PLOS ONE

RESEARCH ARTICLE

Genetic characterization of bla_{NDM}-harboring plasmids in carbapenem-resistant *Escherichia coli* from Myanmar

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Citation: Sugawara Y, Akeda Y, Sakamoto N, Takeuchi D, Motooka D, Nakamura S, et al. (2017) Genetic characterization of bla_{NDM}-harboring plasmids in carbapenem-resistant *Escherichia coli* from Myanmar. PLoS ONE 12(9): e0184720. https://doi.org/10.1371/journal.pone.0184720

Editor: Yeng-Tseng Wang, Kaohsiung Medical University, TAIWAN

Received: June 7, 2017

Accepted: August 29, 2017

Published: September 14, 2017

Data Availability Statement: The whole genome sequence data are available from the DDBJ database under accession numbers DRX064631 (M105), DRX064632 (M107), DRX064633 (M109), DRX064634 (M110), DRX064635 (M213), DRX064636 (M214), DRX064638 (M216), and DRX064639 (M217). The plasmid sequences are available from the DDBJ database under accession numbers AP018136 (pM105_FII), AP018137 (pM105_mF), AP018138 (pM107_FII), AP018139 (pM109_FII), AP018140 (pM110_FII), AP018141 (pM110_X3), AP018142 (pM213_X3),

Abstract

The bacterial enzyme New Delhi metallo-β-lactamase hydrolyzes almost all β-lactam antibiotics, including carbapenems, which are drugs of last resort for severe bacterial infections. The spread of carbapenem-resistant *Enterobacteriaceae* that carry the New Delhi metallo-β-lactamase gene, bla_{NDM}, poses a serious threat to public health. In this study, we genetically characterized eight carbapenem-resistant *Escherichia coli* isolates from a tertiary care hospital in Yangon, Myanmar. The eight isolates belonged to five multilocus-sequence types and harbored multiple antimicrobial-resistance genes, resulting in resistance against nearly all of the antimicrobial agents tested, except colistin and fosfomycin. Nine plasmids harboring bla_{NDM} genes were identified from these isolates. Multiple bla_{NDM} genes were found in the distinct Inc-replicon types of the following plasmids: an IncA/C_2 plasmid harboring bla_{NDM-1} ($n = 1$), IncX3 plasmids harboring bla_{NDM-4} ($n = 2$) or bla_{NDM-7} ($n = 1$), IncFII plasmids harboring bla_{NDM-4} ($n = 1$) or bla_{NDM-5} ($n = 3$), and a multireplicon F plasmid harboring bla_{NDM-5} ($n = 1$). Comparative analysis highlighted the diversity of the bla_{NDM}-harboring plasmids and their distinct characteristics, which depended on plasmid replicon types. The results indicate circulation of phylogenetically distinct strains of carbapenem-resistant *E. coli* with various plasmids harboring bla_{NDM} genes in the hospital.

Introduction

Carbapenems are broad-spectrum antibiotics used as the last line of defense against multidrug-resistant bacteria; however, infections with carbapenem-resistant *Enterobacteriaceae* (CRE) have been increasingly reported since the early 2000s [1], [2]. CRE are resistant to most commonly prescribed antibiotics; therefore, CRE infections are associated with poor prognosis

AP018143 (pM214_AC2), AP018144 (pM214_FII), AP018145 (pM216_AC2), AP018146 (pM216_X3), and AP018147 (pM217_FII).

Funding: This study was supported by the Japan Initiative for Global Research Network on Infectious Diseases (J-GRID) from Ministry of Education, Culture, Sport, Science and Technology in Japan (MEXT), and Japan Agency for Medical Research and Development (AMED, URL: http://www.amed.go.jp/en/). The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

and pose a serious threat in clinical settings [3]. Carbapenem resistance in *Enterobacteriaceae* is primarily due to carbapenemases; plasmid-borne β-lactamases that hydrolyze carbapenems [4]. Several types of carbapenemases, such as KPC, OXA-48, VIM, IMP, and New Delhi metallo-β-lactamase (NDM), have been identified worldwide [5].

Routine worldwide surveillance is essential to understand and prevent CRE transmission; however, in many countries, including Myanmar, surveillance is limited or non-existent. Recently, Myat *et al*. reported the isolation of three *Escherichia coli* and three *Klebsiella pneumoniae* strains harboring bla_{NDM} genes in a screening of 592 blood cultures in three hospitals in Yangon, Myanmar [6]. No other carbapenemase-encoding genes were identified in their study, suggesting that NDM rather than other carbapenemases is prevalent in Myanmar. The bla_{NDM} genes are usually located adjacent to or in between mobile genetic elements, including transposons and insertion sequences, which facilitate transposition between replicons [7], [8]. Accordingly, bla_{NDM} genes are currently found in an array of plasmid replicon types, such as IncF, IncX3, IncL/M, and IncH, as well as in plasmids with a broad-host-range, including IncA/C_2. Consequently, the bla_{NDM} gene has spread from the putative original reservoir, *Acinetobacter*, to enteric bacteria [9]. Additionally, accumulation of nucleotide substitutions in bla_{NDM} has produced several variants from the originally identified enzyme NDM-1 [10]. Organisms harboring bla_{NDM} genes have been detected not only in hospitals, but also in the environment in some Asian countries [11], [12]. Therefore, it is necessary to characterize the bacterial host, the bla_{NDM}-harboring plasmid, and the genetic environment associated with bla_{NDM} in order to understand the gene acquisition mechanism, track its spread, and investigate possible preventive measures [9].

In this study, we genetically characterized eight carbapenem-resistant *E. coli* isolates from a tertiary care hospital in Yangon, Myanmar, using whole-genome sequencing (WGS). Comparative analysis highlighted the diversity of nine plasmids carrying bla_{NDM}, providing insight into an evolutionary relationship with the already identified bla_{NDM} plasmids.

