem resistant bacteria that was conducted over a period of 20 months, from September 2012 to April 2014, in three different hospitals in three distant cities in Colombia (Bogota, Cali, and Bucaramanga) (supplementary data set 1, Supplementary Material online). The other nine clinical isolates were obtained from two, previously described clinical outbreaks generated by bla_{NDM-1} positive *K. pneumoniae* and *P. rettgeri*, respectively, reported in Colombia and Mexico (Barrios et al. 2013; Escobar Perez et al. 2013). Clinical and epidemiological features of the all bla_{NDM-1} positive isolates are listed in supplementary data set 1, Supplementary Material online. *Escherichia coli* transconjugants were obtained from six representative samples using as donor the bla_{NDM-1} positive clinical isolate and as recipient the sodium azide-resistant *E. coli* J53 strain. Equal amounts of a four hours Luria–Bertani (LB) (Oxoid Limited) broth culture of both donor and recipient, were mixed and 100 µl were placed onto a LB agar plate, then conjugation was allowed for 16 h at 37 °C. Subsequently, the NDM-1-positive sodium azide-resistant *E. coli* transconjugants were selected using LB agar plates supplemented with ceftazidime (30 µg/ml) and sodium azide (100 µg/ml) (Sigma–Aldrich Co. LLC.). The *E. coli* species (*uidA* gene) and the bla_{NDM-1} gene were verified in the transconjugants by PCR. Possible donor strain contamination in the transconjugants was ruled out by PCR using specific primers to the genes *khe* for *K. pneumoniae, gyrB* for *A. baumannii*, and *dnaA* for *P. rettgeri* (see supplementary table S1, Supplementary Material online). Otherwise indicated, all NDM-1-positive bacteria were routinely grown in brain hearth infusion agar or broth supplemented with ceftazidime (30 µg/ml) as a selective pressure for guarantee of plasmid permanence.

Whole Genome Sequencing

Total DNA was extracted from 21 bla_{NDM-1} positive clinical isolates and six transconjugants (supplementary data set 1, Supplementary Material online) using the PureLink® Genomic DNA mini kit from ThermoFisher. Multiplexed total DNA libraries were prepared using the Nextera XT Library Preparation Kit and 300-bp paired end sequencing was performed on the Illumina MiSeq platform using the MiSeq v3 600-cycle reagent kit. Sequencing reads were trimmed and filtered using cutadapt v1.1.7 (Martin 2011) to remove adapters, and PRINSEQ-lite v0.20.4 (Schmieder and Edwards 2011) to remove any low quality reads with average read quality less than Q20, low quality trailing ends with base quality less than Q20 and short reads <87 bp. Reads were then de novo assembled using SPAdes v3.5.0 (Bankevich et al. 2012) with default settings and the assemblies were improved to high-quality draft genome standard (Chain et al. 2009) by scaffolding using SSPACE v2.0 (Boetzer et al. 2011), gap filling using GapFiller v1.10 (Boetzer and Pirovano 2012) and removal of contigs shorter than 300 bp. Details of the sequencing data, assemblies and accession numbers for each of these genomes are listed in supplementary data set 1, Supplementary Material online. The bla_{NDM-1} gene variant was verified by comparing the genome assemblies against the reported sequence (accession NC_015872) using BLASTn (Altschul et al. 1990). Assembly for each strain was searched for matches to any known bla_{NDM}-positive plasmids, by BLASTn (Altschul et al. 1990) against an extensive database compiled from all fully sequenced bla_{NDM}-carrying plasmids deposited in the NCBI nucleotide repository (a total of 141 complete plasmids as at 18 May 2017; supplementary data set 2, Supplementary Material online).

Phylogenetic Analysis of *Providencia rettgeri* Clinical Isolates

Since a MLST scheme for the phylogenetic characterization of *P. rettgeri* isolates does not exist, was built a phylogenetic tree based on the core-genome SNPs determined from the assembled contigs of the 14 *P. rettgeri* genomes sequenced in this study, plus the draft genome of *P. rettgeri* Dmel1 (NZ_AJSB00000000.1), the most complete published *P. rettgeri* genome available at the time, as an out-group control. To build the phylogenetic tree, partially assembled genomes were annotated using Prokka v1.11 (Seemann 2014) and an alignment of concatenated core genes (genes present in all genomes with ≥90% of nucleotide identity) was created with Roary (Page et al. 2015) using PRANK (Loytynoja 2014). Poorly aligned positions and divergent regions were eliminated using Gblocks (Talavera and Castresana 2007).

Table 1
General features of bla_{NDM-1}-positive plasmids harboured in the strains included in this study.

Plasmid	Size (bp)	Inc Group	Host	Resistance Gene Profile	GenBank Accesion No	Reference
pNDM-BJ01	47,274	Not assigned	*Acinetobacter* spp.	*aph(3′)-VIa*, ***bla*$_{NDM-1}$**	NC_019268	Hu et al. (2012)
p6234-178kb	178,193	IncA/C2	*K. pneumoniae*	*aph(3′)-VIa*, *aacA29*, *aadA2*, ***bla*$_{NDM-1}$**, *bla*$_{CARB-2}$, *mph(E)*, *msr(E)*, *catB3*, *cmlA1*, *sul2*, *sul1*	NZ_CP010391	Rojas et al. (2016)
p16Pre36-NDM	244,116	Not assigned	*P. rettgeri*	*aadA1*, *aph(3′)-Ia*, ***bla*$_{NDM-1}$**, *sul2*, *sul1*, *tet(B)*, *dfrA1*	KX832927	This study
p16Pre36-2	43,191	Not assigned	*P. rettgeri*	*aac(3)-IIa*, *bla*$_{TEM-1B}$	KX832926	This study
pRB151-NDM	108,417	Not assigned	*P. rettgeri*	***bla*$_{NDM-1}$**	CP017672	Marquez-Ortiz et al. (2017)
p06-1619-NDM	54,712	Not assigned	*P. rettgeri*	*aph(3′)-VIa*, ***bla*$_{NDM-1}$**	KX832928	This study
p06-1619-2	90,666	Not assigned	*P. rettgeri*	No resistance genes	KX832929	This study
pPrY2001	113,295	Not assigned	*P. rettgeri*	*aph(3′)-VIa*, *armA*, *aacA4*, ***bla*$_{NDM-1}$**, *aac(6′)Ib-cr*, *mph(E)*, *msr(E)*, *sul1*	NC_022589	Mataseje et al. (2014)

Note.—Plasmids sequenced in this study are shown in bold letters. The bla_{NDM-1}-positive pPrY2001 plasmid reported previously in a *P. rettgeri* from Canada was also included.

Finally, the phylogenetic tree was created using RAxML version 8.2.9 (Stamatakis 2014) running 1,000 bootstrap replicates under the generalized time reversible model (GTRCAT). Finally, the consensus tree was plotted using Dendroscope (Huson and Scornavacca 2012). Branch lengths are expressed in units of changes/nucleotide position (scale bar).

Complete Plasmid Sequencing

Total DNA was extracted from three representative *P. rettgeri* isolates (16Pre36, RB151, and 06-1619) using the UltraClean® Microbial DNA Isolation Kit (Mo Bio Laboratories, Inc.). BluePippin (Sage Science) 20-kb size-selected libraries were prepared, then sequenced using one SMRT cell each on the PacBio RS II platform (Pacific Biosciences) using P6-C4 chemistry. Sequencing reads were processed and de novo assembled using the HGAP 3 program of SMRT Analysis v2.3 (Chin et al. 2013) with default parameters. To check the assemblies, the filtered PacBio subreads were mapped to the genome assemblies using BWA-MEM (http://bio-bwa.sourceforge.net/bwa.shtml). The assembly was visually inspected and manually verified using Tablet v1.15.09.01 (Milne et al. 2013). Misassembled terminal repeat overlap sequences, known to be an error of the HGAP assembly of circular molecules (Chin et al. 2013; Hunt et al. 2015), were identified and subsequently trimmed manually. Circularization results were verified using Circlator (Hunt et al. 2015), confirming that the manual assembly correction correlated with the automated method. The complete sequences of five plasmids were confirmed: two plasmids for the strain 16Pre36, one plasmid for strain RB151 and two plasmids for strain 06-1619 (table 1). As the sequence start point of assemblies are arbitrary, the position one of each plasmid was shifted according to the *repA* gene (pRB151-NDM and p16Pre36-2), pPrY2001 (p16Pre36-NDM and p06-1619-2) or pNDM-BJ01 (p06-1619-NDM) to facilitate comparative genomics. The plasmids were annotated using Prokka v1.11 (Seemann 2014) and manual curation of the automated annotation was facilitated using Artemis (Rutherford et al. 2000). Antibiotic resistance genes were identified using ARIBA (https://github.com/sanger-pathogens/ariba/wiki) and insertion sequence (IS) elements and transposons (Tn) were identified using ISfinder (Siguier et al. 2012) and BLASTn (Altschul et al. 1990). Presence of class 1, 2 or 3 integrons was determined in silico using the primers reported by Marquez et al. (2008).

