Spreading Patterns of NDM-Producing *Enterobacteriaceae* in Clinical and Environmental Settings in Yangon, Myanmar

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ABSTRACT The spread of carbapenemase-producing *Enterobacteriaceae* (CPE), contributing to widespread carbapenem resistance, has become a global concern. However, the specific dissemination patterns of carbapenemase genes have not been intensively investigated in developing countries, including Myanmar, where NDM-type carbapenemases are spreading in clinical settings. In the present study, we phenotypically and genetically characterized 91 CPE isolates obtained from clinical ($n =$ 77) and environmental ($n =$ 14) samples in Yangon, Myanmar. We determined the dissemination of plasmids harboring genes encoding NDM-1 and its variants using whole-genome sequencing and plasmid analysis. IncFII plasmids harboring bla_{NDM-5} and IncX3 plasmids harboring bla_{NDM-4} or bla_{NDM-7} were the most prevalent plasmid types identified among the isolates. The IncFII plasmids were predominantly carried by clinical isolates of *Escherichia coli*, and their clonal expansion was observed within the same ward of a hospital. In contrast, the IncX3 plasmids were found in phylogenetically divergent isolates from clinical and environmental samples classified into nine species, suggesting widespread dissemination of plasmids via horizontal transfer. Half of the environmental isolates were found to possess IncX3 plasmids, and this type of plasmid was confirmed to transfer more effectively to recipient organisms at a relatively low temperature (25°C) compared to the IncFII plasmid. Moreover, various other plasmid types were identified harboring bla_{NDM-1}, including IncFIB, IncFII, IncL/M, and IncA/C$_2$, among clinical isolates of *Klebsiella pneumoniae* or *Enterobacter cloacae* complex. Overall, our results highlight three distinct patterns of the dissemination of bla_{NDM}-harboring plasmids among CPE isolates in Myanmar, contributing to a better understanding of their molecular epidemiology and dissemination in a setting of endemicity.

KEYWORDS *Enterobacteriaceae*, Myanmar, bla_{NDM}, carbapenemase, carbapenems, plasmid-mediated resistance

Carbapenemases are β-lactamases that hydrolyze almost all types of β-lactams, including carbapenems, which are the last line of defense against multidrug-resistant bacteria. Recent years have witnessed a rapid increase in the occurrence of *Enterobacteriaceae* species resistant to carbapenems; this resistance is mainly conferred by carbapenemase genes encoded by plasmids (1). Such carbapenemase-producing *Enterobacteriaceae* (CPE) are resistant to most of the commonly prescribed antibiotics, and infections by these pathogens are associated with poor prognosis, thereby raising serious concerns during treatment in clinical settings. Moreover, CPE have also been

found in environmental samples such as sewage, tap water, and foodstuffs, posing a severe threat to public health (2–4).

The widespread dissemination of CPE may have been expedited by two mechanisms. The first is a clonal expansion of an organism carrying a carbapenemase-encoding plasmid, as represented by the spread of *Klebsiella pneumoniae* clonal complex 258 producing *K. pneumoniae* carbapenemase (KPC), mainly in the United States and some other countries (5, 6). The other potential mechanism is via the horizontal transfer of carbapenemase-encoding plasmids to naive *Enterobacteriaceae* present in the human and animal gut or even in the environment (7–9). Further, carbapenemase genes often readily transfer between replicons via a transposon-mediated mechanism, resulting in the emergence of novel carbapenemase-encoding plasmids or chromosomal carriage of the gene (10). Recently, whole-genome sequencing (WGS) has been exploited to conduct a high-resolution analysis of the dissemination of carbapenemase genes in clinical settings. However, to date, these analyses have mainly been conducted in the United States and European countries, and few studies have investigated the route of dissemination of carbapenemase genes in resource-poor settings (11–13), where CPE are often endemic (14). In South and Southeast Asian countries, the New Delhi metallo-β-lactamase (NDM) gene bla_{NDM} is widely distributed, representing a main public health concern (6, 15). NDM-producing *Enterobacteriaceae* are also reported to be rapidly spreading in Balkan countries and China (14), and they have been frequently found in European countries and occasionally in the USA (6). Although bla_{NDM-1} and its variants have been found in various *Enterobacteriaceae* species harbored by different types of plasmids (16), there is a paucity of knowledge on the patterns of their dissemination. Thus, further investigation is needed to initiate effective control measures to prevent their further spread.

In a survey conducted in a tertiary-care hospital and two private hospitals in Yangon, Myanmar, *Escherichia coli* and *K. pneumoniae* isolates carrying bla_{NDM} genes were isolated (17). *E. coli* isolates carrying the bla_{NDM} genes were also found in another hospital in Yangon (18), suggesting that organisms carrying these genes are spreading in the region. We previously conducted WGS of eight carbapenem-resistant *E. coli* clinical isolates at a single tertiary-care hospital in Yangon; they were found to be phylogenetically distinct and to possess various types of bla_{NDM}-harboring plasmids (19). To further understand the routes of dissemination and diversity of bla_{NDM}-harboring bacteria, in the present study, we conducted a larger-scale WGS and plasmid analysis of 77 clinical and 14 environmental CPE isolates obtained in Yangon.

RESULTS

Characteristics of clinical and environmental CPE isolates. During a 21-month surveillance in a tertiary-care hospital in Yangon, a total of 2,262 *Enterobacteriaceae* were isolated from clinical specimens, 91 (4%) of which showed resistance to meropenem. We excluded isolates that originated from the same specimens and that were identified as same species and isolates without carbapenemase genes from further analyses. As a result, 77 isolates carrying carbapenemase genes, including 8 *E. coli* isolates that we previously reported (19), were selected for further analysis using Illumina sequencing. These included *E. coli* ($n = 43$), *K. pneumoniae* ($n = 17$), *Klebsiella quasipneumoniae* ($n = 1$), *Citrobacter freundii* ($n = 3$), *Citrobacter amalonaticus* ($n = 1$), and *Enterobacter cloacae* complex ($n = 12$) (Fig. 1; Tables S1 and S2). We identified four variants of bla_{NDM} genes, namely, bla_{NDM-1} ($n = 19$), bla_{NDM-4} ($n = 14$), bla_{NDM-5} ($n = 40$), and bla_{NDM-7} ($n = 5$), among which bla_{NDM-5} was the most prevalent and was found in more than half of the total isolates (51.9%). Another type of carbapenemase gene, $bla_{OXA-181}$, was found in three isolates (two *E. coli* isolates and one *Enterobacter xiangfangensis* isolate) that coexisted with bla_{NDM-1} or bla_{NDM-5}.