Materials and methods

Bacterial isolates

E. coli isolates were obtained from clinical blood or urine specimens collected from patients at Yangon General Hospital, Yangon, Myanmar, from April to August 2015. Ethical approval for the collection of patient specimens was obtained from the Ethics Committee of Osaka University Graduate School of Medicine and the Department of Medical Research, Myanmar, with a waiver of informed consent. All samples were anonymized before analysis. Specimens were cultured on blood agar plates at 37˚C overnight, and each single colony formed was subjected to species identification and antimicrobial-susceptibility testing using a VITEK2 automated system (Sysmex bioMérieux, Marcy l'Etoile, France). *Enterobacteriaceae* strains with minimum inhibitory concentration (MIC) $>$ 2 μg/mL for meropenem were collected and stored at −80˚C in phosphate-buffered saline supplemented with 25% glycerol. Isolates were also grown on M-ECC [13], CHROMagar ECC (CHROMagar Microbiology, Paris, France) supplemented with 0.25 μg/mL meropenem and 70 μg/mL $ZnSO_4$, and analyzed using an API 20E system (Sysmex bioMérieux) and an EIKEN dry plate (Eiken Chemical, Tokyo, Japan) to reconfirm species identity and antimicrobial susceptibility.

WGS analysis

Isolates were subjected to WGS using PacBio RSII (Pacific Biosciences, Menlo Park, CA, USA) and MiSeq systems (Illumina, San Diego, CA, USA). To prepare genomic DNA, bacterial

isolates were cultured overnight in brain-heart-infusion broth (BD Bacto, Franklin Lakes, NJ, USA) supplemented with 0.25 μg/mL meropenem, and DNA was isolated using a PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA). To prepare libraries for PacBio RSII, 10 μg DNA was sheared using g-TUBE (Covaris, Woburn, MA, USA) to obtain 10,000–20,000-bp fragments, which were ligated to SMRTbell adapters (Pacific Biosciences). The fragments were processed with a DNA template prep kit 1.0 and a DNA/polymerase binding kit (P6v2; Pacific Biosciences) according to manufacturer's instructions. Then, the fragments were sequenced with a DNA sequencing kit C4 (Pacific Biosciences), and the sequence reads were assembled *de novo* using HGAP version 2.1.0 with default parameters [14]. The sequences were submitted to the DDBJ/Genbank/EMBL database under accession numbers DRX064631 (M105), DRX064632 (M107), DRX064633 (M109), DRX064634 (M110), DRX064635 (M213), DRX064636 (M214), DRX064638 (M216), and DRX064639 (M217). To prepare libraries for MiSeq analysis, 200 ng of genomic DNA was sheared using Covaris S220 (Covaris) and processed with KAPA library preparation kits (KAPA Biosystems, Boston, MA, USA). Paired-end sequencing with 250-bp reads was performed using a MiSeq version 2 500 cycle kit (Illumina). Reads were mapped to the sequence generated from PacBio RSII using mapping software in CLC Genomics Workbench version 7.5 (CLC Bio, Aarhus, Denmark), and the consensus sequence was extracted for further analysis. The plasmid sequences were submitted to the DDBJ/Genbank/EMBL database under accession numbers AP018136 (pM105_FII), AP018137 (pM105_mF), AP018138 (pM107_FII), AP018139 (pM109_FII), AP018140 (pM110_FII), AP018141 (pM110_X3), AP018142 (pM213_X3), AP018143 (pM214_AC_2), AP018144 (pM214_FII), AP018145 (pM216_AC_2), AP018146 (pM216_X3), and AP018147 (pM217_FII).

A phylogenetic tree of the *E. coli* isolates based on single-nucleotide polymorphisms (SNPs) was constructed using CSI Phylogeny 1.3 [15]. Multilocus-sequence typing, plasmid-replicon typing, and identification of resistance genes were carried out using MLST 1.8 [16], PlasmidFinder 1.3 [17], and ResFinder 2.1 [18], respectively. Genomic sequences were annotated with RASTtk [19] and MiGAP [20], and genetic structure was compared in Easy-Fig [21]. A maximum-likelihood tree for IncFII plasmids was constructed using MEGA7 [22].

Bacterial conjugation and transformation

All bla_{NDM}-harboring plasmids identified in this study were transferred to laboratory strains by conjugation or transformation and the presence of bla_{NDM} and the plasmid-replicon type was confirmed by PCR [23–25].

Bacterial conjugation was carried out as described previously [26], with some modifications. Luria-Bertani (LB) broth cultures of *E. coli* isolates in the early exponential phase were mixed with the recipient strain, *E. coli* ML4909 [27], at a 1:10 ratio. The bacterial mixture was pelleted by centrifugation, transferred onto nitrocellulose membranes on an LB agar plate, and incubated at 37˚C for 6 h. Transconjugants were selected on the LB plate supplemented with 2 μg/mL meropenem and 100 μg/mL rifampicin.

For transformation, plasmids were extracted from overnight cultures of the isolates using the Plasmid Midi kit (Qiagen, Hilden, Germany). HST08 (Takara Bio, Shiga, Japan), a derivative of *E. coli* K12, was electroporated with the extracted plasmids using a Gene Pulser Xcell System (Bio-Rad, Hercules, CA, USA), and transformants were selected on brain-heart-infusion agar (BD Bacto) supplemented with 0.25 μg/mL meropenem.