Comparative Genomics

We used mapping of consensus data from the MiSeq libraries to explore our set of samples for the presence (or residues) of Colombian and Mexican bla_{NDM-1}-positive sequenced plasmids (table 1) and other related bla_{NDM-1}-positive (pPrY2001 and pNDM-BJ01) and bla_{NDM-1}-negative (p06-1619-2) plasmids. For use in the mapping consensus, a reference database was generated using the concatenated complete sequence of the plasmids p6234-178kb, p16Pre36-NDM, pRB151-NDM, p06-1619-NDM, p06-1619-2, pPrY2001 and pNDM-BJ01, broken in fragments of 300 bp (x axis). This reference database was mapped with SHRiMP2 (David et al. 2011) and Nesoni (https://github.com/Victorian-Bioinformatics-Consortium/nesoni) against the total MiSeq reads from each sample (y axis). The presence of $\geq$ 90% nucleotide identity when comparing each 300-bp window from the reference plasmids against the consensus generated from MiSeq reads was determined and visualized as black blocks using SeqFindR (http://github.com/mscook/seqfindr). It was included as internal control MiSeq simulated reads to the reference plasmids, generated with ART read simulator (Huang et al. 2012). Pairwise plasmid comparisons, verification of SeqFindR results and figures were performed by using BLASTn

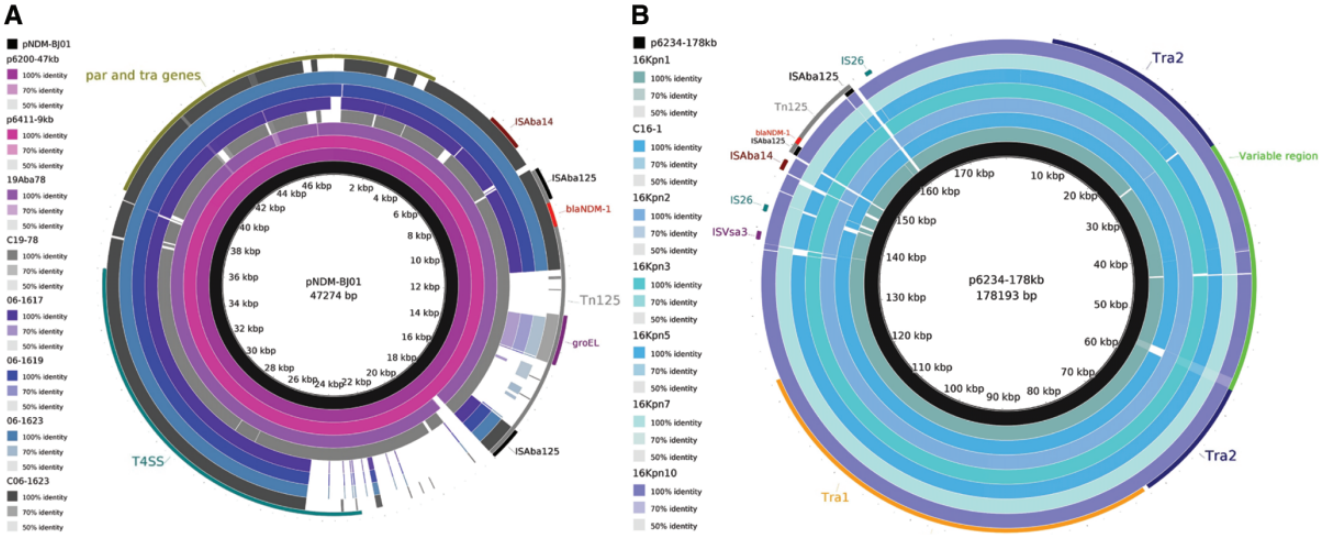

Fig. 1.—*bla*$_{NDM-1}$-plasmids circulating among *Acinetobacter baumannii* and *Klebsiella pneumoniae* in Colombia. (*A*) BLASTn comparison of WGS assemblies of *A. baumanni* 19Aba78 from this study and its *Escherichia coli* transconjugant C19-78, the *Providencia rettgeri* NDM-positive isolates from Mexico (06-1617, 06-1619 and 06-1623) and the *E. coli* transconjugant C06-1623 against the plasmid pNDM-BJ01. Also were included the plasmids p6200-47kb and p6411-9kb previously reported in Colombia. (*B*) BLASTn comparison of WGS assemblies of *K. pneumoniae* isolates from this study and the *E. coli* transconjugant C16-1 against the plasmid p6234-178kb. Regions Tra1 and Tra2 common to IncA/C2 plasmids are highlighted (Fernandez-Alarcon et al. 2011); the Tra2 region is disrupted by a variable region. Black circles correspond to the reference plasmids p6234-178kb (*A*) and pNDM-BJ01 (*B*), included as internal control.

(Altschul et al. 1990), ACT (Carver et al. 2005), Easyfig (Sullivan et al. 2011), and BRIG (Alikhan et al. 2011).

Results

The *bla*$_{NDM-1}$ Gene Is in a Conjugative Element in the Colombian and Mexican Isolates

From a surveillance study in Colombia a total of 12 NDM-positive isolates were collected from 12 patients (one NDM-positive strain per patient). Among these 12 isolates, which were mostly from outpatients with wound or urinary tract infections, 11 were *P. rettgeri* (five from Bucaramanga and six from Bogota) and one was *A. baumannii* isolated in Cali (supplementary data set 1, Supplementary Material online). Additionally, six *K. pneumoniae* and three *P. rettgeri* from previously reported (Barrios et al. 2013; Escobar Perez et al. 2013) clinical outbreaks in a neonatal unit in Bogota (Colombia) and an intensive care unit in Monterrey (Mexico), respectively, were also included in this study. The *K. pneumoniae* and *P. rettgeri* from Bogota were isolated in the same hospital (supplementary data set 1, Supplementary Material online). Analyses of PCR products confirmed the *bla*$_{NDM-1}$ variant was present in all isolates. Thus, in total, 21 NDM-1-positive isolates from Colombia and Mexico were available to investigate in this study.

To explore if the *bla*$_{NDM-1}$ gene in the Latin American isolates could be transferred between bacteria, conjugation experiments were performed using six representative NDM-1-positive isolates as donor strains: one representing the *K. pneumoniae* from Bogota, one the *A. baumannii* from Cali, one the *P. rettgeri* from Bogota, two representing the *P. rettgeri* from Bucaramanga and one the *P. rettgeri* from Mexico, with the sodium azide-resistant *Escherichia coli* strain J53 used as the recipient strain. *Escherichia coli* NDM-1-positive transconjugants were obtained from all six of the donor strains (supplementary data set 1, Supplementary Material online). These results indicate that the *bla*$_{NDM-1}$ gene is located in a conjugative element in the Colombian and Mexican isolates, and that it can be transferred to other strains allowing dissemination within and between genera. To determine the relationship among the conjugative *bla*$_{NDM-1}$-positive genetic structures and among the NDM-1-positive strains circulating in Latin America WGS was performed on the set of 21 Latin American NDM-1-positive clinical isolates as well as the six NDM-1-positive *E. coli* transconjugants (supplementary data set 1, Supplementary Material online).

Acinetobacter baumannii 19Aba78 Harbors *bla*$_{NDM-1}$ within a pNDM-BJ01-Like Plasmid

The WGS assembly of *A. baumannii* isolate 19Aba78 from Cali, Colombia, had 100% nucleotide identity over 99% of the pNDM-BJ01 length (fig. 1*A* and supplementary data set 2, Supplementary Material online), suggesting that *bla*$_{NDM-1}$ is located on a pNDM-BJ01-like plasmid, as has been broadly reported in *Acinetobacter* spp. (Hu et al. 2012;

Sun et al. 2013; Espinal et al. 2015). The 19Aba78 pNDM-BJ01-like plasmid assembled into seven contigs, so the location of all these contigs together on a single plasmid cannot be confirmed from the current assembly. Of note however, bla_{NDM-1} is located within Tn*125* adjacent to plasmid sequences that are identical to pNDM-BJ01, on a single contig in the 19Aba78 assembly (see supplementary fig. S1A, Supplementary Material online), supporting the presence of bla_{NDM-1} on a pNDM-BJ01-like plasmid. Additionally, the *E. coli* transconjugant C19-78 allowed confirmation of bla_{NDM-1} located on a pNDM-BJ01-like plasmid (fig. 1*A* and supplementary data set 2, Supplementary Material online). All contigs that mapped to the genome sequence of the recipient strain, *E. coli* J53 (accession AICK00000000), were removed from the WGS assembly of the transconjugant C19-78, and all remaining contigs were found to map to pNDM-BJ01, confirming the pNDM-BJ01-like plasmid harbored by C19-78 had no insertions or additional sequences (see supplementary fig. S1A, Supplementary Material online).

Two other NDM-1-positive strains of *Acinetobacter* spp., isolated from other Colombian cities (Neiva and Pasto, 520 km away from each other, and 320 and 390 km away from Cali, respectively) were recently reported (Rojas et al. 2016). The bla_{NDM-1}-positive plasmids from these strains, p6200-47kb and p6411-9kb were also found to be pNDM-BJ01-like, each with 99% nucleotide identity over 100% of the pNDM-BJ01 length (fig. 1*A* and supplementary data set 2, Supplementary Material online). In contrast to the similarity between the bla_{NDM-1}-positive plasmids found in the three Colombian NDM-1-positive strains of *Acinetobacter* spp., the strains themselves were of different, unrelated sequence type (ST). The Neiva *A. baumannii* (harboring p6200-47kb) was ST322 and the Pasto *A. nosocomialis* (harboring p6411-9kb) was ST464 (Rojas et al. 2016), but the Cali *A. baumannii* isolate 19Aba78 belongs to ST239, as determined by the Pasteur MLST scheme for *A. baumannii* (Diancourt et al. 2010). These observations of the 19Aba78 isolate and the *E. coli* transconjugant C19-78 thus contribute further evidence of dissemination of closely related pNDM-BJ01-like plasmids among unrelated *Acinetobacter* spp. isolates, as has been reported in Asia and Latin America (Hu et al. 2012; Sun et al. 2013; Waterman et al. 2013; Zhang et al. 2013; Wang et al. 2014; Brovedan et al. 2015; Espinal et al. 2015; Feng et al. 2015; Jones et al. 2015; Li et al. 2015; Rojas et al. 2016), and also its capability to transfer to *Enterobacteriaceae*.