Most of the clinical isolates were not susceptible to commonly used clinical antibiotics, such as levofloxacin (95.1%), minocycline (79.2%), and amikacin (76.6%) (Table S3), whereas the majority of the isolates were susceptible to colistin (97.4% [75/77]) and
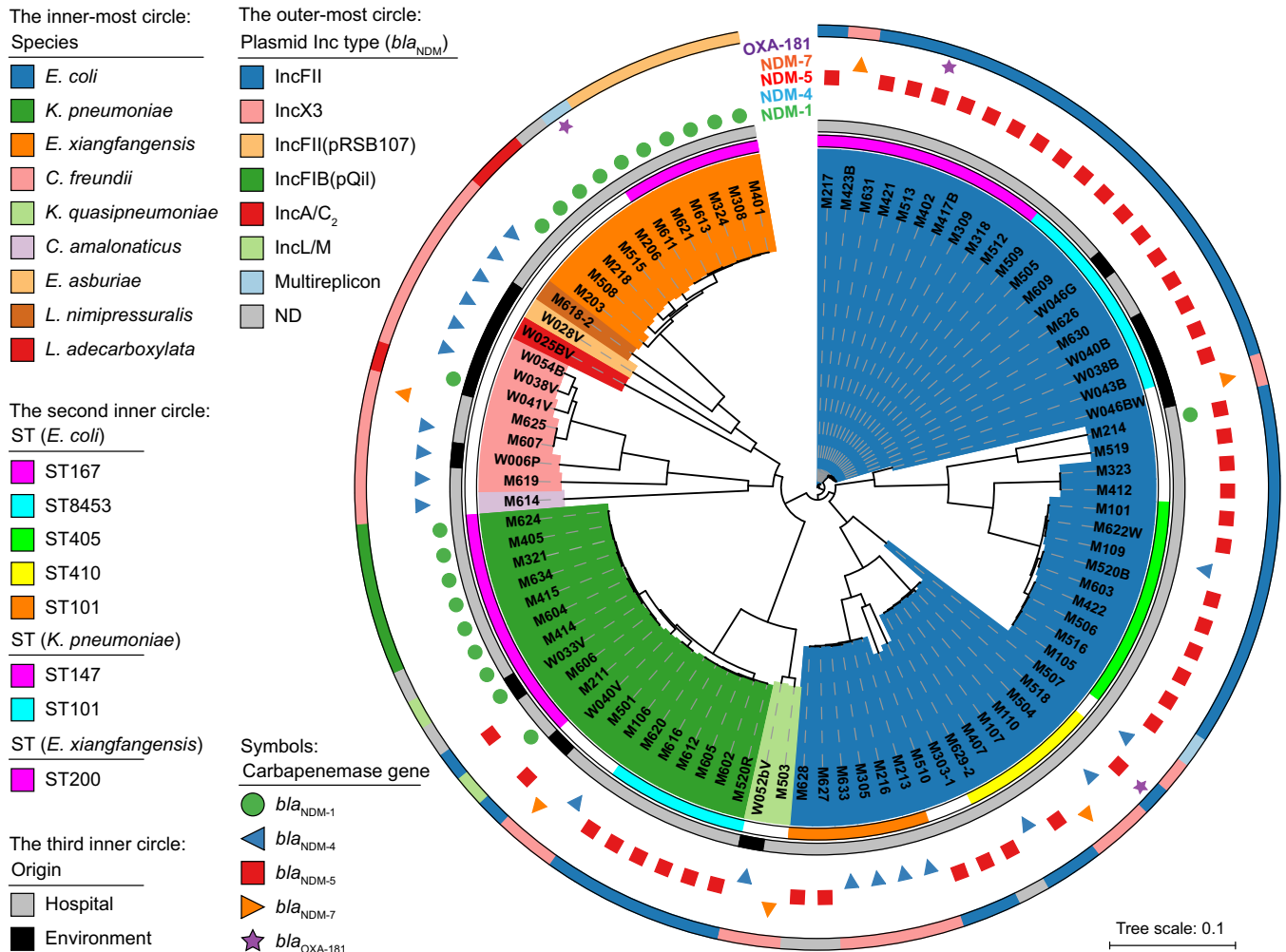
FIG 1 Diversity of CPE isolates from Myanmar. The innermost colored regions show the species of the bacterial isolates. The next inner circle shows major MLST sequence types (STs) found more than two among the isolates. The third inner circle indicates the origin of each isolate. The next outer region indicates the carbapenemase gene carried by each isolate. The outermost circle shows the Inc replicon type of bla_{NDM}-harboring plasmids carried by each CPE isolate. Isolate M214 carried an IncA/C$_2$ plasmid harboring bla_{NDM-1} in addition to an IncFII plasmid harboring bla_{NDM-5}. The genome sequence of *E. coli* K-12 MG1655 (GenBank accession number NC_000913.3) was used as a reference for the construction of the phylogenetic tree. ND, not determined; NA, not applicable.

fosfomycin (63.6% [49/77]). The colistin resistance gene *mcr* was not detected in any of our Myanmar isolates.

A total of 54 sewage samples were collected from six locations adjacent to the hospital and were screened for the presence of isolates harboring carbapenemase genes. Of the 206 carbapenem-resistant isolates, there were 17 *Enterobacteriaceae* (8.3%), 14 of which carried the bla_{NDM} variants bla_{NDM-1}, bla_{NDM-4}, bla_{NDM-5}, and bla_{NDM-7} and 3 possessing none of the four major carbapenemase genes, namely, the bla_{NDM}, bla_{KPC}, bla_{IMP}, and bla_{OXA-48}-like genes. These environmental CPE isolates comprised eight species, including one isolate each of *Enterobacter asburiae* and *Leclercia adecarboxylata* that were found only in the environmental samples (Fig. 1; Table S1 and S2). The antimicrobial susceptibility profile of the environmental isolates was similar to that of the clinical isolates; however, there was a higher frequency of environmental isolates that were susceptible to aztreonam, aminoglycosides, quinolones, and chloramphenicol (Table S3). We also screened 54 drinking water samples and obtained three carbapenem-resistant *Enterobacter cloacae* isolates; however, the above-named four carbapenemase genes were not detected.