Results and discussion

Characterization of carbapenem-resistant *E. coli*

Carbapenem-resistant *E. coli* strains ($n = 8$) were isolated at Yangon General Hospital, Yangon, Myanmar, from April to August 2015. Patients that tested positive for carbapenem-resistant *E. coli* were admitted to the following wards: hematology ($n = 5$), surgery ($n = 2$), and physical medicine ($n = 1$). Six isolates were obtained from blood specimens derived from patients in the hematology (M105, M109, M110, M214, and M217) and physical medicine (M107) wards, and two were isolated from urine derived from patients in the surgery ward (M213 and M216). All eight isolates were non-susceptible to not only β-lactams, including carbapenems, but also most other antibiotics tested, including aminoglycoside, quinolone, and chloramphenicol (Table 1). Interestingly, isolates from urine were highly resistant to chloramphenicol (MIC > 128), unlike isolates from blood (MIC: 8–32). Colistin appeared to be the only drug effective against all of the isolates examined, with fosfomycin representing a viable alternative for all strains, except for M110 (MIC: 16). By database searches using ResFinder and PlasmidFinder, all isolates were found to carry different types of plasmids encoding several antimicrobial-resistance determinants, including β-lactamases, supporting their multidrug-resistance phenotype (Fig 1B and S1 Table). Phylogenetic analysis based on genome-wide SNPs showed that the isolates belong to different previously defined phylogroups [28], with some belonging to pathogenic phylogroups D and F and others belonging to the commensal and less virulent phylogroups A and B1 (Fig 1A). The isolates were further classified into five multilocus-sequence types; no predominant sequence types were found (Fig 1B). Notably, the five isolates from the hematology ward are not closely related genetically, except for M105 and M109, indicating that different resistant strains disseminated in the ward. M105 and M109 differ in the types of NDM and bla_{NDM}-harboring plasmid (Fig 1B), suggesting that they had acquired the plasmids independently. This is in contrast to clonal expansion of other drug-resistant

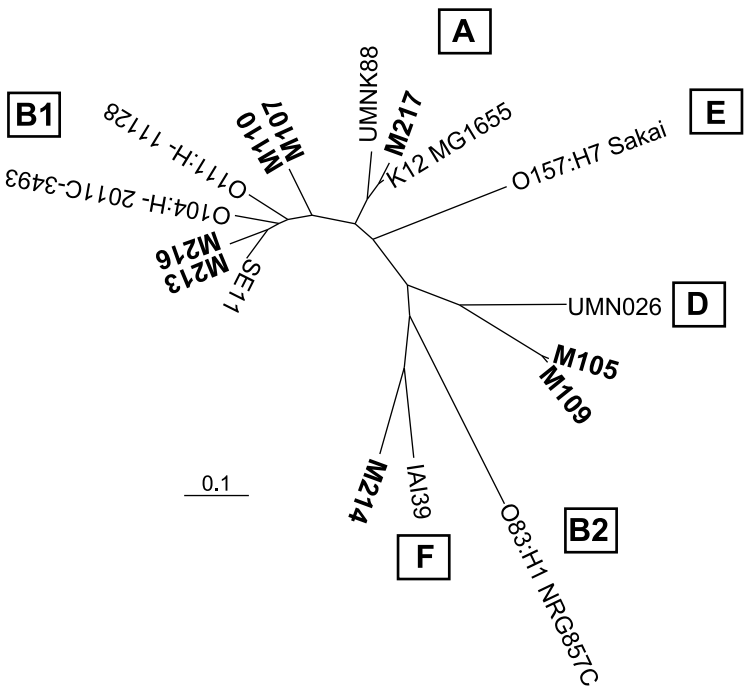
Table 1. MICs of antimicrobial agents for *E. coli* clinical isolates from Myanmar.

	MICs (μg/ml) for strain:							
Antimicrobial	M105	M107	M109	M110	M213	M214	M216	M217
Meropenem	8	4	8	4	>16	>16	8	8
Doripenem	8	2	4	4	>16	>16	>16	8
Imipenem	4	2	4	4	8	>16	>16	8
Ceftazidime	>64	>64	>64	>64	>64	>64	>64	>64
Cefmetazole	>32	>32	>32	>32	>32	>32	>32	>32
Ampicillin/SBT[a]	>64/128	>64/128	>64/128	>64/128	>64/128	>64/128	>64/128	>64/128
Piperacillin/TAZ[a]	>4/512	>4/512	>4/512	>4/512	>4/512	>4/512	>4/512	>4/512
Aztreonam	8	>32	>32	>32	>32	32	>32	32
Gentamicin	>8	>8	>8	>8	>8	>8	>8	>8
Amikacin	>32	>32	>32	≦4	>32	>32	>32	>32
Ciprofloxacin	>2	>2	>2	>2	>2	>2	>2	>2
Levofloxacin	>4	>4	>4	>4	>4	>4	>4	>4
Chloramphenicol	32	8	16	16	>128	16	>128	16
Minocycline	>8	>8	>8	>8	>8	>8	>8	2
ST[a]	>2/38	>2/38	>2/38	≦2/38	>2/38	>2/38	>2/38	>2/38
Colistin	≦0.25	≦0.25	≦0.25	0.5	0.5	1	0.5	0.5
Fosfomycin	≦4	≦4	≦4	16	≦4	≦4	≦4	≦4

[a] SBT; Sulbactam, TAZ; Tazobactam, ST; Sulfamethoxazole/Trimethoprim

https://doi.org/10.1371/journal.pone.0184720.t001

A



B

Strain	MLST	NDM type (*bla*)	Plasmid replicon type				
			IncFII	IncX3	IncA/C_2	Multireplicon IncF	Others
M110	410	NDM-7				FIA-FIB-Q1	
M107	410	NDM-5				FIA-FIB-Q1	
M217	167	NDM-5					I1
M213	101	NDM-4				FIA-FIB-FII	I1, R-Y
M216	101	NDM-4				FIA-FIB-FII	I1
M214	354	NDM-1, 5	5		1	FIA-FIB-FII-Q1	
M105	405	NDM-5				FIA-FIB-Q1	
M109	405	NDM-4				FIA-FIB-Q1	

bla_{NDM} genes
Other β-lactamases
Aminoglycoside resistance genes
Quinolone resistance genes
Macrolide resistance genes
Phenicol resistance genes
Sulphonamide and Trimethoprim resistance genes
Tetracycline resistance genes