The bla_{NDM-1}-Positive Plasmids Circulating in *K. pneumoniae* from Colombia Are Closely Related IncA/C2 Plasmids

Analysis of the WGS assemblies for the six *K. pneumoniae* isolates showed they all have sequences with high similarity to p6234-178kb (>99% nucleotide identity over >98% of the p6234-178kb length, fig. 1*B* and supplementary data set 2, Supplementary Material online), an IncA/C2 plasmid harboring bla_{NDM-1} from a recently reported *K. pneumoniae* isolated in Neiva (Rojas et al. 2016), which is 300 km away from Bogota in Colombia. It therefore seems likely that the bla_{NDM-1} gene is located on a closely related conjugative plasmid in all seven of these Colombian *K. pneumoniae* strains. Despite the plasmid similarities, the *K. pneumoniae* strains from Bogota (16Kpn1, 16Kpn2, 16Kpn3, 16Kpn5, 16Kpn7 and 16Kpn10) are all ST1043 (Escobar Perez et al. 2013), and not related to the ST392 of the *K. pneumoniae* isolate from Neiva (Rojas et al. 2016). The sequences that mapped to p6234-178kb, assembled into several contigs for each of the six Bogota *K. pneumoniae* strains (supplementary data set 1, Supplementary Material online), so the order of the contigs and their genomic location could not be determined. However, the WGS assembly of the NDM-1-positive *E. coli* transconjugant C16-1, obtained using *K. pneumoniae* strain 16Kpn1 as the donor, also showed 99% nucleotide identity over 98% of the length of p6234-178kb (fig. 1*B* and supplementary data set 2, Supplementary Material online), reinforcing the evidence that bla_{NDM-1} is likely to be located on such a related conjugative plasmid in the Bogota outbreak. To rule out the possibility that any related sequences were located in the recipient strain, all contigs that mapped to the chromosome of *E. coli* J53 were removed from the genome assembly of the transconjugant C16-1, and all remaining contigs were found to map to p6234-178kb, with no additional sequences relative to p6234-178kb (see supplementary fig. 1*B*), and only one confirmed difference due to the absence of an IS*5075* element. Thus, this data demonstrates that highly related NDM-1-positive IncA/C2 conjugative plasmids are circulating among *K. pneumoniae* with different genetic backgrounds (ST392 and ST1043) in two distant cities in Colombia.

Providencia rettgeri from Colombia and Mexico Harbor bla_{NDM-1} in Different Not Reported Plasmids

Comparison of the WGS assemblies of the Colombian *P. rettgeri* isolates against the database of complete bla_{NDM-1}-positive plasmid sequences (supplementary data set 2, Supplementary Material online), revealed that there were no matches over the full length of any known plasmid. The most closely related plasmid was the Inc group unclassified pPrY2001, from a Canadian *P. rettgeri* strain (Mataseje et al. 2014), with >99% nucleotide identity over 69–77% of the length of pPrY2001 (supplementary data set 2 and fig. S2, Supplementary Material online). By way of exception, 16Pre47 (isolated in Bogota) and RB152 (isolated in Bucaramanga) had significant nucleotide identity over only 22% and 15% of the length of pPrY2001, respectively (supplementary data set 2, Supplementary Material online). All four NDM-1-positive *E. coli* transconjugants derived from *P. rettgeri* donors, regardless of the relationship of the respective donor strain with the pPrY2001 plasmid, had sequences that

matched to only a small section of pPrY2001 (<15% of the pPrY2001 length covered with >99% nucleotide identity). The main region of identity to pPrY2001 for all four transconjugants, as well as for 16Pre47 and RB152, was limited to the Tn*125* remnant (see supplementary fig. S2, Supplementary Material online).

The *P. rettgeri* isolates from Colombia also had significant sequence matches with the IncA/C2 plasmid p6234-178kb (supplementary data set 2, Supplementary Material online), and in general to all the IncA/C2 NDM-positive plasmids, with >99% nucleotide identity over ~50% of the length of IncA/C2 plasmids (supplementary data set 2, Supplementary Material online). However, the key characteristics of IncA/C2 plasmids were not found in the *P. rettgeri* genome assemblies, as neither the *repA* gene, which is highly conserved among IncA/C2 plasmids, nor any marker for known incompatibility groups (except 16Pre46 with an IncN match), could be identified (see supplementary fig. S3, Supplementary Material online). Furthermore, the corresponding representative *E. coli* transconjugant(s) of *P. rettgeri* isolates from Bogota and Bucaramanga differed in their sequence coverage of IncA/C2 plasmids. *Providencia rettgeri* isolates from Bogota had >99% nucleotide identity over ~65% of the length of p6234-178kb (except 16Pre45 with identity to only 28% of the length), as did the corresponding representative *E. coli* transconjugant C16-36, suggesting the bla_{NDM-1}-positive plasmids circulating in Bogota in *P. rettgeri* and in *K. pneumoniae* may be related. By contrast, although the *P. rettgeri* isolates from Bucaramanga had >99% nucleotide identity over ~60% of the length of p6234-178kb (except RB152 with only 26% coverage), the corresponding representative *E. coli* NDM-1-positive transconjugants CRB151 and CRB152 mapped just to 9% of p6234-178kb (supplementary data set 2, Supplementary Material online), the 9% associated with just the Tn*125* remnant. Thus, the high relatedness to the IncA/C2 plasmids observed in the donor strains but not the transconjugants, suggests that the Bucaramanga *P. rettgeri* isolates probably have the bla_{NDM-1} gene located in a plasmid that is unrelated to p6234-178kb, as well as a bla_{NDM}–negative structure that is related to the bla_{NDM-1}-positive plasmid circulating among *P. rettgeri* and *K. pneumoniae* in Bogota.

Interestingly, the Mexican *P. rettgeri* isolates (06-1617, 06-1619 and 06-1623) and the corresponding representative *E. coli* transconjugant (C06-1623) showed a high level of similarity to the *Acinetobacter* spp. pNDM-BJ01-like plasmids (>99% of nucleotide identity over 74–80% of the length of pNDM-BJ01; fig. 1*A* and supplementary data set 2, Supplementary Material online). This suggests that a pNDM-BJ01-like plasmid harboring bla_{NDM-1} is circulating among the *P. rettgeri* from Mexico, but with a truncated Tn*125* and downstream deletion, relative to pNDM-BJ01 (fig. 1*A*). Additionally, the Mexican *P. rettgeri* isolates showed close relatedness to the pPrY2001 plasmid (>99% of nucleotide identity over 64–76% of the length of pPrY2001); however,

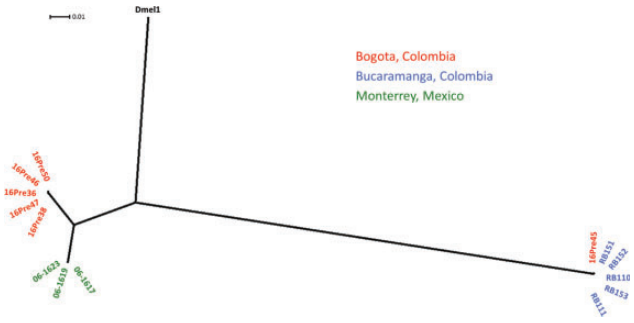

Fig. 2.—Phylogenetic tree of *Providencia rettgeri* isolates based on core-genome SNPs. A Maximum Likelihood (ML) tree was built based on the SNPs in the core-genome assemblies of the NDM-1-positive *P. rettgeri* strains reported in this study (red, blue and green) with the *P. rettgeri* Dmel1 included as an outgroup control. Branch lengths are expressed in units of changes/nucleotide position (scale bar).

the C06-1623 *E. coli* transconjugant did not. It mapped to only 11% of pPrY2001, which corresponded to the Tn*125* remnant (see supplementary fig. S2, Supplementary Material online), suggesting that there is another bla_{NDM}-negative genetic structure in the Mexican *P. rettgeri* that is related to pPrY2001. No known Inc group was identified in any of the genomes for the *P. rettgeri* isolates from Mexico (except 06-1623, with a match to the IncT group), as is also the case for pNDM-BJ01 (see supplementary fig. S3, Supplementary Material online), further supporting the evidence that bla_{NDM-1} is located on a pNDM-BJ01-like plasmid in these strains.

Phylogenetic Concordance with the Geographic Origin of *P. rettgeri*

To investigate the genetic relationship in the *P. rettgeri*, the major NDM-1-positive pathogen identified in the Colombian clinical surveillance (supplementary data set 1, Supplementary Material online), we used the WGS assemblies to build a phylogenetic tree based on the core genome SNPs among the 14 NDM-1-positive *P. rettgeri* isolates included in this study, and found that all but one of the isolates clustered according to the city of origin (fig. 2). The exceptional isolate, 16Pre45, clustered together with the strains from Bucaramanga, even though it was isolated in Bogota. Despite the genetic relationship, no epidemiological link with the Bucaramanga region was identified for the patient harboring 16Pre45 (supplementary data set 1, Supplementary Material online). These results suggest that bla_{NDM-1} dissemination in *P. rettgeri* in Colombia and Mexico is following a clonal behavior according to the geographic origin.