One of the other remarkable features of the Myanmar isolates was the prevalence of the extended-spectrum β-lactamase gene $bla_{CTX-M-15}$, which was found in 70.3%
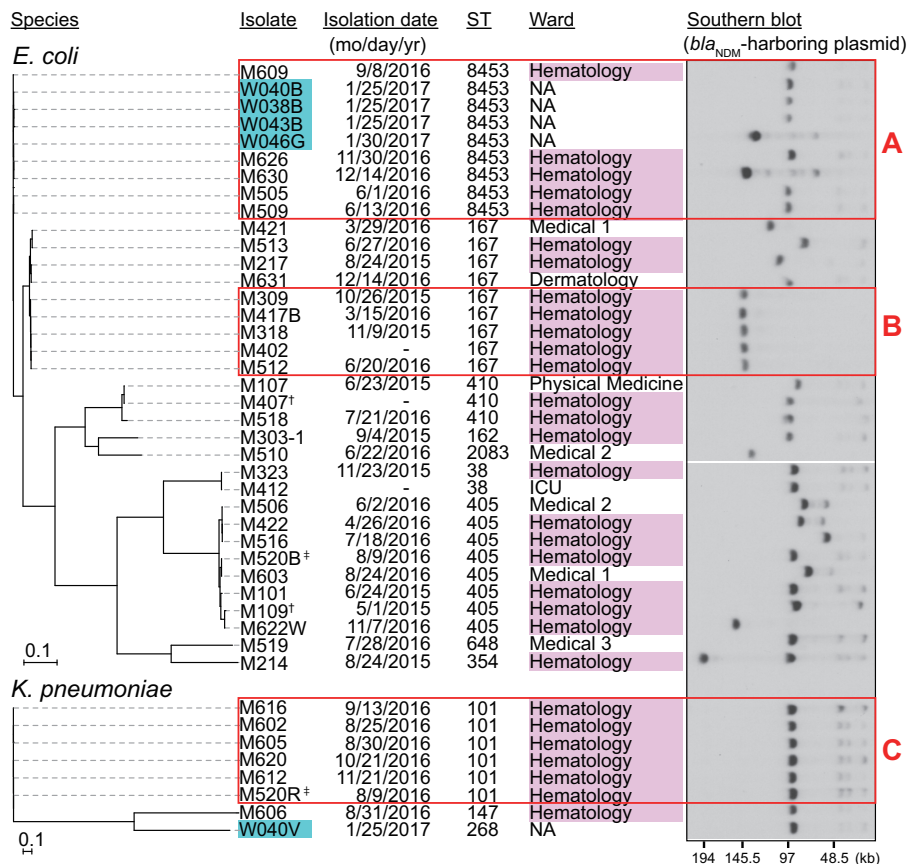
Species	Isolate	Isolation date (mo/day/yr)	ST	Ward	Southern blot (bla$_{NDM}$-harboring plasmid)
E. coli	M609	9/8/2016	8453	Hematology	
	W040B	1/25/2017	8453	NA	A
	W038B	1/25/2017	8453	NA	
	W043B	1/25/2017	8453	NA	
	W046G	1/30/2017	8453	NA	
	M626	11/30/2016	8453	Hematology	
	M630	12/14/2016	8453	Hematology	
	M505	6/1/2016	8453	Hematology	
	M509	6/13/2016	8453	Hematology	
	M421	3/29/2016	167	Medical 1	
	M513	6/27/2016	167	Hematology	
	M217	8/24/2015	167	Hematology	
	M631	12/14/2016	167	Dermatology	
	M309	10/26/2015	167	Hematology	B
	M417B	3/15/2016	167	Hematology	
	M318	11/9/2015	167	Hematology	
	M402	-	167	Hematology	
	M512	6/20/2016	167	Hematology	
	M107	6/23/2015	410	Physical Medicine	
	M407†	-	410	Hematology	
	M518	7/21/2016	410	Hematology	
	M303-1	9/4/2015	162	Hematology	
	M510	6/22/2016	2083	Medical 2	
	M323	11/23/2015	38	Hematology	
	M412	-	38	ICU	
	M506	6/2/2016	405	Medical 2	
	M422	4/26/2016	405	Hematology	
	M516	7/18/2016	405	Hematology	
	M520B‡	8/9/2016	405	Hematology	
	M603	8/24/2016	405	Medical 1	
	M101	6/24/2015	405	Hematology	
	M109†	5/1/2015	405	Hematology	
	M622W	11/7/2016	405	Hematology	
	M519	7/28/2016	648	Medical 3	
	M214	8/24/2015	354	Hematology	
K. pneumoniae	M616	9/13/2016	101	Hematology	
	M602	8/25/2016	101	Hematology	
	M605	8/30/2016	101	Hematology	C
	M620	10/21/2016	101	Hematology	
	M612	11/21/2016	101	Hematology	
	M520R‡	8/9/2016	101	Hematology	
	M606	8/31/2016	147	Hematology	
	W040V	1/25/2017	268	NA	

0.1

194 145.5 97 48.5 (kb)

FIG 2 Phylogenetic relationships among the CPE isolates with IncFII-type plasmids harboring bla$_{NDM}$. All of the isolates, except for two bla$_{NDM-4}$ carriers (†), harbored the bla$_{NDM-5}$ gene on the IncFII-type plasmid. Environmental isolates are marked in cyan. bla$_{NDM}$-harboring plasmids were detected by S1-PFGE-Southern blot analysis with a bla$_{NDM}$ probe. Red rectangles indicate closely related isolates. Cluster A, 7 to 20 SNPs, >99.9% ANI; cluster B, 6 to 22 SNPs, >99.9% ANI; cluster C, 8 to 28 SNPs, >99.9% ANI. SNPs between the isolates in cluster B and the other ST167 isolates were 833 to 1,051. Genome sequences of *E. coli* K-12 MG1655 and *K. pneumoniae* AATZP (GenBank accession number CP014755.1) were used as references for construction of the phylogenetic trees of respective species. Isolate M214 carried an IncA/C$_2$ plasmid harboring bla$_{NDM-1}$ (176 kb) in addition to an IncFII plasmid harboring bla$_{NDM-5}$ (95 kb). ‡, M520B and M520R were isolated from the same specimen. -, unknown.

(64/91) of the isolates that include five different species (*E. coli*, *K. pneumoniae*, *K. quasipneumoniae*, *C. freundii*, and *E. xiangfangensis*) isolated from clinical and environmental samples (Table S2).