Fig 1. Phylogeny of carbapenem-resistant *E. coli* isolated in Myanmar and features of their bla_{NDM}-harboring plasmids. (A) Phylogenetic tree of carbapenem-resistant *E. coli* isolates based on WGS. Reference genomes and several other available genomes were included for comparison. Phylogroups of the reference strains are indicated in boxes. (B) Multilocus-sequence types, NDM types, plasmid-replicon types, and antimicrobial-resistance profiles determined by database search. In strain M214, NDM types are indicated by numbers on black-shaded boxes.

https://doi.org/10.1371/journal.pone.0184720.g001

bacteria, such as methicillin-resistant *Staphylococcus aureus*, that cause nosocomial infections. A database search with WGS and Sanger-sequencing sequence data indicated that the isolates harbor different bla_{NDM} variants (bla_{NDM-1}, bla_{NDM-4}, bla_{NDM-5}, and bla_{NDM-7}) (Fig 1B) carried by nine distinct plasmids, of which eight belong to the known replicon types IncA/C_2

(n = 1), IncX3 (n = 3), and IncFII (n = 4). The remaining plasmid is comprised of replication genes of three replicon types (IncFIA, FIB, and Q1). Additionally, isolate M214 harbors two different types of bla_{NDM}-carrying plasmids (IncFII and A/C_2).

IncA/C_2 plasmids

IncA/C_2 plasmids are broad-host-range plasmids that are predominantly found among NDM-1 producers [29,30]. Indeed, a plasmid we designated as pM214_A/C_2 represents an IncA/C_2 plasmid harboring bla_{NDM-1} (Fig 2A). A BLAST search indicated that pM214_A/C_2 shares the highest degree of similarity with pNDM-1_Dok01 (GenBank accession no. AP012208.1), a bla_{NDM-1} plasmid isolated from a clinical *E. coli* isolate in Japan, but of Indian origin, with 99% identity and 93% query coverage [31]. Both plasmids encode bla_{NDM-1} in the ARI-A region, a resistance island designated for IncA/C_2 [32]; however, the gene clusters containing bla_{NDM-1} are inverted between the two plasmids. In pM214_A/C_2, the resistance island consists of two parts: an IS*Aba125*-mediated composite transposon (Tn*125*) and a Tn*1548*-like transposon. The former region, which harbors bla_{NDM-1}, completely matches that in pNDM-BJ01 (GenBank accession no. JQ001791.1) (Fig 2B), which was isolated from a clinical isolate of

