Characterization of Plasmids in Extensively Drug-Resistant *Acinetobacter* Strains Isolated in India and Pakistan

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The *bla*_{NDM-1} gene is associated with extensive drug resistance in Gram-negative bacteria. This probably spread to *Enterobacteriaceae* from *Acinetobacter* spp., and we characterized plasmids associated with *bla*_{NDM-1} in *Acinetobacter* spp. to gain insight into their role in this dissemination. Four clinical NDM-1-producing *Acinetobacter* species strains from India and Pakistan were investigated. A plasmid harboring *bla*_{NDM-1}, pNDM-40-1, was characterized by whole-genome sequencing of *Acinetobacter bereziniae* CHI-40-1 and comparison with related plasmids. The presence of similar plasmids in strains from Pakistan was sought by PCR and sequencing of amplicons. Conjugation frequency was tested and stability of pNDM-40-1 investigated by real-time PCR of isolates passaged with and without antimicrobial selection pressure. *A. bereziniae* and *Acinetobacter haemolyticus* strains contained plasmids similar to the pNDM-BJ01-like plasmids identified in *Acinetobacter* spp. in China. The backbone of pNDM-40-1 was almost identical to that of pNDM-BJ01-like plasmids, but the transposon harboring *bla*_{NDM-1}, Tn125, contained two short deletions. *Escherichia coli* and *Acinetobacter pittii* transconjugants were readily obtained. Transconjugants retained pNDM-40-1 after a 14-day passage experiment, although stability was greater with meropenem selection. Fragments of pNDM-BJ01-like plasmid backbones are found near *bla*_{NDM-1} in some genetic contexts from *Enterobacteriaceae*, suggesting that cross-genus transfer has occurred. pNDM-BJ01-like plasmids have been described in isolates originating from a wide geographical region in southern Asia. *In vitro* data on plasmid transfer and stability suggest that these plasmids could have contributed to the spread of *bla*_{NDM-1} into *Enterobacteriaceae*.

Acinetobacter baumannii is a successful nosocomial pathogen, and extensively drug-resistant strains are increasingly prevalent (1, 2). Other *Acinetobacter* spp. are found in the environment and can cause opportunistic infections (2). There is evidence that the gene encoding New Delhi metallo-β-lactamase-1 (NDM-1) evolved in an *Acinetobacter* background through fusion between the aminoglycoside resistance gene *aphA6* and a β-lactamase-encoding progenitor of *bla*_{NDM-1} (3). Subsequently, *bla*_{NDM-1} and its closely related variants have spread rapidly among many genera of Gram-negative bacteria (4, 5), and they are found on plasmids of several different incompatibility types and chromosomally (4–6). NDM enzymes hydrolyze all β-lactams except aztreonam and are commonly found with other resistance mechanisms, mediating resistance to almost all clinically available antimicrobials (5, 7).

*bla*_{NDM} genes are prevalent in clinical *Enterobacteriaceae* isolates in South Asia. Many cases of infection or colonization with NDM-producing *Enterobacteriaceae* around the world have been linked to travel to the Indian subcontinent (5, 8). Studies within Indian hospitals have identified NDM-1-producing *Acinetobacter* spp. causing infections in intensive care units (9–11). Similar cases have been reported in Europe, but most patients probably became colonized during travel to the Balkans or North Africa (12–14). In China, *bla*_{NDM-1} has been observed many times in several *Acinetobacter* spp., including *A. baumannii*, from clinical, environmental, and farm animal samples but is less commonly reported in *Enterobacteriaceae* (15–22).

The immediate genetic contexts of *bla*_{NDM} (the genes flanking *bla*_{NDM}) in *Acinetobacter* spp. are well conserved. An intact *ISAbal25* is normally present upstream (3, 14, 16). Downstream

there are usually a conserved set of genes from the bleomycin resistance gene, *ble*, to *ISCR27*. Most examples have an intact *ISAbal25* further downstream from *ISCR27*, thus capturing the entire context in a Tn125 transposon (13, 14, 16). In Chinese isolates, *bla*_{NDM-1} is usually found on plasmids, with all sequenced examples being closely related to pNDM-BJ01 from *A. lwoffii* WJ10621, despite being reported in many different *Acinetobacter* spp (15, 16, 19, 23). In most isolates identified outside Asia, *bla*_{NDM-1} is found on the chromosome (12–14).