Isolates carrying IncFII-type bla$_{NDM}$-harboring plasmids. The Inc types of bla$_{NDM}$-harboring plasmids carried by the isolates were determined by PlasmidFinder, BLAST, and Southern blot analysis. The IncFII-type plasmid was the most prevalent type of plasmid detected in our isolates, carried by *E. coli* and *K. pneumoniae* (Table S2). Almost all of the bla$_{NDM-5}$ genes detected (41/45) were found on this plasmid type, which also carried the bla$_{NDM-4}$ gene in two of the *E. coli* isolates. The IncFII plasmids were around 90 kb; however, several of the bla$_{NDM-5}$-harboring plasmids showed different sizes, ranging from 50 to 150 kb (Fig. 2).

The *E. coli* isolates carrying IncFII-type plasmids (n = 35) were diverse, with nine different sequence types (STs) detected, including a novel ST (Fig. 2). The single nucleotide polymorphism (SNP)-based phylogenetic analysis identified two clusters consisting of highly related isolates, designated A and B. The isolates of cluster A differed from each other by 7 to 20 SNPs and were assigned to ST8453, a single locus variant of ST167. This group included five clinical isolates obtained from the hematology ward of the hospital and four environmental isolates, suggesting the spread of organisms with IncFII plasmids both inside and outside the hospital. Cluster B included

five ST167 isolates that differed by 6 to 22 SNPs, which all harbored a relatively larger plasmid (137 kb) than the other IncFII-type plasmids found in this study (Fig. 2). All of the isolates included in cluster B were also obtained from the hematology ward, suggesting that clonal expansion of the organisms occurred in this ward of the hospital.

Of the eight *K. pneumoniae* isolates carrying IncFII-type plasmids, six were geno-typed as ST101 (Fig. 2, cluster C) and were closely related (8 to 28 SNPs between isolates). Moreover, all of these isolates were also obtained from the hematology ward, suggesting their clonal spread. Notably, *K. pneumoniae* M520R and *E. coli* M520B were isolated from the same patient (Fig. 2, marked with a double dagger). This was the first detection of the IncFII plasmid in a *K. pneumoniae* isolate during the surveillance period; thus, it is likely that transfer of the IncFII plasmid occurred from M520B to M520R within a patient.

Genomic structure of the IncFII-type plasmid pM309-NDM5. We determined the genomic structure of the *bla*$_{NDM-5}$ gene-harboring plasmid pM309-NDM5, carried by a cluster B isolate (M309), using a long-read sequencer (Fig. S1A). The plasmid possessed IncFIA replication gene in addition to IncFII (FAB formula, F36:A4:B− or F36:A20:B−). Its multidrug resistance region appeared to consist of two parts. One harbored the *bla*$_{NDM-5}$ gene and was entirely conserved in pM214_FII, a typical IncFII plasmid (F2:A-:B-) detected among our isolates (19) (Fig. S1B, region A). This genomic region was flanked by two intact IS*26* sequences and coharbored other genes encoding β-lactamases and those conferring resistance to aminoglycoside, macrolide, sulfon-amide, and trimethoprim. The other resistance region was bracketed by derivatives of Tn*5403* and Tn*2* and harbored *bla*$_{CTX-M-15}$ and other resistance genes against tetracy-cline, aminoglycoside, and chloramphenicol (Fig. S1B, region B). Several plasmids, including *E. coli* plasmid pLZ135-CTX (GenBank accession number MF353155.1), were found to possess this resistance region by a database search. pLZ135-CTX shared a common type of plasmid backbone (F36:A4:B- or F36:A20:B-) with pM309-NDM5, and these plasmids were highly homologous (96% coverage and 99% nucleotide identity) except for the multidrug resistance region harboring *bla*$_{NDM-5}$. In pM309-NDM5, the left and right sides of the resistance region containing *bla*$_{NDM-5}$, including the two IS*26* sequences, were bracketed by derivatives of Tn*2* (ΔTn*2*) and IS*Sba14* (ΔIS*Sba14*), respectively. Of note, both of these gene configurations found at boundaries between the resistance region and the plasmid backbone, i.e., ΔTn*2*-IS*26* and IS*26*-ΔIS*Sba14*, were also conserved in pLZ135-CTX.

Isolates carrying IncX3-type *bla*$_{NDM}$-harboring plasmids. IncX3 was the second most prevalent plasmid type detected among *bla*$_{NDM}$-harboring plasmids. The IncX3-type plasmids harbored *bla*$_{NDM-4}$ or *bla*$_{NDM-7}$ and were found in 24 isolates (26.4% of all isolates) consisting of 17 clinical and 7 environmental isolates, comprising nine different species. Unlike the IncFII plasmids, the clinical isolates harboring IncX3-type plasmids were obtained from 10 different wards of the hospital, and only two of them exhibited a close relationship, showing substantial phylogenetic diversity (Fig. 3). Half of the 14 environmental isolates examined possessed IncX3-type plasmids with a size of approximately 50 kb, except for those found in three *C. freundii* isolates that exhibited variable sizes. Thus, IncX3 plasmids have disseminated widely among *Enterobacteriaceae* species through horizontal transfer.