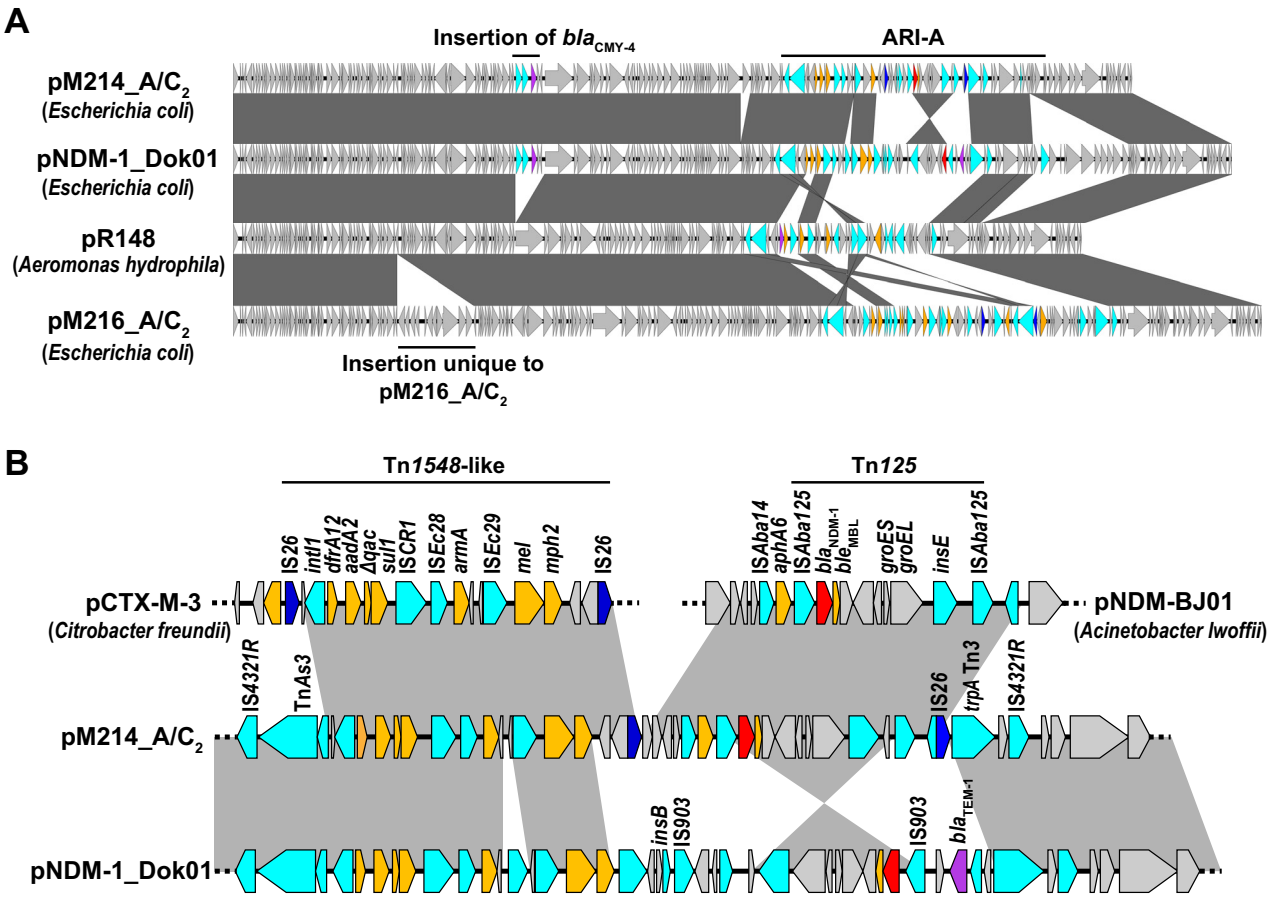


Fig 2. IncA/C_2 plasmids harboring bla_{NDM-1}. (A) Comparison of IncA/C_2 plasmids isolated in this study with similar and/or putative backbone plasmids pNDM-1_Dok01 and pR148. Homologous regions are shaded dark gray. Genes are indicated in colors as follows: bla_{NDM-1}, red; other β-lactamases, purple; other antimicrobial-resistance genes, orange; IS*26*, blue; other mobile genetic elements, cyan; other accessory genes, light gray. The origins of the indicated plasmids are shown in parentheses. pM214_ A/C_2 and pNDM-1_Dok01 harbor the class C β-lactamase gene, bla_{CMY-4}. (B) Schematic representation of the genetic context of bla_{NDM-1} in pM214_ A/C_2 and in other plasmids.

https://doi.org/10.1371/journal.pone.0184720.g002

Acinetobacter lwofii [33]. Notably, Tn*125* is often detected in *Acinetobacter*; however, the intact form of this transposon has never been found in *Enterobacteriaceae* [34]. This gene cluster is bracketed by IS*26*-insertion sequences in pM214_ A/C_2, suggesting that the plasmid acquired bla_{NDM-1} from pNDM-BJ01 or a closely related plasmid via a single IS*26*-mediated gene transfer. Additionally, the Tn*1548*-like transposon is located adjacent to Tn*125* (Fig 2B). This 15,525-bp fragment contains a class 1 integron comprising several antimicrobial-resistance genes and is identical to an unrelated plasmid (pCTX-M-3, GenBank accession no. AF550415.2) [35], except for one nucleotide. This cluster is partially conserved in other IncA/C_2 plasmids, such as pNDM-1_Dok01 and pM216_A/C_2, isolated from strain M216. Therefore, a Tn*1548*-like transposon might have contributed to the ARI-A island in the putative ancestor of these plasmids, with prototypic features being well conserved in pM214_A/C_2. However, pM216_A/C_2 encodes neither bla_{NDM-1} nor bla_{CMY-4}, both of which are present in pM214_A/C_2, but not in the putative precursor plasmid pR148 (GenBank accession no. JX141473.1) [36]. Moreover, the gene array in the ARI-A resistance island significantly differs from that in pM214_A/C_2 (Fig 2A), suggesting that these two plasmids were derived from different precursors.

IncX3 plasmids

We detected three IncX3 plasmids encoding NDM variants: the bla_{NDM-4}-harboring plasmids pM213_X3 and pM216_X3 and the bla_{NDM-7}-harboring plasmid pM110_X3. The three plasmids are highly similar to previously reported IncX3 plasmids: the bla_{NDM-4}-harboring plasmid pJEG027 (GenBank accession no. KM400601.1) [37], the bla_{NDM-5}-harboring plasmid pNDM_MGR194 (KF220657.1) [38], and the bla_{NDM-7}-harboring plasmids pKpN01-NDM7 (CP012990.1) [39] and pOM26-1 (KP776609.1), suggesting they share a common ancestor (Fig 3A and 3B). Notably, pJEG027 was isolated from a *K. pneumoniae* isolate from an Australian patient hospitalized in Myanmar before returning to Australia [37]. In these IncX3 plasmids, the bla_{NDM} genes are encoded in three distinct structures with various lengths of upstream insertion sequences as shown in Fig 3A. Additionally, all of the plasmids found in the survey lack a 92-bp fragment upstream of *taxD* as compared with pJEG027 and pNDM_MGR194 (Fig 3A, arrow) and as previously observed in the bla_{NDM-7} plasmids pKpN01-NDM7 and pOM26-1 [37]. The IncX3 plasmids do not carry antimicrobial-resistance genes other than bla_{NDM} and apparently represent early steps in the evolution and spread of the bla_{NDM} gene [38]. Nonetheless, these plasmids encode distinct NDM variants. Therefore, it is likely that bla_{NDM} variants have evolved via nucleotide substitutions within the IncX3 plasmid [37], [39]. This process might be driven by selection for stronger resistance, given that NDM-4, -5, and -7 exhibit higher β-lactamase activity than does NDM-1 [40–42].

IncFII plasmids

IncFII was the most predominant type of bla_{NDM}-harboring plasmid in the isolates investigated. Here, we isolated the bla_{NDM-4}-harboring plasmid pM109_FII and the bla_{NDM-5}-harboring plasmids pM107_FII, pM214_FII, and pM217_FII (Fig 4A). In contrast to IncX3 plasmids, these plasmids carry other β-lactamases, as well as resistance genes against several antibiotics, including aminoglycosides, macrolides, sulphonamides, and trimethoprims. The overall genetic structure of these plasmids resembles that of known bla_{NDM} IncFII plasmids, such as pGUE-NDM (GenBank accession no. JQ364967.1) [43], pMC-NDM (HG003695.1) [44], and pCC1409-1 (KT725789.1)/pCC1410-1 (KT725788.1) [45], which were found in clinical isolates originating from India, Congo, and United Arab Emirates, respectively. Nevertheless, the genetic structure of the IncFII plasmids is relatively diverse as compared to those of IncX3