The *Acinetobacter* plasmids from China share features which suggest that they could have contributed to the acquisition of *bla*_{NDM} by *Enterobacteriaceae*. We set out to see if similar plasmids were present in *Acinetobacter* spp. from India and Pakistan, where some studies have shown a high prevalence of *Enterobacteriaceae*

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TABLE 1 Bacterial isolates used in this study

Isolate	Species	Source	Location where isolated	Yr isolated
CHI-40-1	<i>Acinetobacter bereziniae</i>	Clinical isolate (pus)	Tamil Nadu, India	2005
73261-EC	<i>Acinetobacter haemolyticus</i>	Fecal screening	Karachi, Pakistan	2012
70114-EC	<i>Acinetobacter haemolyticus</i>	Fecal screening	Karachi, Pakistan	2012
69122-EW	<i>Acinetobacter haemolyticus</i>	Fecal screening	Karachi, Pakistan	2012
74312-EC	<i>Acinetobacter schindleri</i>	Fecal screening	Karachi, Pakistan	2012
73668-ECT	<i>Acinetobacter townieri</i>	Fecal screening	Karachi, Pakistan	2012

producing *bla*_{NDM}. We describe the whole-genome sequence (WGS) of an *A. bereziniae* isolate from Chennai, India, with such a plasmid. We further show that closely related plasmids carrying *bla*_{NDM-1} are present in *Acinetobacter* spp. isolated in Karachi, Pakistan. We also investigated the conjugation efficiencies and stabilities of these plasmids in different recipients to further explore their potential as vectors in the dissemination of *bla*_{NDM-1}.

MATERIALS AND METHODS

Bacterial strains studied. A full list of isolates used in this study is given in Table 1. *A. bereziniae* CHI-40-1 (11) was investigated in detail in the current study. Other *Acinetobacter* species isolates were from fecal screening samples collected at the Civil Hospital Karachi, Pakistan, in 2012. Samples from consecutive patients admitted to the hospital were collected at admission and discharge, and isolates growing on selective plates containing ertapenem were further analyzed. The isolates studied here represent all of the *bla*_{NDM-1}-positive *Acinetobacter* spp. isolated from 717 fecal swabs processed as of January 2013. *Escherichia coli* UAB190 (24) and *A. pittii* AG3528 were used as recipients in mating experiments.

Identification and antimicrobial susceptibility testing. Initial bacterial identification was performed by matrix-assisted laser desorption ionization–time of flight mass spectroscopy (MALDI-TOF) (Bruker, Billerica, MA, USA). Confirmation of the identification was by phylogenetic analysis of 16S rRNA gene sequences for Pakistan isolates, together with ribosomal multilocus sequence typing (rMLST) for CHI-40-1 (see Fig. S1 in the supplemental material). Antimicrobial susceptibility testing was performed by Etest (bioMérieux, LaPlane, France) and MIC test strip (Liofilchem, Roseto degli Abruzzi, Italy). Interpretation was according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints (version 3.1).

WGS and analysis. *A. bereziniae* CHI-40-1 was sequenced using an Illumina HiSeq platform at the Wellcome Trust Sanger Institute, Cambridge. A unique index-tagged insert library was prepared to allow processing of the sample data following multiplex sequencing with other libraries on eight channels of an Illumina Genome Analyzer GAII cell to give 100-bp paired-end reads, as previously described (25). Reads were assembled *de novo* using the Velvet Assembly Tool (version 1.2.10) (26). Plasmid contigs were identified using Blast searches against the sequence of plasmid pNDM-BJ01 from *Acinetobacter lwoffii* strain WJ10621 (accession number JQ001791) (16). Links between contigs were confirmed by PCR and sequencing of amplicons. Detailed comparison between pNDM-40-1 and closely related plasmids was performed using nucleotide alignments created in Geneious (27). Antibiotic resistance genes were identified using RESFinder. Annotation was by transfer of annotations for genes with close nucleotide identity from reference sequences in Geneious.

PFGE and in-gel hybridization. Genomic DNA was prepared in 1% low-melting-point agarose plugs as described previously (28). Briefly,

plugs were made using a bacterial cell suspension in Tris-EDTA (TE) buffer at a standard optical density of 1.8 to 2.0 at a wavelength of 600 nm (28). Plugs were treated with ApaI (Thermo Scientific, Waltham, MA, USA) or S1 nuclease (Thermo Scientific) and pulsed-field gel electrophoresis (PFGE) performed as described previously (28, 29). Autoradiographs were prepared by in-gel hybridization of pulsed-field gels with gene probes, made using a random primer method to label *bla*_{NDM-1} or *traA* PCR products with [³²P]CTP, as previously described (28).