Temperature-dependent transmissibility of IncFII- and IncX3-type *bla*$_{NDM}$-harboring plasmids. We assessed the efficiency of the conjugal transfer of the IncFII- and IncX3-type plasmids using the *E. coli* HST08 transformants. Although there was no significant difference in the transfer efficiency of the two plasmids, the optimal tem-peratures for conjugal transfer differed (Fig. 4). For the IncFII plasmids, the conjugal transfer was most efficient at 35°C, whereas the most appropriate temperature for transfer of the IncX3 plasmids was 25°C, which may explain their ability for broad dissemination in the environment. The means of the efficiency of transfer at 37°C for IncFII and IncX3 plasmids were 1.6×10^{-2} and 1.2×10^{-3}, respectively.
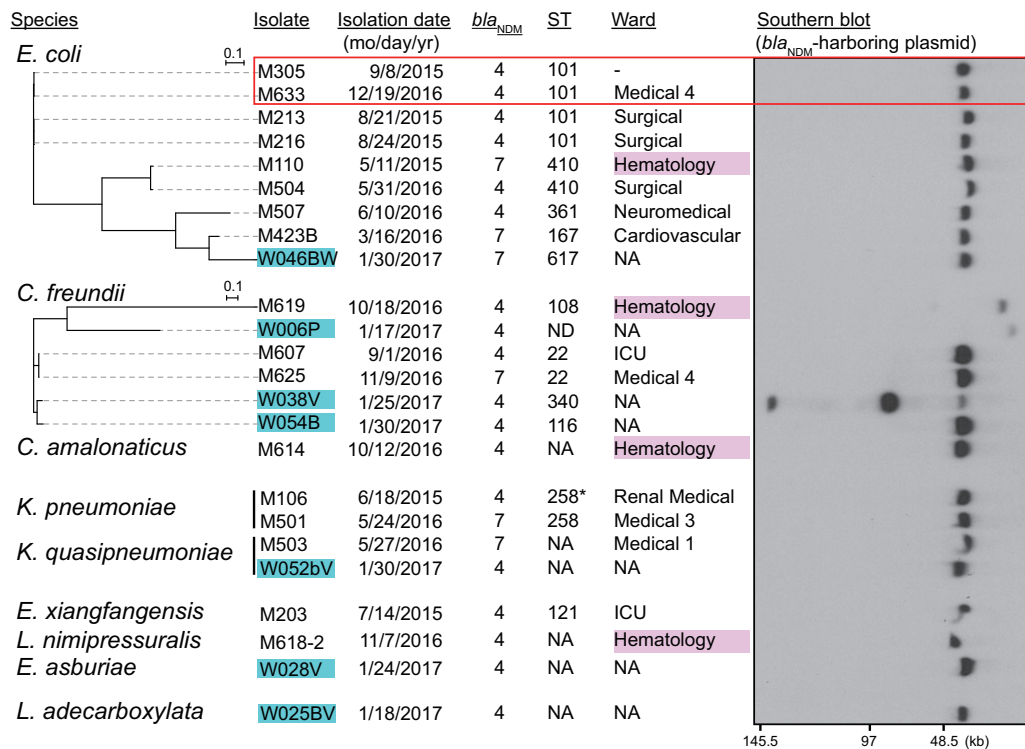
FIG 3 Phylogenetic relationships among CPE isolates with IncX3-type plasmids harboring bla_{NDM}. Environmental isolates are marked in cyan. The red rectangle indicates closely related isolates: 18 SNPs, >99.9% ANI. Genome sequences of *E. coli* K-12 MG1655 and *C. freundii* CFNIH1 (GenBank accession number CP007557.1) were used as references for construction of the phylogenetic trees of respective species. ST258* is a single locus variant of ST258.

Isolates carrying other plasmid types harboring bla_{NDM-1}. We further identified 21 isolates with a bla_{NDM-1}-harboring plasmid, which were mainly dominated by *K. pneumoniae* (n = 9) and *E. xiangfangensis* (n = 10), with only 1 *E. coli* isolate detected in this group. We found five different replicon types among the plasmids harboring bla_{NDM-1}: IncFIB(pQil), IncL/M, IncFII(pRSB107), IncA/C$_2$, and a multireplicon-type plasmid harboring the IncFII(K), IncQ1, and IncR replication genes (Fig. S2A), thereby demonstrating the high diversity of bla_{NDM-1}-harboring plasmids (Fig. 5).
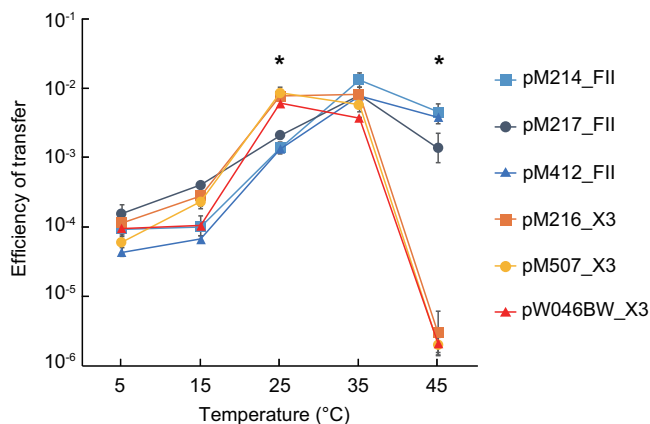
FIG 4 Comparison of the efficiencies of transfer of IncFII- and IncX3-type plasmids harboring bla_{NDM}. The effect of incubation temperature on the efficiency of transfer of IncFII- or IncX3-type plasmids from their transformants to a recipient, *E. coli* ML4909, was assessed by a conjugation assay. *E. coli* HST08 transformed with IncFII plasmids harboring bla_{NDM-5} (pM214_FII, pM217_FII, or pM412_FII) and IncX3 plasmids harboring bla_{NDM-4} (pM216_X3 or pM507_X3) or bla_{NDM-7} (pW046BW) was used as the donor. Measurements were performed in triplicate (n = 3). *, $P < 0.05$ (t test).
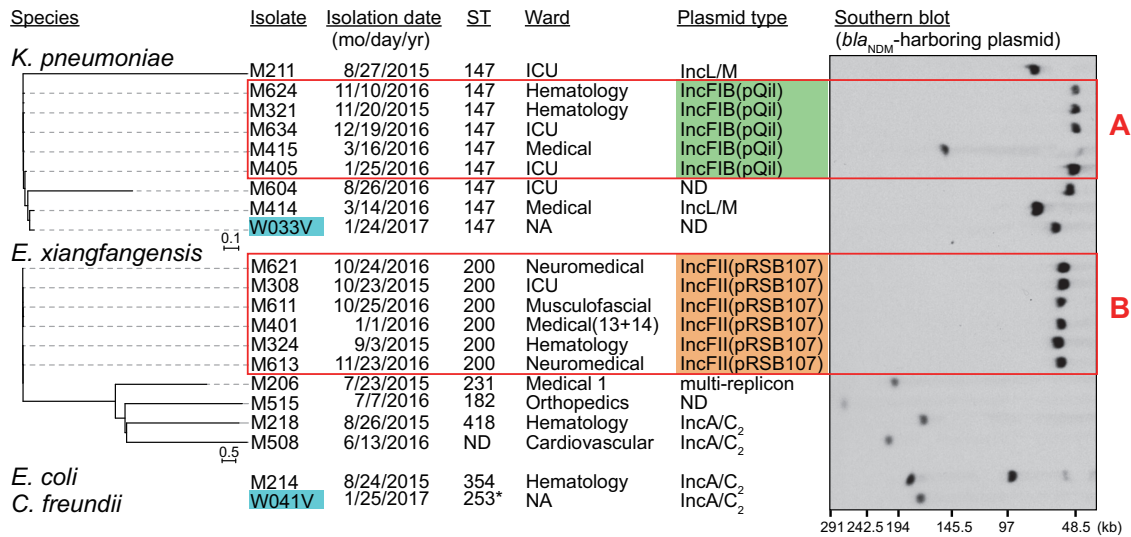
FIG 5 Phylogenetic relationship among CPE isolates carrying the bla_{NDM-1} gene. Environmental isolates are marked in cyan. Red rectangles denote closely related isolates. Cluster A, 20 to 72 SNPs, >99.9% ANI; cluster B, 8 to 30 SNPs, >99.8% ANI. Isolate M214 carried two bla_{NDM}-harboring plasmids: the IncA/C₂ plasmid harboring bla_{NDM-1} (176 kb) and the IncFII plasmid harboring bla_{NDM-5} (95 kb). Genome sequences of *K. pneumoniae* AATZP or *E. xiangfangensis* LMG27195 (GenBank accession number NZ_CP017183.1) were used as references for construction of the phylogenetic trees of the respective species. ST253* is a single locus variant of ST253.