General Features of the Complete bla_{NDM-1}-Positive Plasmids from Latin American *P. rettgeri*

The complete sequences of plasmids from one representative isolate for each of the three *P. rettgeri* clusters (16Pre36 from

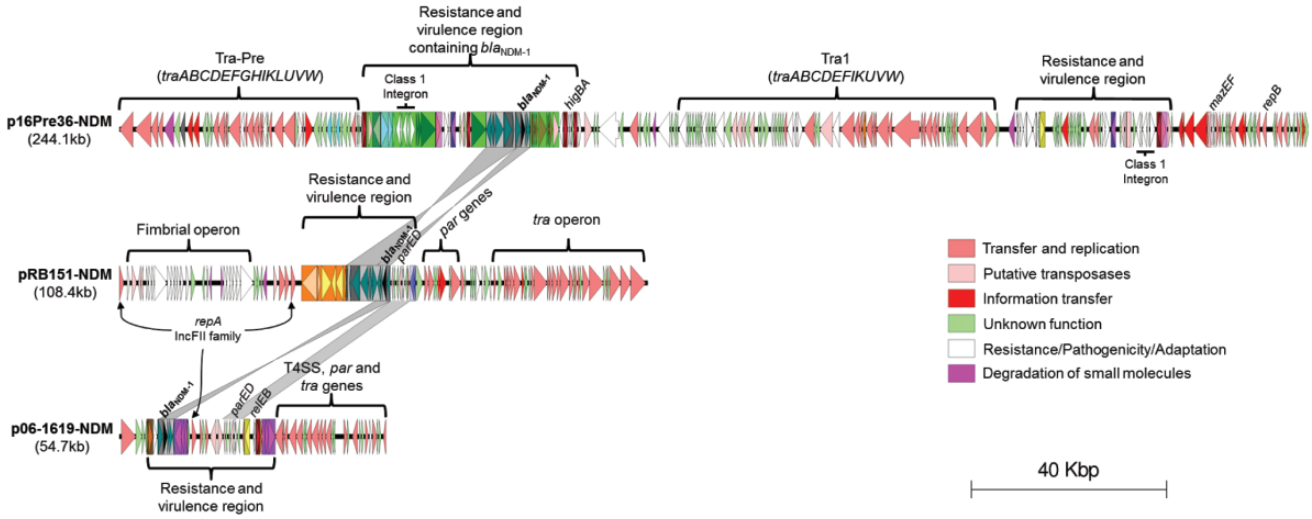

Fig. 3.—General description of the bla_{NDM-1}-positive plasmids from three representative *Providencia rettgeri* isolates sequenced in this study, including regions encoding genes for transposition and for replication, and virulence and resistance regions. Plasmid p16Pre36-NDM has two tra regions, one reported only for a *P. rettgeri* isolate (Tra-Pre) and the other reported in different IncA/C2 plasmids (Tra1). Plasmids pRB151-NDM and p06-1619-NDM have putative *repA* genes belonging to the IncFII family. Insertion sequences or transposons are shown as rectangles containing their respective CDS for transposition and accessory genes. Gray bars between pairs of sequences indicates >90% nucleotide identity in a window of 400 bp. The scale bar indicates sequence length.

Bogota, RB151 from Bucaramanga and 06-1619 from Monterrey), were obtained by PacBio sequencing. The number of plasmids in each strain varied: 16Pre36 had two plasmids (p16Pre36-NDM and p16Pre36-2), RB151 had one plasmid (pRB151-NDM) and 06-1619 had two plasmids (p06-1619-2 and p06-1619-NDM) (table 1). Among these representative *P. rettgeri* isolates, the bla_{NDM-1} gene was located on unrelated plasmids (fig. 3). None of the plasmids belonged to any reported incompatibility group using Carattoli et al. (2014) schemes (see supplementary fig. S3, Supplementary Material online), however, annotation of the plasmids pRB151-NDM and p06-1619-NDM, showed they have two and one *repA* genes, respectively (fig. 2), which are closely related to each other (>85% nucleotide identity over the full *repA* sequence). The putative plasmid replication proteins encoded by these *repA* genes each had a significant match to the IncFII RepA protein family in Pfam (Finn et al. 2016). However, these three *repA* genes showed a poor relation (<51% nucleotide identity over the full *repA* sequence) to the reported IncFII *repA* genes (Carattoli et al. 2014), and plasmids pRB151-NDM and p06-1619-NDM are not related to any reported IncFII plasmid (not shown). In p06-1619-NDM the *repA* gene is located inside a putative mobile element, which possibly brought *repA* from another plasmid (fig. 3).

Plasmid p16Pre36-NDM was found to encode two different putative conjugative transfer-associated regions (fig. 3). One (described here as Tra-Pre), has so far only been reported in the bla_{NDM-1}-positive plasmid pPrY2001; the other, is a common transfer-associated region of IncA/C2 plasmids described as Tra1 by Fernandez-Alarcon et al. (2011), which is frequently found in widely disseminated plasmids in a broad host range (Sekizuka et al. 2011; Doublet et al. 2012; Diene et al. 2013; Tijet et al. 2015; Wang et al. 2015; Wasyl et al. 2015; Rojas et al. 2016), including the IncA/C2 p6234-178kb-like bla_{NDM-1}-positive plasmids in the Colombian *K. pneumoniae* strains (supplementary data set 1, Supplementary Material online). At 244,116 bp, p16Pre36-NDM is the largest and most variable of the bla_{NDM-1}-positive plasmids sequenced in this study, and among the largest bla_{NDM-1}-positive plasmids ever reported (supplementary data set 2, Supplementary Material online). It has two resistance regions each containing a toxin–antitoxin system and a class 1 integron. Both class 1 integrons have the genes *dfrA1-aadA1-qacEΔ1-sul1* associated with resistance to quaternary ammonium compounds, aminoglycosides, sulphonamides and trimethoprim. This plasmid also carries the additional resistance genes *aph(3′)-Ia*, *sul2* and *tet(B)*. One of the resistance regions contains a Tn*125* remnant (with its bla_{NDM-1} gene intact) inside a shuffled Tn*21* element, with the two Tn*21* inverted repeats flanking the Tn*125* remnant (fig. 4). This shuffled Tn*21* element has its typical components IS*1353*, IS*1326*, the *mer* operon and a class 1 integron (Liebert et al. 1999), but they are rearranged and the *mer* operon is separated from the rest of the Tn*21* by the Tn*125* remnant (fig. 4). Tn*21*-like elements are implicated in the global dissemination of antibiotic resistance genes among *Enterobacteriaceae* and *Pseudomonas* (Liebert et al. 1999) and have been reported to generate mosaic structures (Yurieva et al. 1997; Noguchi et al. 2000; Partridge et al. 2001; Valverde et al. 2006). Moreover, the ΔTn*125* (having just one copy of the IS*Aba125* element) surrounding bla_{NDM-1} in p16Pre36-NDM has suffered a rearrangement that has not been previously reported: the genes

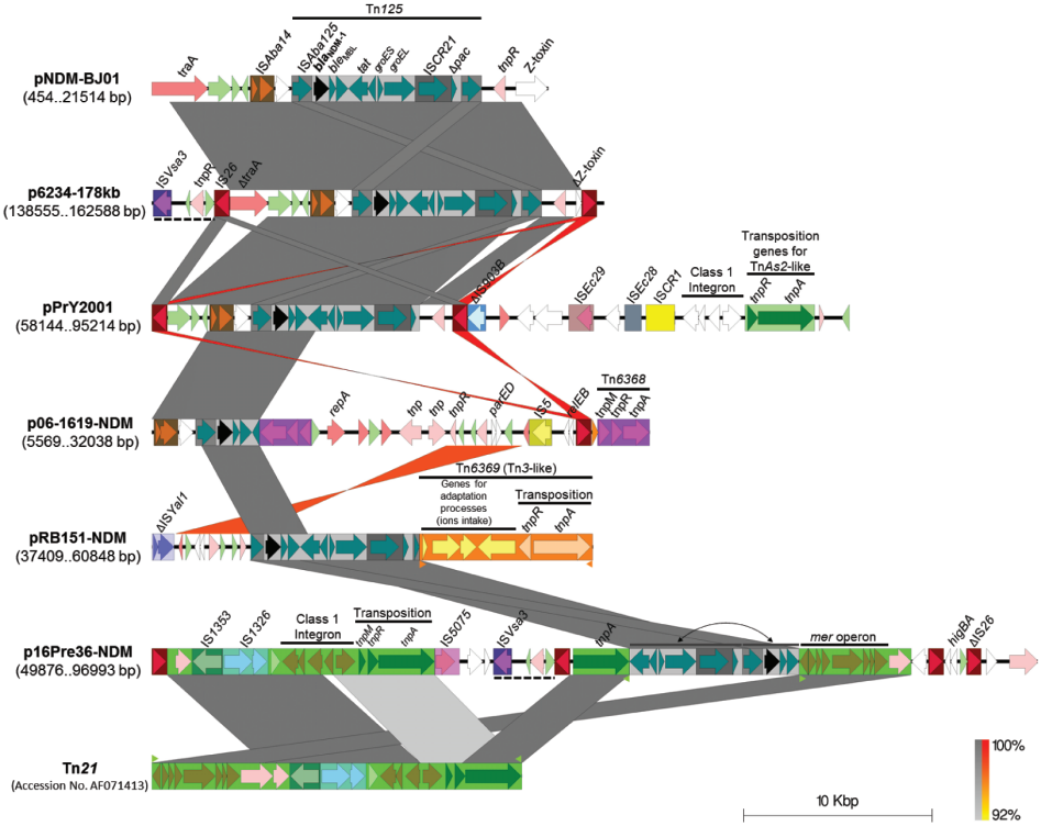

Fig. 4.—Comparison of variable bla_{NDM-1}-containing regions from plasmids p16Pre36-NDM, pRB151-NDM, p06-1619-NDM, p6234-178kb, pPrY2001 and pNDM-BJ01. Insertion sequences or transposons are shown as rectangles containing their respective CDS for transposition and accessory genes in different colors. Outside orange and green triangles correspond to the inverted repeats of a putative Tn*3*-like (Tn*6369*) and a Tn*21*, respectively. The prototype sequence of the Tn*21* was included (Liebert et al. 1999). Dashed lines indicate the 2,928-bp sequence containing IS*Vsa3* carried by p6234.178kb (once) and p16Pre36-NDM (twice). Gray and red (inverted matches) shading between pairs of sequences indicates >90% of nucleotide identity in a window of 400 bp. The scale bar indicates sequence length.