Conjugation and passage experiments. Conjugation experiments were performed as described previously using a plate mating assay at 30°C (4), with *A. bereziniae* CHI-40-1 and *A. haemolyticus* 69122-EW as donors and *E. coli* UAB190 and *A. pittii* AG3528 (both rifampin resistant) as recipients. Selection was performed on Brilliance UTI Clarity agar (Oxoid Ltd., Basingstoke, United Kingdom) supplemented with rifampin (Sigma-Aldrich, St. Louis, MO, USA) for recipient selection, ampicillin (Sigma-Aldrich) and rifampin for UAB190 background transconjugants, or meropenem (AstraZeneca, London, United Kingdom) and rifampin for AG3528 background transconjugants. For each experiment, 5 isolated colonies were subcultured to selective media. Pure growths on subculture were tested by MALDI-TOF to confirm the species background, and the presence of *bla*_{NDM-1} was confirmed by PCR. Mating efficiency was calculated as the number of transconjugants per recipient cell. Transconjugants obtained from mating between CHI-40-1 and each recipient background were subjected to S1 PFGE and in-gel hybridization with *bla*_{NDM-1} and *traA*.

A passage experiment was performed on *A. bereziniae* CHI-40-1 and its transconjugants, *E. coli* UAB190_{NDMP2} and *A. pittii* AG3528_{NDMP1}. Cultures from selective plates were inoculated into Luria-Bertani (LB) broth (Thermo Scientific) with and without antibiotic selection and incubated overnight at 37°C. The following day, cultures were reinoculated into a fresh broth with the same selection as the starting culture. Columbia blood agar plates (E&O Laboratories, Bonnybridge, Scotland) were inoculated daily to check purity, and cultures were stored each day in LB broth with 10% glycerol at –80°C. This procedure was repeated for 14 consecutive days. Antibiotic selection was with meropenem at 10 µg/ml for CHI-40-1 and AG3528_{NDMP1} and at 1 µg/ml for UAB190_{NDMP2}. Stored cultures were investigated by S1 PFGE and probing for *bla*_{NDM-1} and *traA* as well as real-time quantitative PCR (qPCR) (see below).

PCR. A full list of PCR and sequencing primers used together with full PCR conditions is given in Table S2 in the supplemental material. The presence of plasmids similar to pNDM-BJ01 was confirmed by PCR with primers described by Hu et al. (16). Sequencing of PCR amplicons was used to resolve gaps in the pNDM-40-1 sequence and to primer walk the *bla*_{NDM-1} context in 69122-EW. PCR amplicons for sequencing were purified using the QIAquick gel extraction kit (Qiagen, Limburg, Netherlands) as per the manufacturer's instruction, and products were submitted to Eurofins MWG Operon (Ebersberg, Germany) for sequencing.

Real time-quantitative PCR (qPCR) was performed to quantify changes in *bla*_{NDM-1} and *traA* copy number present in bacterial cells over the course of the passage experiment. The single-copy chromosomal gene *rpoB* was used as the reference gene. Dual-labeled probes with fluorescent dye and quenchers were synthesized by Eurofins MWG Operon. *bla*_{NDM-1} and *traA* fluorescence cycle threshold (*C*_T) values were compared to *rpoB* *C*_T values, and quantification was performed by the $\Delta\Delta C_T$ method (30). Regression analysis was performed using Excel 2007. A validation experiment showed that ΔC_T values were linear over the range of values detected in the passage experiment. All experiments were performed in triplicate.

Nucleotide sequence accession numbers. Accession numbers for pNDM-40-1 from *A. bereziniae* CHI-40-1 and the partial sequence for pNDM-69122 from *A. haemolyticus* 69122-EW are [KF702385](#) and [LN611576](#), respectively. *A. bereziniae* CHI-40-1 assembly contigs are deposited under study accession number PRJEB7120, contig accession numbers CDEL01000001 to CDEL01000324.