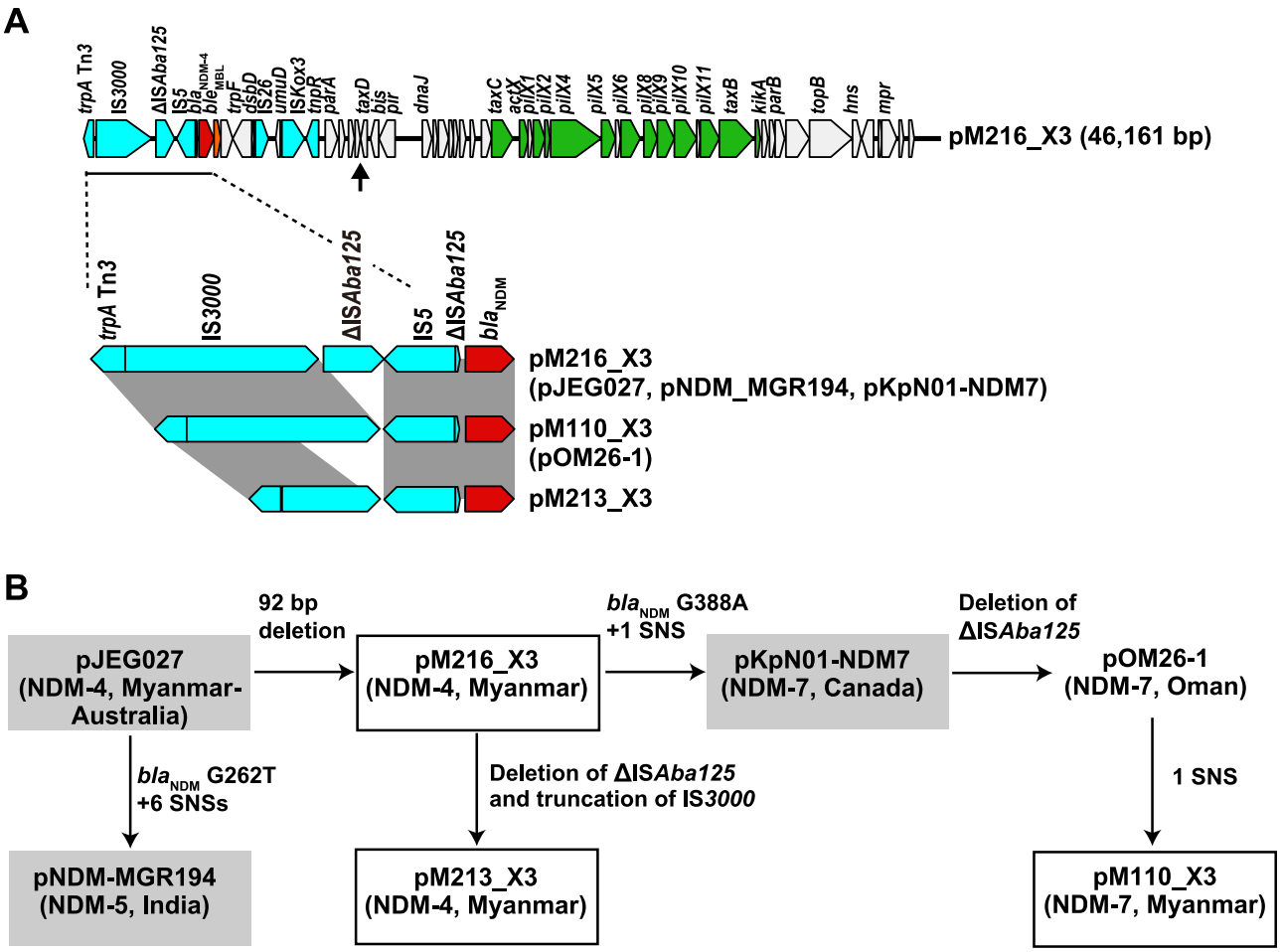


Fig 3. IncX3 plasmids harboring bla_{NDM}. (A) Schematic representation of the bla_{NDM-4} plasmid pM216_X3 and comparison of the genetic context of bla_{NDM} in IncX3 plasmids. Genes are indicated in colors as defined in Fig 2, except that genes involved in conjugal transfer are depicted in green. A 92-bp deletion that is found in pM110_X3, pM213_X3, pM216_X3, pKpN01-NDM7, and pOM26-1 is indicated by the arrow. (B) Evolutionary relationship of fully sequenced IncX3 plasmids harboring bla_{NDM} as inferred from genetic variances. Encoded NDM variants are shown in parentheses along with geographical origin. The plasmids identified in this study are boxed. pJEG027, pNDM-MGR194, and pKpN01-NDM7 were isolated from *K. pneumoniae* isolates and are shaded gray. pOM26-1 was isolated from an *E. coli* isolate. SNS, single nucleotide substitution.

https://doi.org/10.1371/journal.pone.0184720.g003

plasmids, as we found several insertions/deletions and clear traces of genetic mobilization via IS*26*. pM109_FII, p214_FII, and p217_FII contain a complete transfer operon and were successfully transferred to *E. coli* ML4909 by conjugation. However, a fragment was missing from pM107_FII (Fig 4A, arrowhead), rendering the transfer operon incompetent.

Concerning the genetic context of bla_{NDM}, pM109_FII is distinct from other IncFII Myanmar plasmids, but similar to the bla_{NDM-1} plasmid pGUE-NDM (Fig 4B). Both plasmids share a 12-kbp resistance-gene region surrounding bla_{NDM}. In pM109_FII, an additional gene cassette bracketed by two IS*26* sequences containing the class A β-lactamase gene bla_{TEM-1} and the aminoglycoside resistance-coding gene *rmtB* is located downstream of this region. Interestingly, this gene cassette was also found in the bla_{NDM} region of other plasmids, including pMC-NDM (Fig 4B). pM109_FII differs from other IncFII Myanmar plasmids in that it lacks the macrolide-resistance gene *ermB* and the toxin-antitoxin module *pemI-pemK* (Fig 4A and 4B).