tat-dct-groES-groEL-IS*CR21*-Δ*pac* that are found upstream of the bla_{NDM-1} in p16Pre36-NDM, have always been previously reported downstream (fig. 4). These odd rearrangements of the ΔTn*125* and the Tn*21* in p16Pre36-NDM were confirmed by mapping of the PacBio reads to the assembled plasmid (no regions of low read coverage or quality were found that could suggest an assembly issue); by investigation of the MiSeq assembly (the same rearrangement was identified on a single contig, see supplementary fig. S4A, Supplementary Material online); and by using PCR to confirm the location of the ΔTn*125* inside the Tn*21* (see supplementary fig. S4B, Supplementary Material online). The entire ΔTn*21* region in p16Pre36-NDM is flanked upstream and downstream by copies of IS*26* (fig. 4), thus it is possible that this entire region of DNA may be mobilized as a composite transposon. IS*26* intramolecular replicative transposition has been previously identified as the source of reorganization of plasmids carrying multidrug-resistant determinants as could have happened here (He et al. 2015).

Compared with p16Pre36-NDM the pRB151-NDM (from Bucaramanga, Colombia) is a less complex plasmid, with a size of 108,417 bp. This is a novel plasmid backbone, unrelated to any previously reported (without any significant match against the NCBI nucleotide database), that encodes a putative conjugative transfer machinery, plasmid replication and partition proteins, a restriction-modification system and a putative fimbrial operon (fig. 3). It possesses only one resistance and virulence region, and that contains the bla_{NDM-1} gene as the plasmid's only antibiotic resistance determinant (table 1). The variable region has a ΔTn*125* harboring the bla_{NDM-1} with a typical structure except that the two flanking IS*Aba125* are both truncated (fig. 4). Downstream of the ΔTn*125* in the resistance region is a novel transposon, registered as Tn*6369* in the Tn Number Registry (Roberts et al. 2008), whilst upstream there is another putative mobile element (or its remains) encoding two putative transposon resolvases and a ParED toxin–antitoxin system that is also present in the p06-1619-NDM plasmid, plus an upstream ΔIS*Yal1* (fig. 4).

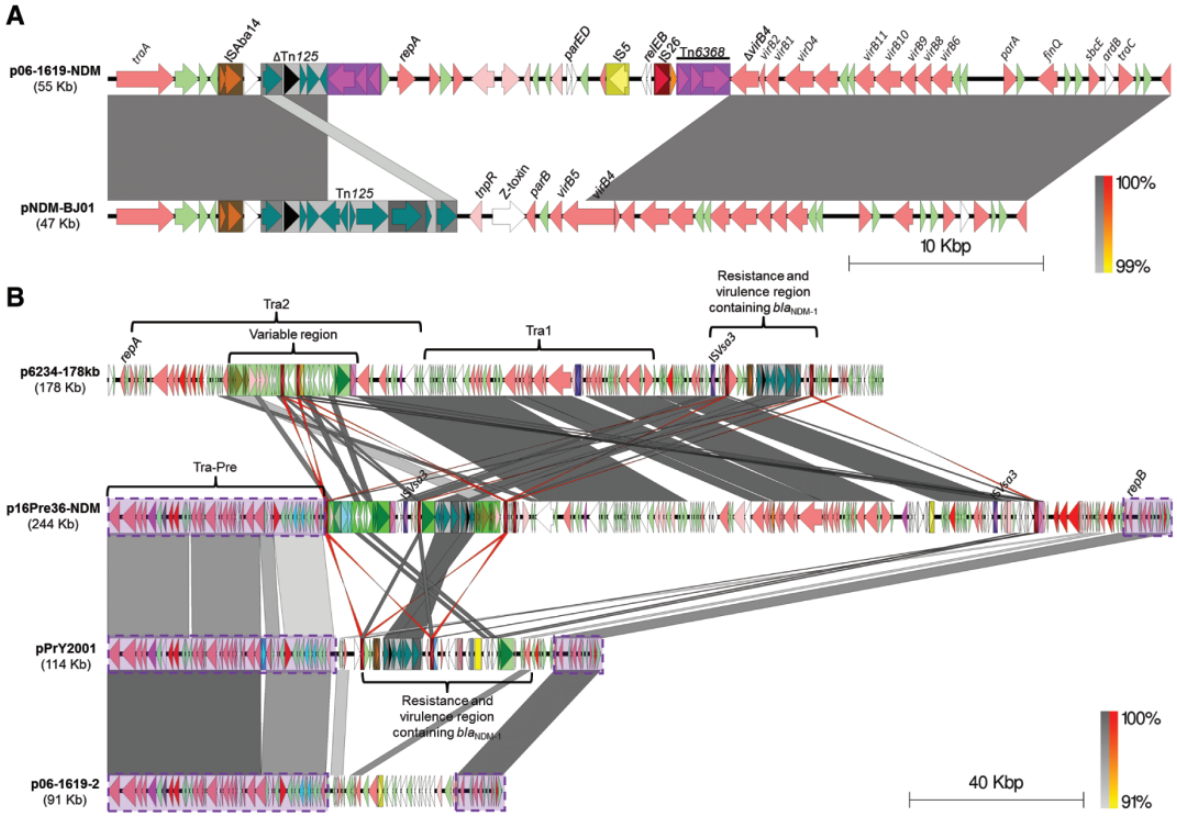

Fig. 5.—BLASTn comparison of (*A*) pNDM-BJ01 with the related p06-1619-NDM plasmid from *Providencia rettgeri*, and (*B*) pPrY-like plasmids (p16Pre36-NDM, pPrY2001 and p06-1619-2) from *P. rettgeri* with the IncA/C2 related p6234-178kb plasmid reported in *Klebsiella pneumoniae*. Conserved pPrY-like region is highlighted in purple rectangles with dashed lines. Gray and red (inverted matches) shading between pairs of sequences indicate >90% of nucleotide identity in a window of 400 bp. The scale bar indicates sequence length.

The p06-1619-NDM plasmid (from Mexico) has a size of 54,712 bp, and belongs to the conserved pNDM-BJ01-like family of plasmids (from *Acinetobacter* spp.), with the same backbone (99% nucleotide identity over 74% of the pNDM-BJ01 length, fig. 5*A*). However, it has a putative mobile genetic element (MGE) flanked by identical copies of a novel transposon with intact transposition genes, registered as Tn*6368*, that has inserted downstream of the bla_{NDM-1} gene. The insertion of this MGE, has truncated the Tn*125* after the *tat* gene upstream (fig. 4), as well as deleted the genes *parB* and *virB5* and truncated *virB4* downstream, so truncating the T4SS locus (fig. 5*A*). These genes are implicated in the mating pair formation and DNA partitioning process (Christie et al. 2005; Schumacher and Funnell 2005; Kusiak et al. 2011). Their loss in this plasmid may not affect its conjugation capability given that in this study we were able to obtain an *E. coli* transconjugant from a Mexican *P. rettgeri* strain harboring the p06-1619-NDM plasmid (fig. 1*A* and supplementary fig. S1E, Supplementary Material online); although, it is also possible that the other plasmid in this strain, p06-1619-2, which contains the putative *P. rettgeri* conjugation machinery (Tra-Pre), could act as a helper for the conjugation process, as has been reported for other antibiotic resistance plasmids (Dery et al. 1997; Bennett 2008; Al-Marzooq et al. 2015). As well as the deletions described here, insertion of the putative novel mobile element has given the plasmid two toxin–antitoxin systems (*parED* and *relEB*), *repA* and the IS elements IS*5* and IS*26* (fig. 5*A*).