TABLE 2 Antimicrobial MICs for *Acinetobacter* species recipients and transconjugants

Strain	MIC (mg/liter) ^a												
	ATM ^b	CAZ ^b	IPM	MEM	TZP	AMK	GEN	TOB	CIP	CST	FOF	SXT	TGC ^b
CHI-40-1	48 (R)	≥256 (R)	≥32 (R)	≥32 (R)	96 (R)	48 (R)	≥256 (R)	12 (R)	≥32 (R)	0.75 (S)	8	≥32 (R)	0.75 (R)
69122-EW	3 (S)	≥256 (R)	≥32 (R)	≥32 (R)	24 (R)	32 (R)	2 (S)	8 (R)	≥32 (R)	0.75 (S)	32	0.25 (S)	1 (R)
73668-ECT	16 (R)	≥256 (R)	≥32 (R)	≥32 (R)	48	6 (S)	192 (R)	ND	≥32 (R)	0.5 (S)	4	0.25 (S)	0.5 (I)
74312-EC	48 (R)	≥256 (R)	≥32 (R)	≥32 (R)	≥256 (R)	48 (R)	96 (R)	12 (R)	≥32 (R)	0.5 (S)	16	≥32 (R)	0.75 (R)
AG3528	16 (R)	3 (S)	0.75 (S)	0.75 (S)	2	2 (S)	0.75 (S)	0.75 (S)	4 (R)	1 (S)	16	0.125 (S)	ND
UAB190	0.125 (S)	0.25 (S)	0.38 (S)	0.047 (S)	2	2 (S)	8 (R)	1.5 (S)	0.006 (S)	0.5 (S)	3	0.094 (S)	ND
AG3528 _{NDMP1}	16 (R)	≥256 (R)	≥32 (R)	≥32 (R)	96	3 (S)	0.75 (S)	0.75 (S)	3 (R)	1 (S)	16	0.125 (S)	ND
UAB190 _{NDMP2}	0.064 (S)	≥256 (R)	24 (R)	4 (I)	256	2 (S)	8 (R)	1.5 (S)	0.006 (S)	0.5 (S)	3	0.094 (S)	ND

^a Antimicrobial susceptibility results based on EUCAST pharmacokinetic/pharmacodynamic (PK/PD) non-species-specific breakpoints. R, resistant; I, intermediate resistance profile; S, sensitive; ATM, aztreonam; CAZ, ceftazidime; IPM, imipenem; MEM, meropenem; TZP, piperacillin-tazobactam; AMK, amikacin; GEN, gentamicin; TOB, tobramycin; CIP, ciprofloxacin; CST, colistin sulfate; FOF, fosfomycin; RIF, rifampin; SXT, co-trimoxazole; TGC, tigecycline; ND, not determined.

^b Species-specific *Acinetobacter* species breakpoints not available.

RESULTS

Species identification, antimicrobial susceptibility, and resistance genes present in *A. bereziniae* CHI-40-1. The clinical *Acinetobacter* isolate from Chennai was found to be an *A. bereziniae* strain by rMLST. Five NDM-1-producing *Acinetobacter* isolates from the fecal screening study were obtained from five different patients and included three species (Table 1; see Fig. S1 in the supplemental material). Three *Acinetobacter haemolyticus* isolates were found to be representatives of a single strain by ApaI restriction digestion and PFGE. All strains were extensively drug resistant (Table 2). In keeping with this, genes associated with resistance to β -lactams (*bla*_{OXA-58}), aminoglycosides (*strA*, *strB*, and *aacC2*), macrolides [*msr*(E) and *mph*(E)], trimethoprim (*dfrA1*), and sulfonamides (*sul1* and *sul2*) were identified in *A. bereziniae* CHI-40-1.

Characterization of plasmid pNDM-40-1. S1 PFGE and *bla*_{NDM-1} probing showed that *A. bereziniae* CHI-40-1 harbored multiple plasmids, with *bla*_{NDM-1} present on plasmids corresponding to bands of ~45 kb and ~250 kb (Fig. 1a and b). How-

ever, *bla*_{NDM-1} was present on a single ApaI restriction fragment of ~45 kb (Fig. 1c and d).

The *de novo* assembly of the *A. bereziniae* CHI-40-1 WGS produced 324 contigs, with a mean GC content of 38% and a combined size of 4.78 Mb. A 45,827-bp plasmid harboring *bla*_{NDM-1}, pNDM-40-1, was closed by PCR and sequencing of amplicons. The GC content of the plasmid backbone is 36.2%, and that of the variable region (from IS*Aba14* to the end of Tn*I25*, nucleotides [nt] 5427 to 16280) is 52.5%.