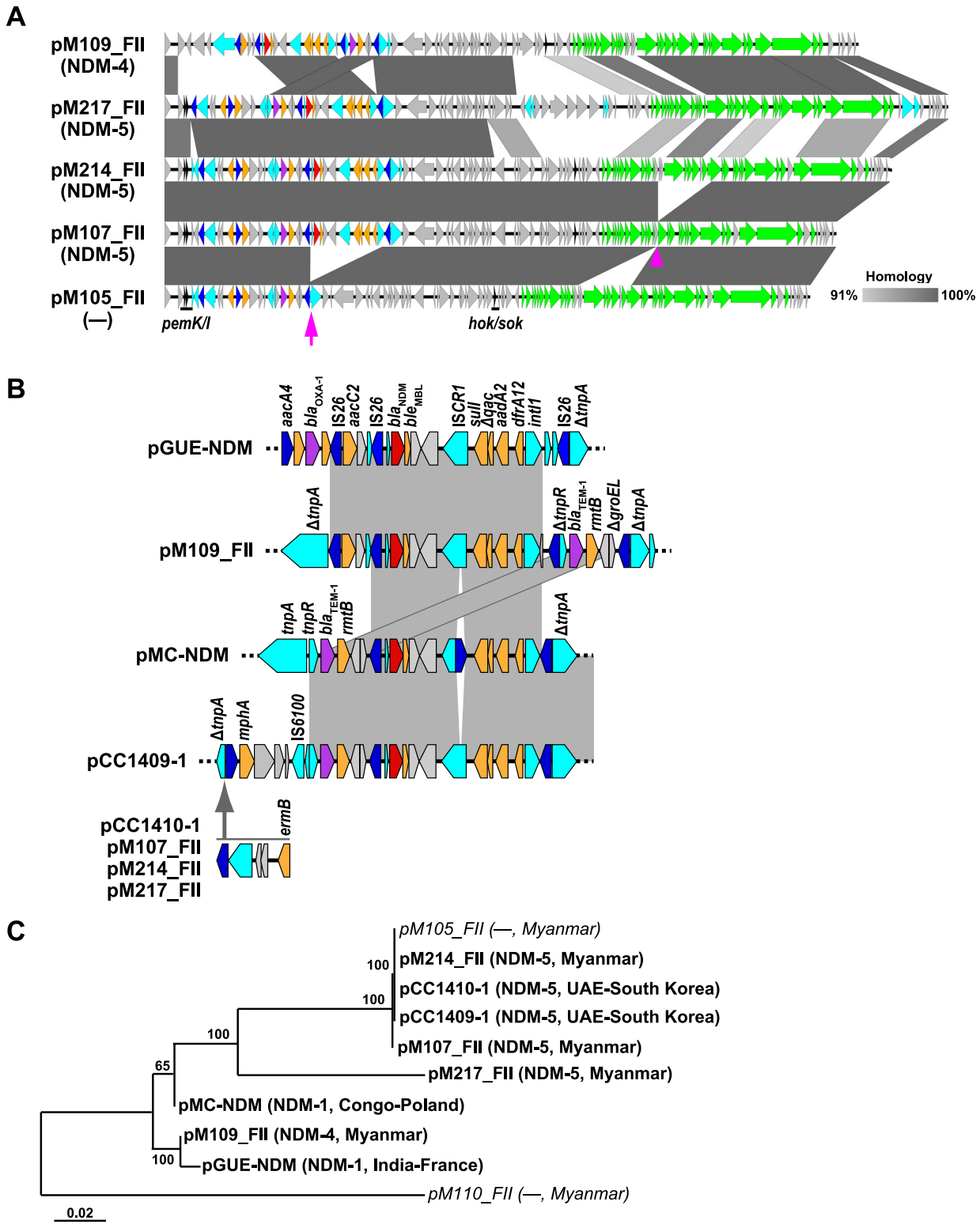


Fig 4. IncFII plasmids harboring bla_{NDM}. (A) Comparison of IncFII plasmids harboring bla_{NDM} in Myanmar isolates. Homologous regions are shaded gray, with the percent identity shaded according to the color bar. The arrowhead indicates a deletion in pM107_FII that encompasses several genes involved in conjugal transfer. The arrow indicates a deletion of a gene cluster carrying bla_{NDM-5} in pM105_FII. Genes are indicated in colors as defined in Fig 2, except that genes involved in conjugal transfer and toxin-antitoxin

modules are illustrated in green and black, respectively. (B) Schematic representation of the genetic context of bla_{NDM}. Identical regions are shaded gray, whereas arrows denote gene-cluster insertions. (C) Maximum-likelihood tree of IncFII plasmids harboring bla_{NDM}. Encoded NDM variants are shown in parentheses along with geographical origin. IncFII plasmids lacking bla_{NDM} and found in Myanmar isolates were also included and are indicated in italics. Bootstrap support values based on 1000 replications are indicated at the branching points. All the plasmids, except for pCC1409-1 and pCC1410-1, which were identified in *K. pneumoniae* isolates, were isolated from *E. coli*.

https://doi.org/10.1371/journal.pone.0184720.g004

pM107_FII and pM214_FII clustered with pCC1409-1 and pCC1410-1 in the phylogenetic tree, whereas pM217_FII was assigned to a different branch (Fig 4C). The transfer operon in the former four plasmids differs slightly from that in other IncFII plasmids, with pM214_FII and pM217_FII sharing 81% nucleotide identity in this region. Interestingly, the transfer operon in pM214_FII is completely identical to that in known plasmids, such as pC15-1a [46]. Therefore, the transfer region in pM214_FII and related plasmids might be unrelated to other plasmids, possibly as a result of recombination.

Additionally, we found two IncFII plasmids lacking bla_{NDM} (pM110_FII and pM105_FII). pM110_FII encodes resistance genes against quinolone and tetracycline and is phylogenetically distinct from other IncFII plasmids harboring β-lactamases and macrolide-resistance genes (Figs 1B and 4C). pM105_FII, a plasmid identified in strain M105, clustered with the bla_{NDM-5}-harboring plasmids pM107_FII and pM214_FII. Indeed, pM105_FII is very similar to pM214_FII, except for the region surrounding bla_{NDM-5} (Fig 4A, arrow), with 99.3% nucleotide identity outside of this region. Therefore, it is likely that pM105_FII had been encoded previously, but recently lost bla_{NDM-5} along with other neighboring genes. This region is bracketed by two IS*26* sequences, implying that IS*26* was involved in the loss of this fragment [47]. Additionally, the M105 isolate encodes bla_{NDM-5} on a multireplicon IncF plasmid designated as pM105_mF, and, notably, the genetic context of bla_{NDM-5} in this plasmid is identical to that in pM214_FII and related plasmids (Fig 5). Furthermore, the resistance island in pM107_FII and pM214_FII bracketed by two IS*26* sequences is conserved in pM105_mF (Fig 5, yellow). This implies that pM105_mF likely acquired bla_{NDM-5} from the pM105_FII precursor or related IncFII plasmids via IS*26*.