Pairwise comparisons show there is no relationship between the three different *P. rettgeri* bla_{NDM-1}-positive plasmids circulating in Colombia and Mexico, apart from the Tn*125* remains and the presence of multiple copies of the IS*26* element (fig. 3). Only the p16Pre36-NDM plasmid shows some relationship with p6234-178kb-like plasmids found in Colombian *K. pneumoniae* strains (supplementary data set 2, Supplementary Material online, and fig. 5*B*). Both p16Pre36-NDM and p6234-178kb share the same Tra1 region, although the Tn*125* (harboring bla_{NDM-1}) is located within a different genetic context (figs. 4 and 5*B*). These similarities explain the genetic relationship found among the bla_{NDM-1}-positive IncA/C2 plasmids and the *P. rettgeri* from Bogota (supplementary data set 2, Supplementary Material online). However, p6234-178kb does not have the Tra-Pre region found in p16Pre36-NDM, and has a more complex antibiotic resistance gene profile (table 1) due to the presence

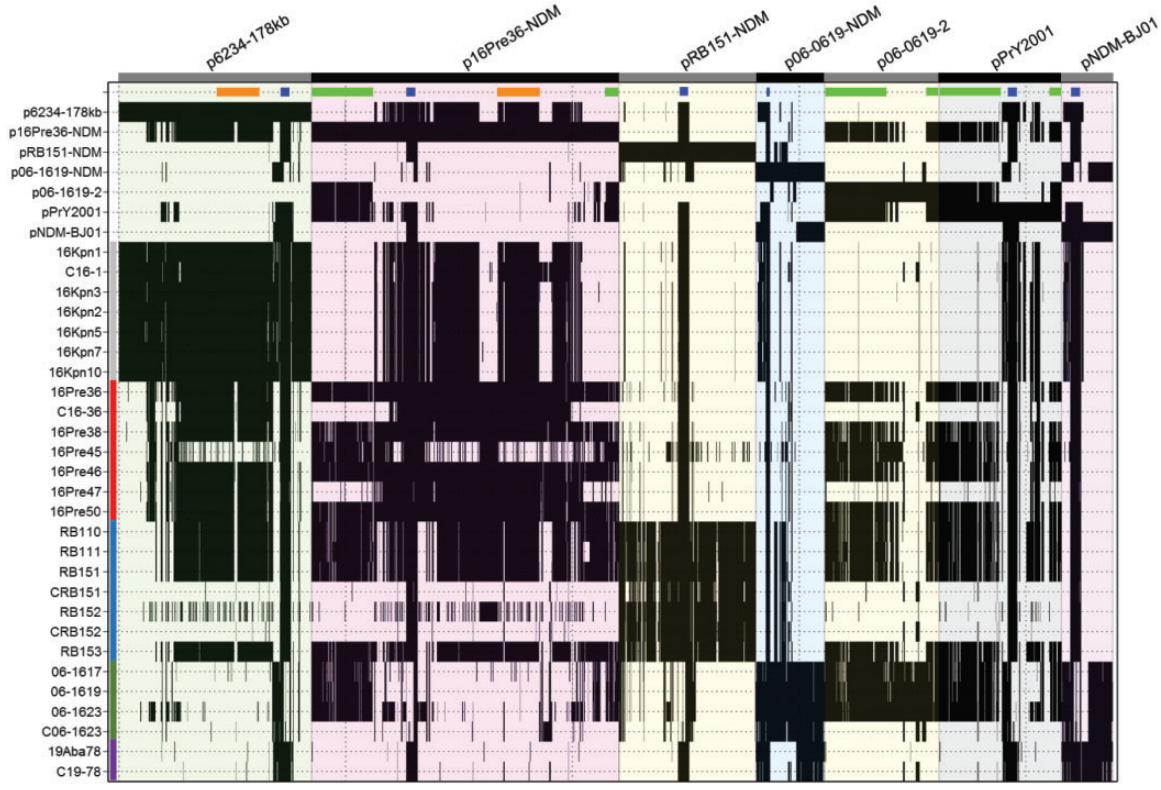

Fig. 6.—Presence of bla_{NDM-1}-plasmids in the clinical isolates and transconjugants. Complete sequence of *bla*NDM-1-plasmids circulating among Colombian and Mexican NDM-1-positive isolates and some related bla_{NDM-1}-positive (pPrY2001 and pNDM-BJ01) and bla_{NDM}-negative (p06-1619-2) plasmids are shown along the *x* axis. Black shading indicates a match of ≥90% nucleotide identity in a window of 300 bp, calculated by comparing the query sequence (*x* axis, reference plasmids) against the consensus from mapped reads for each strain (*y* axis). Horizontal orange and light green bars represent the Tra1 and pPrY-like regions, respectively; blue rectangles represent the Tn*125* (or remnants) region in each plasmid. Vertical bars correspond to: gray for *Klebsiella pneumoniae* from Bogota (Colombia), red for *Providencia rettgeri* from Bogota (Colombia), blue for *P. rettgeri* from Bucaramanga (Colombia), green for *P. rettgeri* from Monterrey (Mexico), purple for *Acinetobacter baumannii* from Cali (Colombia). Simulated reads for the reference plasmids were included as an internal control.

of an additional class 1 integron containing most of those genes.

Surprisingly, despite no previously reported epidemiological connection, a genetic link between *P. rettgeri* strains from Colombia, Mexico and Canada was found through analysis of the plasmids the strains harbor. We found the putative *P. rettgeri* conjugation machinery (Tra-Pre) and some additional regions, first identified in pPrY2001 from the Canadian *P. rettgeri* strain 09ACRGNY2001, were also present in the bla_{NDM}-negative p06-1619-2 plasmid from Mexico and in the bla_{NDM-1} positive p16Pre36-NDM plasmid from Bogota, Colombia (fig. 5*B*).

Genomic Epidemiology of the bla_{NDM-1}-Positive Plasmids Circulating in Colombia and Mexico

To gain a better understanding of the dissemination dynamics of bla_{NDM-1}—positive plasmids in Latin America, the MiSeq sequencing reads for all isolates included in the study were mapped against the fully sequenced bla_{NDM-1}-positive plasmids from the representative Colombian and Mexican strains, as well as the related bla_{NDM-1}-positive plasmids pNDM-BJ01, pPrY2001 and the bla_{NDM}-negative p06-1619-2 (fig. 6).

The reads from four out of six *P. rettgeri* strains from Bogotá were found to map well over the entire sequence of p16Pre36-NDM (fig. 6), so are likely have bla_{NDM-1} in a plasmid closely related to p16Pre36-NDM. Of the remaining two *P. rettgeri* strains from Bogota, one, 16Pre47, lacked the Tra-Pre locus and the mapped reads covered only one section of the p16Pre36-NDM sequence, suggesting that 16Pre47 carries a much smaller variant of that plasmid; the other, 16Pre45, is positive for the Tra-Pre region, but has no reads mapping to several other regions of p16Pre36-NDM, so it is possible that it has a pPrY-like plasmid or an unrelated plasmid harboring the bla_{NDM-1} gene. Additionally, the reads for the *E. coli* transconjugant strain C16-36, generated from 16Pre36, did not map to the whole of the donor plasmid sequence, covering only a 138,178 bp section of p16Pre36-NDM, from position 67,813–206,190 bp (fig. 6 and supplementary fig.

S1C, Supplementary Material online). This 138-kb region encodes the Tra1 region and ΔTn*125* harboring the bla_{NDM-1} and has a 2,928-bp direct repeat sequence at each end (99% nucleotide identity to each other), which encodes IS*Vsa3* (known as an ISCR2-like element). The mapping profile (fig. 6) showed no C16-36 reads mapped to the Tra-Pre region common to pPrY2001 and its related plasmids (fig. 5*B*). Using primers specific to the Tra-Pre region a PCR product was generated for the donor strain 16Pre36 but not the transconjugant C16-36 (data not shown), confirming the absence of the Tra-Pre region in the transconjugant was due to transfer of only part of the donor plasmid and not a problem with the WGS data. Taken together, these data indicate that p16Pre36-NDM contains a smaller, self-mobilizing transposable element, 138 kb in size, that can be transferred via conjugation to a new host, possibly via the self-encoded Tra1 conjugative apparatus, common to IncA/C2 plasmids.

The sequencing reads from all five *P. rettgeri* isolates from Bucaramanga and the corresponding representative transconjugants CRB151 and CRB152, mapped to almost the entire pRB151-NDM sequence, suggesting that they harbor bla_{NDM-1} in a plasmid closely related to pRB151-NDM (fig. 6). Plasmid pRB151-NDM does not encode the Tra-Pre and Tra1 regions and the strain RB151 does not have additional plasmids, yet four out of five of the related *P. rettgeri* isolates from Bucaramanga (including RB151) had sequences that mapped to the pPrY2001-like and IncA/C2 Tra1 regions (fig. 6). Investigation of the complete RB151 genome sequence (Marquez-Ortiz et al. 2017) revealed the presence of a genomic island with a high degree of similarity to p16Pre36-NDM, including the Tra-Pre and Tra1 regions, which is chromosomally inserted in strain RB151, flanked by 14.6-kb direct repeats (99% nucleotide identity) (see supplementary fig. S5A, Supplementary Material online). This chromosomal insertion was confirmed by PCR using specific primers (see supplementary fig. S5B, Supplementary Material online).

The read mapping data also showed that all three of the *P. rettgeri* Mexican isolates have both p06-1619-NDM and p06-1619-2 plasmids, and the reads from the representative transconjugant C06-1623 showed good coverage over the entire length of p06-1619-NDM, confirming the presence of bla_{NDM-1} on this plasmid.

Thus, the conjugative transfer-associated region Tra-Pre was observed to be present in 12 out of 14 NDM-1-positive *P. rettgeri* isolates (fig. 6), from Colombia (Bogota and Bucaramanga) and Mexico. In addition to the first report in the Canadian *P. rettgeri* bla_{NDM-1}-positive plasmid pPrY2001, this Tra-Pre region is also found in the partially sequenced genome of a NDM-positive *P. rettgeri* isolated in Israel, strain H1736 (Olaitan et al. 2015) (see supplementary fig. S2, Supplementary Material online). However, the partial assembly of the Israeli isolate prevented determination of whether or not the bla_{NDM-1} gene is located in the same plasmid as the Tra-Pre region. Together, these results validate a genetic link among epidemiologically unrelated isolates of NDM-1-positive *P. rettgeri*.