At the time of writing, complete sequences of nine pNDM-BJ01-like plasmids were available in GenBank. The backbone of pNDM-40-1 is 100% identical at the nucleotide level to those of pNDM-BJ01 (bases 1 to 5684 and 17987 to 47274, accession number JQ001791), pNDM-BJ02 (JQ060896), pAbNDM-1 (JN377410), and pXM1 (AMXH01000087). pNDM-AB (KC503911), pM131-NDM-1 (JX072963), and pNDM-Iz4b (KJ547696) exhibit minor differences from the backbone of pNDM-BJ01 and from one another. pNDM-AB differs the most, because of a 3.5-kb insertion containing the genes *traD* and *insB* and a putative methyltransferase gene (Fig. 2;

FIG 1 (a) Pulsed-field gel of S1 nuclease-digested genomic DNA from *A. bereziniae* CHI-40-1, recipients, and transconjugants; (b) in-gel hybridization with a *bla*_{NDM-1} gene probe; (c) pulsed-field gel of ApaI-digested genomic DNA from CHI-40-1; (d) in-gel hybridization with a *bla*_{NDM-1} gene probe. The molecular size marker is concatemers of λ of ~50 to 1,000 kb.

FIG 2 (A) Gene maps of plasmids pNDM-BJ01, pNDM-40-1, and pNDM-AB; (B) immediate *bla*_{NDM-1} context from pNDM-40-1, *A. haemolyticus* 69122-EW, and related sequences in *Acinetobacter* and *Enterobacteriaceae*. Open reading frames (ORFs) are color coded with the direction of transcription indicated by arrowheads, and truncated remnants of ORFs are shown as rectangles. Red, *bla*_{NDM-1}; orange, other antibiotic resistance; lime green, usually immediately downstream of *bla*_{NDM-1}; blue, from a common context in *Xanthomonas* and *Pseudoxanthomonas*; brown, *ISAbal25*; dark gray, ISCR transposases; light gray, other insertion sequence transposases; yellow, resolvase and zeta-toxin from pNDM-BJ01-like plasmids; dark green, named plasmid backbone genes; white, genes for hypothetical proteins. Regions with a light blue shaded background contain plasmid backbone with close identity among pNDM-BJ01-like plasmids. The pink shaded regions represent genes normally found in the *bla*_{NDM-1} context in *Acinetobacter* spp. Regions from pNDM-AB with a yellow background represent genes with no significant identity to those in pNDM-40-1. *oriT*, origin of transfer; *traD*, conjugal transfer gene; *traA*, MobA/L-type relaxase gene; *res*, resolvase gene; *nuc*, nuclease homologue; *virB1* to *-B11* and *virD4*, putative T4SS genes; *trg*, putative lytic transglycosylase gene; *parA*, putative plasmid partition gene; *rms*, putative type I restriction-modification system methyltransferase subunit gene; *aphA6*, aminoglycoside resistance gene; *ble*, bleomycin resistance gene; *trpF*, phosphoribosylanthranilate isomerase gene; *tat*, twin-arginine translocation pathway signal sequence domain gene; *cutA1*, periplasmic divalent cation tolerance gene; *groES*, cochaperonin gene; *groEL*, chaperonin gene; *oriIS*, origin of insertion of ISCR27; *res*, putative resolvase gene; *msr(E)* and *mph(E)*, macrolide resistance genes.

see Table S3 in the supplemental material). These plasmids were all identified in isolates from China and were found in five different species, including *A. baumannii*. Additionally, several *bla*_{NDM-1}-negative *Acinetobacter* species sequences contain regions with significant identity to the backbones of pNDM-BJ01-like plasmids (see Fig. S3 in the supplemental material).