All nine *K. pneumoniae* isolates carrying bla_{NDM-1} were typed as ST147. IncFIB(pQil)-type plasmids were found in five closely related clinical isolates with 20 to 72 SNPs between them, although the size of the plasmid was different in isolate M415 (Fig. 5, red rectangle A). Despite their sequence similarity, these isolates were obtained from three different wards of the hospital. The IncFIB(pQil)-type bla_{NDM-1}-harboring plasmid carried by *K. pneumoniae* isolate M321, designated pM321-NDM1 (Fig. S2B), was almost identical (two nucleotide substitutions) to pNDM-1fa (20), harbored by the *K. pneumoniae* ST147 isolate AATZP, of Indian origin. Whole-genome comparison further confirmed that M321 and AATZP are closely related, with an average nucleotide identity (ANI) value of 99.95%. Two bla_{NDM-1}-harboring plasmids carried by phylogenetically distant isolates (1,377 SNPs), M211 and M414, were determined as the IncL/M type. Assembled contig sequences of these two isolates were mapped with high confidence (93% coverage and 99% nucleotide identity) to a previously reported IncL/M plasmid carried by a clinical *K. pneumoniae* isolate of Omani origin, pNDM-OM (21), which was recently reclassified as IncM2 (22).

A cluster of closely related *E. xiangfangensis* isolates carrying bla_{NDM-1} (Fig. 5, red rectangle B) differing by 8 to 30 SNPs included isolates obtained from five different wards, suggesting dissemination from a common source. We conducted a more in-depth analysis of two of these plasmids, designated pM308-NDM1 and pM324-NDM1, identified in *E. xiangfangensis* isolates M308 and M324, respectively, using a long-read sequencer. Both plasmids were typed as IncFII(pRSB107) with PlasmidFinder, although the replicon sequence showed only 86.6% identity with the reference (GenBank accession number AJ851089). The sequences of pM308-NDM1 and pM324-NDM1 were highly conserved overall, except for the occurrence of a few insertions or deletions (Fig. S2C). Another four related isolates also possessed this type of plasmid with similar sizes; however, no homologous plasmids were found in the GenBank database.

IncFIB(pQil) was exclusively found in *K. pneumoniae* isolates, whereas the IncFII(pRSB107)-like plasmids were found only in *E. xiangfangensis*. Therefore, interspecies transmission of these plasmids did not occur among the Myanmar isolates. Indeed, these plasmids do not possess the genes necessary for plasmid transfer (Fig. S2B and C).

Plasmid harboring $bla_{OXA-181}$. We determined complete sequences of three $bla_{OXA-181}$-harboring plasmids, which were all IncX3-type plasmids and were highly similar to each other (only 2 to 5 single nucleotide variations/51,479 bp) (Fig. S2D).

DISCUSSION

We have identified phylogenetically divergent CPEs with different types of bla_{NDM}-encoding plasmids in a tertiary-care hospital in Yangon, Myanmar, suggesting multiple independent introductions of these resistant organisms into the hospital, along with their clonal spread. Two types of bla_{NDM}-harboring plasmids, IncFII and IncX3, were prevalent among the CPE isolates, along with various types of plasmids harboring bla_{NDM-1} detected at lower frequencies. These different types of plasmids showed distinct dissemination patterns, which appear to largely depend on the plasmid backbone and bacterial species harboring them.

IncFII plasmids spread among *E. coli* found in the fecal microbiota of humans and animals (23), which could explain the prevalence of *E. coli* carrying the IncFII-type plasmids among our CPE isolates. These plasmids were also found to be highly diverse in size, implying their high plasticity. We previously identified five IncFII plasmids with or without bla_{NDM} from Myanmar *E. coli* isolates and determined their sequences, in which two to four copies of IS26 were found and traces of IS26-mediated insertion and mobilization of gene clusters were observed (19). In that study, the isolate M105 was lacking the IS26-bracketed antimicrobial resistance gene cluster containing bla_{NDM-5} in the IncFII plasmid; instead this cluster was found in a different plasmid backbone, resulting in a novel bla_{NDM-5}-harboring plasmid (19). In this study, we further provide another example of the emergence of a novel plasmid harboring bla_{NDM-5}. The plasmid pM309-NDM5 carrying FIA and FII replicons also possessed the IS26-bracketed multi-drug resistance region containing bla_{NDM-5}. Using a BLAST search, the plasmid backbone of pM309-NDM5 was found homologous to another plasmid with FIA and FII replicons, pLZ135-CTX. Although this plasmid lacked the resistance cluster harboring bla_{NDM-5}, the intact IS26s and their neighboring sequences bracketing the resistance cluster were conserved in both plasmids. Thus, intermolecular homologous recombination could occur between a plasmid harboring the bla_{NDM-5} region, such as pM214_FII, and pLZ135-CTX like plasmid, resulting in the emergence of pM309-NDM5. Nevertheless, plasmids homologous to pLZ135-CTX have not yet been identified among the Myanmar isolates.