Discussion

In this study, we used WGS data to provide a high-resolution picture of bla_{NDM-1} dissemination in Latin America, which led us to interesting findings about the dissemination route of this gene between *Enterobacteriaceae* and *Acinetobacter* species. *Acinetobacter* spp. harboring bla_{NDM-1} in pNDM-BJ01-like plasmids are frequently detected all over the world (Feng et al. 2015; Fu et al. 2015); these (or NDM-positive isolates with bla_{NDM-1} genetic surroundings suggesting the presence of pNDM-BJ01-like plasmids) have even been found in Latin America (Waterman et al. 2013; Pasteran et al. 2014; Brovedan et al. 2015; Quinones et al. 2015; Montana et al. 2016; Rojas et al. 2016). Here, we report an *A. baumannii* clone isolated in Colombia that also harbors bla_{NDM-1} in a pNDM-BJ01-like plasmid. This finding supports observations of such a bla_{NDM-1}-harboring plasmid present in *Acinetobacter* spp. of different genetic backgrounds. However, although the majority of plasmids harboring bla_{NDM} in *Acinetobacter* spp. are pNDM-BJ01-like plasmids, and although there is some evidence to suggest that *Acinetobacter* spp. passed bla_{NDM} on to the *Enterobacteriaceae* (Toleman et al. 2012), pNDM-BJ01-like plasmids do not seem to have good fitness in non-*Acinetobacter* bacteria, or at least in *Enterobacteriaceae*. To date, only one non-*Acinetobacter* harboring a pNDM-BJ01-like plasmid has been reported (Feng et al. 2015), whereas a plethora of *Enterobacteriaceae* hosting diverse, completely unrelated bla_{NDM}-positive plasmids have been found (supplementary data set 2, Supplementary Material online). The mechanisms of bla_{NDM} gene transmission from *Acinetobacter* spp. to *Enterobacteriaceae* are not yet understood.

In this study, we identified a second *Enterobacteriaceae* family member harboring a pNDM-BJ01-like plasmid (p06-1619-NDM), a *P. rettgeri* isolated in an outbreak in Mexico (Barrios et al. 2013). However, the p06-1619-NDM plasmid has suffered a major modification in the variable region, with the insertion of a previously unreported mobile element introducing two toxin–antitoxin systems (flanked by two novel Tn6368 transposons). It seems plausible that the toxin–antitoxin systems have generated a strong dependence on that plasmid as has been previously reported (Kamruzzaman et al. 2017), thereby avoiding transposition of bla_{NDM-1} to another more compatible plasmid and its subsequent elimination. Adding to the advantages conferred by the two addictive systems is the high selective pressure of the environment—an Intensive Care Unit—from which the Mexican *P. rettgeri* strains were isolated. As the other plasmid hosted by these isolates (p06-1619-2) does not have any resistance genes (table 1), selective pressure could force permanent residence of the pNDM-BJ01-like plasmid due to the conferred

resistance to aminoglycosides and beta-lactams, including carbapenems.

Providencia species are frequently found in environmental settings, but are also opportunistic human pathogens, mainly as the causative agents of urinary tract infections (Wie 2015). They are not among the most significant or prevalent human threats, but recently they have been attracting interest due to increasing reports of *P. rettgeri* NDM-positive isolates found around the world (Barrios et al. 2013; Carvalho-Assef et al. 2013; Mataseje et al. 2014; Pollett et al. 2014; Tada et al. 2014; Carmo Junior et al. 2015; Manageiro et al. 2015; Nachimuthu et al. 2015; Wailan, Paterson, et al. 2016). Here, we also found *P. rettgeri* to be the most frequent bacteria harboring the bla_{NDM-1} gene in three hospitals at distant locations from each other in Colombia. Most of the cases correspond to outpatients, suggesting that NDM-1-positive *P. rettgeri* strains are present in the community in Colombia. In spite of the increase in NDM-positive *P. rettgeri* cases, only two completely sequenced bla_{NDM-1}-positive plasmids are in the NCBI nucleotide database for *P. rettgeri*, both of which were isolated in Canada (supplementary data set 2, Supplementary Material online). Here, we report three additional unrelated complete plasmids hosted by *P. rettgeri* (two from Colombia and one from Mexico), providing more information to help elucidate bla_{NDM-1} dissemination in the *Enterobacteriaceae*.

Interestingly, despite the geographic distances between the sites of isolation of NDM-1-positive *P. rettgeri* strains and despite the very different structures of their bla_{NDM-1}-positive plasmids, we found a common feature—a putative conjugative transfer region named here as Tra-Pre—that appears to be stable among *P. rettgeri* from different regions. Supporting this hypothesis of a common, stable feature for bla_{NDM-1}-positive *P. rettgeri*, is the fact that this Tra-Pre region is also found in the partially sequenced genome of the NDM-1-positive *P. rettgeri* H1736, reported in Israel in 2011 (Olaitan et al. 2015).

The Tra-Pre-family plasmids harboring bla_{NDM-1} that are hosted by *P. rettgeri* (pPrY2001 and p16Pre36-NDM) are unrelated to the pNDM-BJ01-like plasmids from *Acinetobacter* species. Nevertheless, it is possible the bla_{NDM-1}-positive Tra-Pre-encoding plasmids emerged in *P. rettgeri* through transposition of bla_{NDM-1} from a pNDM-BJ01-like plasmid (prior to its loss) to a more stable plasmid, as suggested by the coexistence of a bla_{NDM}-negative plasmid containing the Tra-Pre region (p06-1619-2) and a pNDM-BJ01-like plasmid in the *P. rettgeri* from Mexico. This proposed mechanism is further supported by the presence of the Tra-Pre region in almost all *P. rettgeri* isolates in this study (12 out of 14), even isolates in which bla_{NDM-1} is found on an unrelated plasmid, for example in the *P.* rettgeri isolates from Bucaramanga, Colombia. The simultaneous detection in the Bucaramanga *P. rettgeri* isolates of a new plasmid harboring the bla_{NDM-1}, and of a region in the bacterial chromosome encoding both the IncA/C2-related and Tra-Pre regions similar to those found in the bla_{NDM-1}-positive plasmid circulating in Bogota, indicates how a possible transposition of the bla_{NDM-1} region to a new, different backbone may have occurred in this strain. Thus, our study supports the role of gene module transposition in the spread of bla_{NDM-1} among *P. rettgeri* clinical isolates, a role that has been identified as relevant to the evolution of bla_{NDM-1}-positive plasmids (Khong et al. 2016).

We found a further interesting genetic link in the relationship between bla_{NDM-1}-positive plasmids present in *P. rettgeri* (p16Pre36-NDM) and those found in *K. pneumoniae* (p6234-178kb). The isolates harboring p16Pre36-NDM and p6234-178kb were detected in the same Colombian hospital (supplementary data set 1, Supplementary Material online). Although p16Pre36-NDM and p6234-178kb cannot be classified in the same Inc group, they share a large region, commonly found in the IncA/C2 bla_{NDM-1}-positive and negative plasmids from diverse *Enterobacteriaceae*. This region was also found to be inserted in the chromosome of the *P. rettgeri* isolates from Bucaramanga. These results suggest that the complex p16Pre36-NDM plasmid originated in *P. rettgeri*, through the co-integration of a pPrY2001-like plasmid with an acquired IncA/C2 broad host range plasmid from a different *Enterobacteriaceae*. This IncA/C2 plasmid may then have been transferred to (or from) a non-*Providencia Enterobacteriaceae*, such as the *K. pneumoniae* in this study. Conjugation of p16Pre36-NDM to *E. coli* J53 resulted in transfer of only part of the plasmid, a putative self-mobilizable IS*Vsa3* (an IS*CR2*-like element) composite transposon encoding the bla_{NDM-1} and Tra1 regions, but not the Tra-Pre region. The mapping data indicates that the clinical isolate 16Pre47 also only contains this IS*Vsa3* composite transposon, and is missing the remainder of p16Pre36-NDM, suggesting this Tra-Pre-negative strain 16Pre47 may have receive the IS*Vsa3* composite transposon from other *P. rettgeri* strain (through partial conjugation) or from a *K. pneumoniae*. This putative bla_{NDM-1}-positive conjugative transposon may have derived from the p6234.178kb circulating in *K. pneumoniae* in Colombia prior to the isolation of the *P. rettgeri*, given their similarity and the presence of a closely related sequence containing IS*Vsa3* (2,928 bp) in both p6234.178kb and p16Pre36-NDM (figs. 4 and 5). Interestingly, this transposable element was found to be conserved (99% nucleotide identity over the 2,928 bp) in the genomes of a very wide range of bacteria, in a search against the NCBI database, and also IS*Vsa3*-like elements has been recognized as key players in IncA/C plasmids evolution (Toleman and Walsh 2010). Therefore, the novel bla_{NDM-1}-positive putative conjugative transposon identified in this study could facilitate broad dissemination of bla_{NDM-1} through transposition, conjugation and integration of the transferred circular intermediate into the host genome or to other plasmids via homologous recombination.