The *bla*_{NDM-1} gene in pNDM-40-1 is found within a Tn125 transposon (Fig. 2), as with other pNDM-BJ01-like plasmids (12, 13, 16). Tn125 in pNDM-40-1 has two deletions relative to Tn125 in pNDM-BJ01: a 1,298-bp deletion from the 3' end of *ble* to *tat* and a 150-bp deletion within ISCR27 (see Fig. S3 in the supplemental material). pNDM-BJ02 and contig 5 from *Acinetobacter soli* TCM341 lack the 3' *ISAbal25*, while in pAB-D499 and pM131-NDM-1, there is an *ISAbal1* inserted at the 3' end of the element. In pNDM-AB, a large part of the context from *cutA1* to the 3' *ISAbal25* is replaced by the macrolide resistance genes *msr(E)* and *mph(E)* (23). In all pNDM-BJ01-like plasmids, the

aminoglycoside resistance gene *aphA6* and an *ISAbal4* element are found immediately upstream of Tn125 (Fig. 2).

GenBank searches show that *bla*_{NDM-1} contexts in *Enterobacteriaceae* have high degrees of identity with the *bla*_{NDM-1} context from pNDM-BJ01-like plasmids. In most cases, this is restricted to genes that make up part of the full Tn125 element harboring *bla*_{NDM-1}, with at least a fragment of the *ISAbal25* upstream of *bla*_{NDM-1} and the *ble* and *trpF* genes being present in almost all cases. Four sequences from *Enterobacteriaceae* with regions of close identity to the *bla*_{NDM-1} context in pNDM-BJ01, which included part of the plasmid backbone, were available at the time of writing (31) (Fig. 2). These were in the plasmids pPrY2001 from *Providencia rettgeri* (KF295828), pMR0211 from *Providencia stuartii* (JN687470), pNDM-1_Dok01 from *E. coli* (AP012208), and pECL3-NDM-1 from *Enterobacter cloacae* (KC887917). Plasmid pPrY2001 contains the most extensive region of identity. The sequence is nearly identical to that found in pNDM-BJ01 from the

far 3' end of *traA* to the resolvase gene, with the main difference being the absence of the 3' IS*Aba125*.

All pNDM-BJ01-like plasmids share a region with genes coding for a type IV secretion system (T4SS) involved in constructing the conjugation machinery and mediating conjugative transfer of plasmid DNA to recipient bacteria (16). In addition, all pNDM-BJ01-like plasmids contain genes proposed to code for a plasmid partition system (*parA*) and a putative zeta-toxin, which may contribute to plasmid stability through a toxin-antitoxin addiction system. It has not been possible to identify the replicase or the origin of replication of these plasmids. However, the wide range of replication strategies already described means that the lack of an identifiable replicase is not entirely surprising (6, 32).

The *bla*_{NDM-1} context in *A. haemolyticus*. PCR analysis revealed that the *A. haemolyticus* strain, but not the other two *Acinetobacter* species isolates from Karachi, contained several regions of a pNDM-BJ01-like plasmid backbone. The immediate *bla*_{NDM-1} context in *A. haemolyticus* 69122-EW was linked to pNDM-BJ01-like backbone genes *traA* upstream and the resolvase gene downstream (Fig. 2). The immediate context differed from that described in pNDM-BJ01 in that most of Tn125 was missing.

A previously uncharacterized insertion sequence, IS*Aha3*, most similar to IS*Alw1* (95% amino acid [AA] identity between transposases), was inserted between *ble* and the putative resolvase gene. No direct repeats (DRs) were observed, but this was not uncommon for other closely related ISs deposited in ISFinder. It is possible that transposition of IS*Aha3* resulted in deletion of the sequence often found between *ble* and *res* and also resulted in the loss of one of the DRs (33). S1 PFGE and in-gel hybridization showed that *bla*_{NDM-1} was present on ~45-kb plasmids in *A. haemolyticus* 69122-EW, pNDM-69122.

Conjugative transfer and stability of plasmids harboring *bla*_{NDM-1}. Transconjugants were obtained from mating experiments with *A. bereziniae* CHI-40-1 and *A. haemolyticus* 69122-EW donors, in both *E. coli* UAB190 and *A. pittii* AG3528 recipients, at rates of 10⁻⁴ to 10⁻⁵ transconjugants per recipient cell. All putative transconjugants tested were found to be the recipient background species by MALDI-TOF and to be *bla*_{NDM-1} positive by PCR. MICs to all β-lactams except aztreonam were elevated in selected transconjugants (Table 2).

PCR analysis showed that the relaxase gene, *traA*, and other sections of the pNDM-BJ01-like backbone were present in all transconjugant colonies tested. In *A. pittii* AG3528 transconjugants, in-gel hybridization showed that *traA* and *bla*_{NDM-1} were present on ~45-kb plasmids, as expected for pNDM-40-1. However, in *E. coli* UAB190 transconjugants, these genes were both present on either the chromosome or ~90-kb plasmids (Fig. 1b) (*traA* data not shown).