Other plasmids

Multiple replicons might broaden the plasmid host range and enable the maintenance of incompatible replicons [24]. To the best of our knowledge, the pM105_mF plasmid backbone comprising IncFIA-, FIB-, and Q1-replication genes (Fig 1B, designated FIA-FIB-Q1) has not been previously reported. A BLAST search revealed a partial match to pV228-a (Fig 5, dark green), with 99% identity, but query coverage of only 54%. pV228-a, containing IncFIA-, FIB-, and FII-replication genes, has been identified in an *E. coli* strain isolated from a sewage-treatment plant in India [48], and similar multireplicon F plasmids have been found in three other Myanmar isolates (Fig 1B), with one containing an IncFII-replication gene (FIA-FIB-FII-Q1). These plasmids commonly encode several antimicrobial-resistance determinants, such as bla_{TEM-1}, and resistance genes against sulphonamide, trimethoprim, and tetracycline. Collectively, these data indicate that multireplicon plasmids with several antimicrobial-resistance genes might be widespread in Myanmar.

Although it is unclear whether the bla_{NDM-5} gene cluster was transferred directly from a precursor of pM105_FII onto pM105_mF, our findings demonstrated that mobilization of bla_{NDM} between plasmids resulted in a novel resistance plasmid. Additionally, the number of antimicrobial-resistance genes in pM105_mF was higher than that in IncFII plasmids

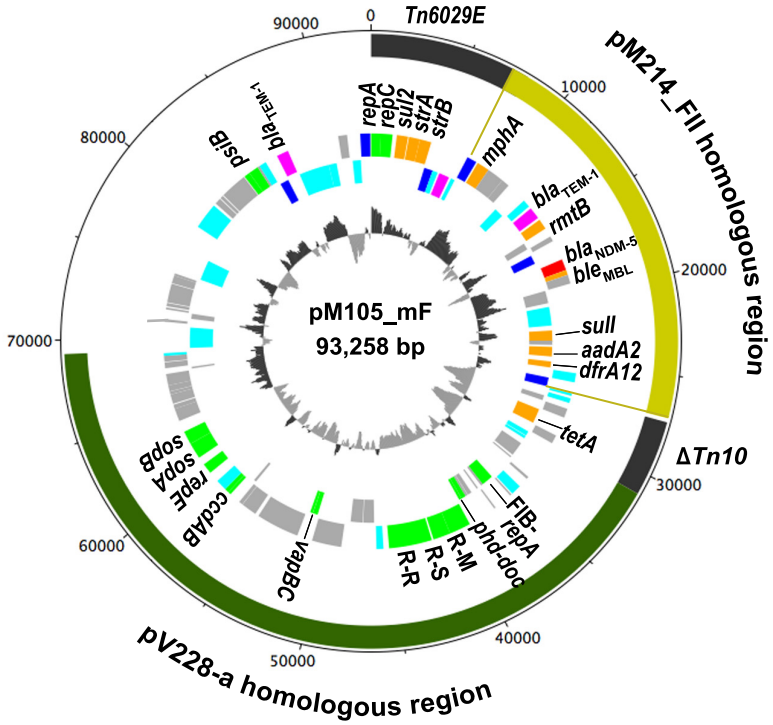


Fig 5. Circular map of plasmid pM105_mF. The outermost circle indicates size in bp. The next circle indicates composite transposons (dark gray) and regions homologous to the bla_{NDM-5} region in pM214_FII (yellow) and pV228-a (dark green). The third and fourth circles show coding sequences transcribed clockwise and anti-clockwise, respectively. Genes are indicated in colors as defined in Fig 2, except that genes involved in plasmid replication and maintenance are illustrated in green. R-M, R-S, and R-R denote restriction-modification genes (M, methylase; S, specificity; and R, restriction). The innermost circle shows GC content.

https://doi.org/10.1371/journal.pone.0184720.g005

harboring bla_{NDM-5} (Fig 1B and S1 Table), suggesting that recombination increased the number of resistance genes encoded on the same plasmid as bla_{NDM}. As shown in Fig 1B, several multidrug-resistant plasmids, such as multireplicon F plasmids, co-exist with bla_{NDM} plasmids in all isolates. Therefore, clinical settings in Myanmar appear to provide a breeding ground for novel resistance plasmids, and thus require more vigilance from national and local public health authorities.

Conclusions

We genetically characterized clinical carbapenem-resistant *E. coli* isolates from Myanmar and discovered multiple-resistance plasmids and several NDM variants in the diverse genetic backgrounds of the bacteria, even within the eight isolates examined. These findings suggest dissemination of NDM via multiple introductions and/or prolonged presence in the hospital allowing for recombination and dissemination via horizontal gene transfer into various genetic backgrounds. The situation seems to be similar to that reported in India, where NDM is already endemic and is found not only in healthcare settings, but also in the community [11], [49]. Therefore, further surveillance, including both the hospital and the community, is warranted to understand the dissemination of CRE in Myanmar.

Supporting information

S1 Table. Antimicrobial resistance determinants carried by the plasmids identified in *E. coli* clinical isolates in Myanmar.
(DOCX)

Acknowledgments

We are grateful to Yoshikazu Ishii, Masato Suzuki and Mizue Anda for helpful discussion and comments. We also thank Yumi Sasaki and Kazuhiro Maeda for technical assistance on antimicrobial susceptibility testing.

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