Most of the clinical isolates carrying the IncFII-type plasmids were obtained from the same hematology ward. Identification of three groups of closely related isolates carrying IncFII plasmids suggests the nosocomial spread of clonal lineages in this ward. These isolates were genotyped as *E. coli* ST167, its single locus variant ST8453, and *K. pneumoniae* ST101. The dose and frequency of the use of antimicrobials tend to be higher in the hematology ward than in other wards of the hospital since infections often become more severe in immunocompromised patients. This situation might allow these multidrug-resistant strains to spread in the ward. Of note, *E. coli* ST8453 isolates were also obtained from sewage samples, demonstrating the spread of clinically relevant organisms in the environment.

In contrast to the IncFII plasmids, we identified IncX3 plasmids harboring bla_{NDM-4} or bla_{NDM-7} in a wider variety of bacterial species of clinical and environmental origins, suggesting the dissemination of these plasmids via horizontal transfer. An IncX3 plasmid could be efficiently transferred in a conjugation assay at 25°C, whereas the optimum temperature was 35°C for the transfer of the IncFII plasmid. The IncX3 plasmids were prevalent in environmental isolates and were found in various *Enterobacteriaceae* isolates; therefore, conjugal transfer in environmental organisms might play a significant role in the dissemination of this plasmid. In this regard, the efficiency of transfer of IncA/C or nontypeable plasmids harboring bla_{NDM-1} from environmental *Enterobacteriaceae* isolates was reported to be better at 30°C than at 37°C (2). Efficient transfer of the IncX3 plasmid at a lower temperature could be one of the underlying mechanisms of its widespread dissemination. IncFII- and IncX3-type plasmids employ

different types of type IV secretion machinery for plasmid transfer. IncFII-type plasmid possesses F-type conjugative pilus, while IncX3-type plasmid harbors P-type pilus homologous to *Agrobacterium tumefaciens* VirB/VirD4 (24). While F-type pili are typically long and flexible, P-type pili are thicker and more rigid; thus, the difference in physical properties of conjugative pilus might be related to temperature sensitivity of plasmid transfer.

The IncX3-type plasmid appears to be an efficient vector for carbapenemase genes, as it has been reported to carry the bla_{NDM}, bla_{OXA-48}-like, and bla_{KPC} genes (25–27) and can disseminate the genes both inside (28) and outside (29, 30) of the clinical setting. IncX3 plasmids also harbor the *hns* gene, a homolog of which was reported to allow plasmids to be transferred to a bacterial host by minimizing their fitness cost (31). The *hns* gene is also involved in the temperature-dependent control of plasmid transfer (32), although the role of this gene, if any, in the temperature effects observed in the present study remains to be elucidated.

The bla_{NDM-1} gene was not found on either of the two most prevalent plasmids detected in our isolates, although it was previously found on IncFII (33, 34) and IncX3 (26) plasmids in other countries. It was found in other types of plasmids, such as IncFIB(pQil), IncFII(pRSB107)-like, IncL/M, and IncA/C$_2$, indicating their independent acquisition of the gene rather than the dissemination of the gene with major transferable plasmids. It was noteworthy that phylogenetically closely related isolates were isolated from different wards. Five *K. pneumoniae* ST147 isolates carrying the IncFIB-(pQil) plasmid were found in four different wards and six *E. xiangfangensis* ST200 isolates carrying the IncFII(pRSB107) plasmid were isolated from six different wards of a single hospital, suggesting their nosocomial spread from a common source and implying their persistent nature in the nosocomial environment. The intensive care unit could be the source of dissemination of these isolates, since two of the *K. pneumoniae* isolates and one of the *E. xiangfangensis* isolates were found in the ward.

We also characterized $bla_{OXA-181}$-harboring plasmids for the first time in Myanmar isolates. The plasmid isolated from *E. xiangfangensis* M206, designated pM206-OXA181, was an IncX3-type plasmid, and its sequence was completely identical to those previously identified in a clinical *E. coli* isolate from China (25) and a porcine *E. coli* isolate from Italy (29), although epidemiological links of these isolates are unlikely.

In conclusion, we have demonstrated the spread of diverse *Enterobacteriaceae* isolates harboring various bla_{NDM}-harboring plasmids in a clinical setting and sewage from its adjacent area in Yangon, Myanmar, in which three patterns of dissemination of bla_{NDM}-harboring plasmids were highlighted (Fig. 6). The IncFII- and IncX3-type plasmids are also spreading in other countries; thus, the implications of our results are not limited to Myanmar. In addition, we identified some novel plasmids, highlighting the vast pool of bla_{NDM}-harboring plasmids in this Southeast Asian country. The presence of these various isolates in a tertiary-care hospital appears to result not only from the nosocomial transmission but also from multiple introductions into the hospital, implying their spread in the community. Further study is warranted to better understand the mechanism of spread of CPE outside clinical settings and to track their dissemination beyond Myanmar.

MATERIALS AND METHODS

Bacterial isolates. *Enterobacteriaceae* isolates were isolated from clinical specimens of patients at Yangon General Hospital, Yangon, Myanmar, from April 2015 to December 2016 as described previously (19). 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. Environmental samples were collected at different locations within 500 m from the hospital in January 2017. Drinking water samples were collected from water storage container in individual households. Sewage samples were collected from a drainage canal collecting the flow of household effluents from nearby apartments. The drainage canal is not connected directly to the hospital drainage system. Bacteria were collected from water samples (15 ml) by centrifugation at 12,000 × *g* for 5 min and inoculated onto CHROMagar ECC (CHROMagar, Paris, France) supplemented with 0.25 μg/ml of meropenem and 70 μg/ml of ZnSO$_4$ (35), to obtain carbapenem-resistant isolates. All the colonies with different morphologies and colors were stored and subjected to further analysis. Species identification was carried out using a matrix-assisted
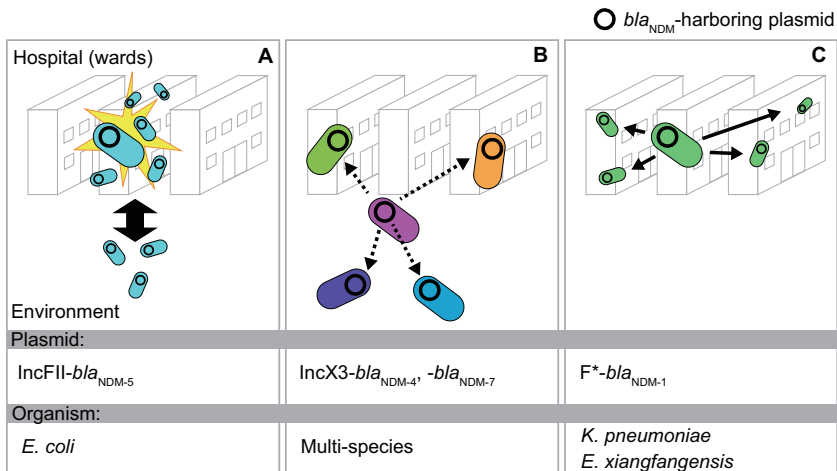
FIG 6 Three different patterns of dissemination of bla_{NDM}-harboring plasmid. (A) Clonal expansion of *E. coli* carrying IncFII-type plasmid harboring bla_{NDM} was observed in the same hematology ward. Closely related isolates were also found in environmental samples. (B) Diverse clinical and environmental isolates possessed IncX3-type plasmids harboring bla_{NDM-4} or bla_{NDM-7}, suggesting dissemination of the plasmids via horizontal plasmid transfer (dotted arrows). (C) Closely related *K. pneumoniae* or *Enterobacter xiangfangensis* carrying bla_{NDM-1}-harboring plasmids were isolated in different wards in the hospital, suggesting clonal expansion among the different wards (arrows). *, the types of bla_{NDM-1}-harboring plasmids are IncFIB(pQil) and IncFII(pRSB107) for *K. pneumoniae* and *E. xiangfangensis*, respectively.