Probing of S1 PFGE gels of CHI-40-1, UAB190_{NDMP2}, and AG3528_{NDMP1} over the course of a 14-day passage showed that *bla*_{NDM-1}-positive bands did not alter in size (see Fig. S4 in the supplemental material). The intensity of the *bla*_{NDM-1} bands in CHI-40-1 was similar over the course of the experiment with and without meropenem selection. The intensity of the *bla*_{NDM-1} bands for both transconjugant strains was stable with antibiotic selection but decreased significantly over the course of the passage without meropenem selection.

Regression analysis of ΔΔ*C*_T values from qPCR experiments (see Fig. S5 in the supplemental material) showed statistically significant falls in the quantities of *bla*_{NDM-1} and *traA* template over

the course of the passage experiment for transconjugant strains without meropenem selection. For the donor strain, CHI-40-1, there was little change. For all strains tested, *bla*_{NDM-1} and *traA* remained detectable throughout the 14-day passage experiment, even in the absence of antibiotic selection.

DISCUSSION

All the complete NDM-1 plasmid sequences from *Acinetobacter* spp. that we were able to identify in GenBank were from Chinese isolates and were similar to pNDM-BJ01. pNDM-BJ01 contains a single *ApaI* restriction site, in keeping with the ~45-kb bands present in both the S1 and *ApaI* gels. We conclude that CHI-40-1 harbors just one plasmid containing *bla*_{NDM-1} and that the 300-kb band represented residual supercoiled plasmid DNA.

The pNDM-BJ01-like plasmids have a GC content similar to that of most *Acinetobacter* species WGSs deposited in GenBank (~40%). All examples of these plasmids harboring *bla*_{NDM-1} have very high levels of identity with one another. The small number of related sequences not associated with *bla*_{NDM-1} are more distantly related to pNDM-BJ01-like plasmids but are also found exclusively in *Acinetobacter* spp. We propose that these findings are compatible with this plasmid lineage having evolved within the *Acinetobacter* genus and with the acquisition of *bla*_{NDM-1} being a relatively recent event.

Most descriptions of conjugative transfer of *bla*_{NDM-1} from *Acinetobacter* spp. *in vitro* are for isolates with pNDM-BJ01-like plasmids (15, 16, 19, 23). Conjugation rates were similar for the pNDM-BJ01-like plasmids studied here into *E. coli* and *A. pittii* recipients. The stability of both *bla*_{NDM-1} and *traA*, coding for the pNDM-BJ01-like relaxase, was similar in *E. coli* and *A. pittii* transconjugants. However, in the *E. coli* transconjugants studied in more detail, this apparently required recombination of pNDM-40-1, or part of it, with the chromosome or another plasmid. Although pNDM-40-1 was therefore no longer likely to function as an autonomously replicating plasmid, this still suggests a potential means by which *bla*_{NDM-1} contexts could have spread from *Acinetobacter* spp. to *Enterobacteriaceae*. This may explain why no complete pNDM-BJ01-like plasmid has yet been described in *Enterobacteriaceae*. However, sequence data now strongly suggest that at least some *bla*_{NDM-1} contexts in *Enterobacteriaceae* are derived from pNDM-BJ01-like plasmids, since several examples demonstrate high levels of identity with the plasmid backbone sequences which flank IS*Aba14*, *aphA6*, and Tn125 containing *bla*_{NDM-1} (31).

Recent reports suggest that NDM-1-producing *Enterobacteriaceae* may be more common than originally demonstrated in China (21, 22), and it is possible that the spread of *bla*_{NDM-1} into *Enterobacteriaceae* could have initiated there. However, the high prevalence in some regions of the Indian subcontinent of NDM-producing *Enterobacteriaceae* has been well documented, and most reports of travel-associated colonization with NDM-producing bacteria involved the subcontinent (5, 8). It is therefore potentially significant that pNDM-BJ01-like plasmids have now been identified from India and Pakistan and from returning travelers to the region (34, 35). Insufficient data are available to establish whether these cases could be linked to recent spread from China or whether these plasmids are prevalent in a wider geographical region including the Indian subcontinent.

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We have no conflicts to declare.

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