The low levels of similarity in the vicinity of Tn*125* (or its remnants) between p16Pre36-NDM and p6234-178kb could

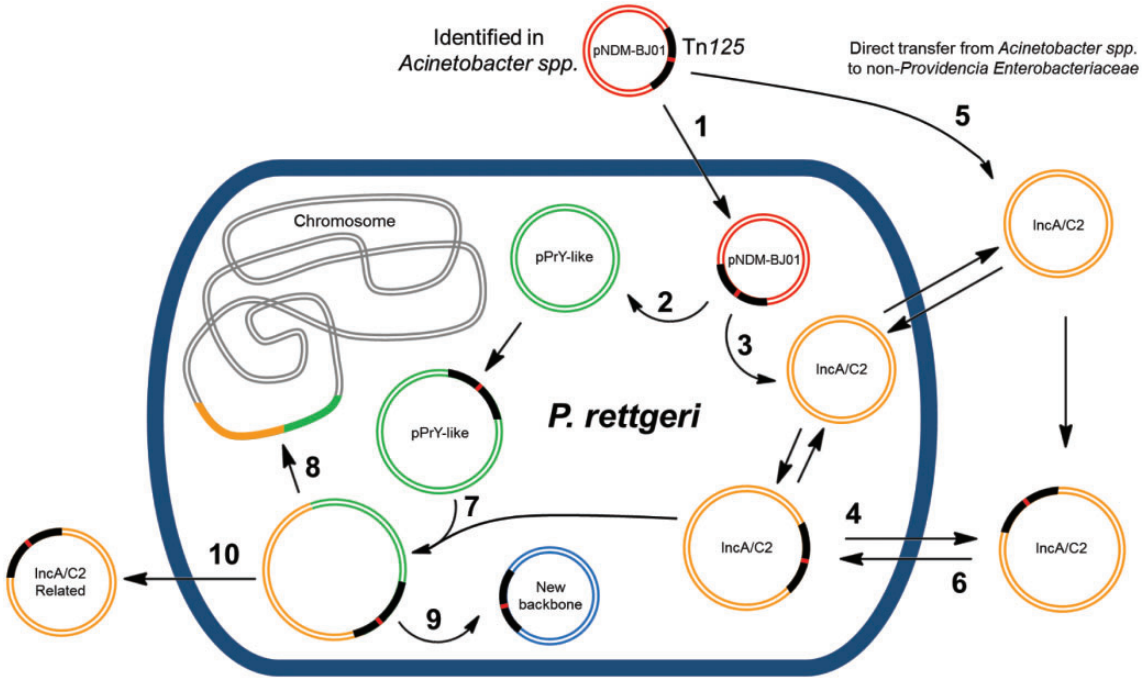

Fig. 7.—Possible roles of *Providencia rettgeri* in bla_{NDM-1}-plasmids evolution in Latin America. In an initial stage pNDM-BJ01-like plasmids are acquired from *Acinetobacter* spp. (*−1*). Shortly after, bla_{NDM-1} is transposed to pPrY-like plasmids (from *P. rettgeri* circulation; *−2*) or IncA/C2 plasmids (from *Klebsiella pneumoniae*, *Escherichia coli* or other *Enterobacteriaceae*; *−3*) via Tn*125* transposition or by mean of other mobile genetic elements surrounding the Tn*125* (or its remnants). IncA/C2 bla_{NDM-1}-plasmids could be transferred to a broad bacteria host range (*−4*). It is also possible that a non-*Providencia Enterobacteriaceae* could capture a pNDM-BJ01-like plasmid and transposes Tn*125* to a broad host range IncA/C2 plasmid (*−5*); later this IncA/C2 bla_{NDM-1}-plasmid could be conjugated to *P. rettgeri* (*−6*). An interesting finding of the present study is the generation in *P. rettgeri* of new plasmids by mean of co-integration of pPrY-like plasmids and IncA/C2 plasmids (*−7*). These chimeric structures can also be transposed to the *P. rettgeri* chromosome (*−8*). The Tn*125* (or its remnants) could be transposed to new plasmid backbones with possible implications upon its dissemination (*−9*). Additionally, by mean of partial conjugation could be disseminated IncA/C2-related (*repA* negative) bla_{NDM-1}-plasmids (*−10*).

be due to the later isolation of the *P. rettgeri* strain harboring p16Pre36-NDM. Although both the *P. rettgeri* and the *K. pneumoniae* strains were isolated at the same hospital (supplementary data set 1, Supplementary Material online), *P. rettgeri* harboring p16Pre36-NDM was isolated more than a year after the *K. pneumoniae* outbreak. The length of time the *P. rettgeri* were present in the hospital under selective pressure, could promote the genetic rearrangements observed in the p16Pre36-NDM plasmid. In an earlier isolate of *K. pneumoniae*, the bla_{NDM-1} surroundings on the p6234-178-kb plasmid are more closely related to those in *Acinetobacter* species.

Dissemination of the IncA/C2 p6234-178kb plasmid in different cities and in strains of different genetic backgrounds, is consistent with the globally observed trend for IncA/C2 (recently designated as IncC; Harmer et al. 2016) group plasmids, which are known to be associated with spread of multidrug resistance genes to different countries and different bacterial hosts (Fricke et al. 2009; Roy Chowdhury et al. 2011; Carattoli et al. 2012), meaning they post a significant risk to human health, particularly as they are capable of disseminating bla_{NDM-1}. The importance of improving existing infection control measures, such as isolation of patients harboring resistant pathogens and hand hygiene, is further substantiated by our findings suggesting that some bla_{NDM-1}-positive plasmids are likely to have originated by co-integration of less stable bla_{NDM-1}-positive plasmids with more stable and disseminative plasmids in environmental bacteria. A case in point is found in the *P. rettgeri* isolates, in which the Tn*125* (or its remains) may have transposed from pNDM-BJ01-like plasmids to pPrY2001-like or IncA/C2-related plasmids that could subsequently be transferred to other bacterial species, including more problematic non-*Providencia Enterobacteriaceae*. An important scenario where all these factors can be found simultaneously is in the mammalian gut where *Enterobacteriaceae* can thrive, aiding the inter-species and inter-genera dissemination of the NDM-1 antibiotic resistance gene among the bacterial community of the gut. Host-specific conditions, such as the inflammatory host response, can also boost horizontal gene transfer and hence microbiota evolution (Stecher et al. 2012), that may have led to the plasmid rearrangements observed here, probably under the selective pressure of the hospital environment. However, direct transfer from *Acinetobacter* spp. to non-*Providencia Enterobacteriaceae* cannot be ruled out, due to conjugation

of pNDM-BJ01-like plasmids from *Acinetobacter* spp. to non-*Providencia Enterobacteriaceae* (mainly *E. coli*) has been demonstrated in this and other studies (Hu et al. 2012; Huang et al. 2015). However, more work needs to be done to better understand the genetic basis of the dissemination of bla_{NDM-1}-positive plasmids in Latin America, by evaluating the stability, fitness cost and conjugation capability of pNDM-BJ01-like and pPrY2001-like plasmids to other *Enterobacteriaceae*.

In our analysis of NDM-1-positive clinical isolates in Latin America, the high variability of bla_{NDM-1}-positive plasmids present in different species, highlights that bla_{NDM-1}-dissemination has not only followed a predominantly clonal evolution, but rather a Russian doll model (Sheppard et al. 2016). In this Russian doll model, a resistance gene such as bla_{NDM-1} resides on nested transmissible units and therefore can move through the environment at multiple different levels, that can be both coincident and independent of one another. For example, bacterial cell hosting resistance gene; plasmid within bacterial cell harboring resistance gene; mobile element within plasmid harboring resistance gene; and mobile element within mobile element harboring resistance gene, with the consequence that the resistance gene may move between plasmids within a bacterial cell via multiple mechanisms (Sheppard et al. 2016). In line with this model, different clones have acquired different bla_{NDM-1}-positive plasmids and related strains have disseminated locally, as for example in the NDM-1 outbreaks in Colombia and Mexico (Barrios et al. 2013; Escobar Perez et al. 2013) and also the cases of the *P. rettgeri* isolated in Bogota and Bucaramanga (Colombia) in the recent surveillance study. These related strains acquired the bla_{NDM-1} from a variety of plasmids, such as IncA/C2-related or pPrY2001-like plasmids, that in turn received bla_{NDM-1} from plasmid co-integration or transposition from another plasmid, or from *Acinetobacter* spp. pNDM-BJ01-like plasmids in an initial dissemination stage. In our Russian doll model, *P. rettgeri* plays an important role as a reservoir of bla_{NDM-1} available for transmission into highly disseminative plasmids due to its high recombination capability supported by the high plasmid variability found in this species (fig. 7). In this study, the presence of pNDM-BJ01-like, Tra-Pre-encoding and IncA/C2-related plasmids or genetic structures in *P. rettgeri*, and their relationship with the plasmids present in *K. pneumoniae* and *Acinetobacter* species, illustrates the evolution route of bla_{NDM-1}-positive plasmids in Latin America, where *P. rettgeri* appears to be crucial for bla_{NDM-1} transmission from *Acinetobacter* spp. to *Enterobacteriaceae*.

Taken together, these findings expose the role of microorganisms such as *P. rettgeri*, that generally are not the target of public health surveillance systems, in the dissemination and storage of resistance genes, highlighting the importance of more comprehensive studies, which do not merely focus on the most frequently occurring pathogens but also encompass the resistance determinants and their mobilization machinery.

Supplementary Material

Supplementary data are available at *Genome Biology and Evolution* online.

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Author Contributions

R.A.M., J.E. and N.K.P. designed research; R.A.M., L.H. and N.K.P. performed research; N.O., C.D., U.G.R., J.S.S., B.E.C., E.M.S., M.B., M.V.M., J.E.C. and A.V. contributed new reagents/analytic tools; R.A.M., J.E. and N.K.P. analyzed data; R.A.M., I.G.C., N.V., J.E. and N.K.P. conceived the study; and R.A.M. and N.K.P. wrote the paper.

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