laser desorption ionization–time of flight mass spectrometry system (MALDI Sepsityper; Bruker Daltonics, Bremen, Germany) and an API 20E system (bioMérieux, Marcy l'Etoile, France). Drug susceptibility testing was performed by the broth microdiilution method using a MicroScan Walkaway Plus system with a Neg EN Combo1J panel (Beckman Coulter, Brea, CA) or an EIKEN dry plate (Eiken, Tokyo, Japan). Clinical breakpoints defined by the Clinical and Laboratory Standards Institute (M100-S22) (36) were used to interpret the results from the drug susceptibility tests. Carbapenemase genes (bla_{NDM}, bla_{KPC}, bla_{IMP}, and bla_{OXA-48}-like) in the bacterial isolates were detected using PCR-dipstick chromatography (37).

WGS and bioinformatics analysis. Isolates were subjected to WGS using the HiSeq 3000 or MiSeq system (Illumina, San Diego, CA). Several isolates (M206, M308, M309, M321, and M324) were additionally analyzed using PacBio RSII (Pacific Biosciences, Menlo Park, CA) to obtain complete plasmid sequences, since the types of bla_{NDM}-harboring plasmids carried by these isolates appeared novel in Myanmar. Genomic DNA was prepared using the DNeasy PowerSoil kit (Qiagen, Hilden, Germany). The genomic DNA library for Illumina sequencing was prepared using KAPA Frag (Kapa Biosystems, Woburn, MA) and TruSeq DNA Nano kit (Illumina). Sequence reads were *de novo* assembled using CLC Genomics Workbench 11.0.1 (CLC Bio, Aarhus, Denmark) and used for further analysis. Library preparation for PacBio RSII sequencing and *de novo* assembly of the obtained sequences were performed as described previously (19). Clonal relatedness of the isolates was assessed using CSI Phylogeny 1.4 (38), and the phylogenetic tree was drawn on iTOL (39). Multilocus sequence typing was conducted using MLST 1.8 (40) or PubMLST (https://pubmlst.org/). The ANI value, calculated on EZBioCloud (41), was used for identification of *Enterobacter* species and for assessing the degree of relatedness between isolates. Plasmid replicon typing, plasmid multilocus sequence typing, and identification of resistance genes were performed using PlasmidFinder 1.3 (42) pMLST 2.0 (42), and ResFinder 2.1 (43), respectively. Plasmids similar to those found in this study were identified by a National Center of Biotechnology Information BLAST search using whole-plasmid sequences or contigs containing bla_{NDM} genes as queries. Assembled contigs from Illumina short reads were mapped to reference plasmids, and then the nucleotide identity and coverage were determined using BLAST on CLC Genomics Workbench. Plasmid sequences were annotated with MiGAP (https://www.migap.org/index.php/en), and the genomic structure was compared in EasyFig (44). Transposons and insertion sequences were determined using ISfinder (45).

Plasmid analysis. The size and replicon types of bla_{NDM}-harboring plasmids were determined by S1 nuclease pulsed-field gel electrophoresis (PFGE) followed by Southern hybridization. PFGE plugs prepared from the clinical or environmental isolates were treated with S1 nuclease (TaKaRa Bio, Shiga, Japan) and subjected to PFGE using the CHEF Mapper XA system (Bio-Rad, Hercules, CA). Separated DNA was transferred to a nylon membrane and probed with a digoxigenin-labeled DNA probe (Roche Diagnostics, Basel, Switzerland) specific to bla_{NDM} and plasmid replicon IncFII, IncX3, IncFII(pRSB107), IncFIB(pQil), or IncL/M (42). Whole-plasmid sequences of $bla_{OXA-181}$-harboring plasmids identified in isolates M513 and M518 were determined as follows. Contigs assembled from Illumina sequencing reads were mapped to the sequence of pM206_OXA181, a $bla_{OXA-181}$-harboring plasmid identified in *E. xiangfangensis* M206. PCR primer pairs were designed to fill the intervals between mapped contigs, and the sequences of the PCR product were determined by Sanger sequencing.

Transformation and conjugation. Transformants with bla_{NDM}-harboring plasmids were obtained by electroporation using the *E. coli* strain HST08 (TaKaRa Bio) as a recipient, as previously described (19).

Bacterial conjugation was performed using the transformants as donors and *E. coli* ML4909 (46) as a recipient. Mating was conducted on nitrocellulose membranes on a Luria-Bertani agar plate by incubation at 5, 15, 25, 35, or 45°C for 2 h. Transconjugants were selected on a brain heart infusion agar plate supplemented with 0.25 μg/ml of meropenem and 100 μg/ml of rifampin. The conjugation frequency was calculated as the number of CFU of the transconjugants/number of CFU of the donor and transconjugants.

Accession number(s). The sequence data and details of the sequenced samples, including the date and location of collection and source of specimen, were submitted to the DDBJ/GenBank/ENA database under BioProject number PRJDB5126.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC .01924-18.

SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.
SUPPLEMENTAL FILE 2, XLSX file, 0.04 MB.

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