AMERICAN SOCIETY FOR MICROBIOLOGY | Microbiology Spectrum

OBSERVATION

Role of Chromosome- and/or Plasmid-Located *bla*$_{NDM}$ on the Carbapenem Resistance and the Gene Stability in *Escherichia coli*

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ABSTRACT The spread of New Delhi metallo-β-lactamase (NDM)-producing *Enterobacterales* represents a public health risk. The horizontal transfer of plasmids encoding the NDM gene, *bla*$_{NDM}$, usually mediates its spread to other bacteria within the family. In contrast, *Enterobacterales* with a chromosome-located *bla*$_{NDM}$ is rarely reported. The phenotypic differences between chromosome- and plasmid-located carbapenemase genes are poorly understood. To determine the significance in terms of the location of drug resistance genes, we examined carbapenemase activity and stability of chromosome- and plasmid-located *bla*$_{NDM}$. *Escherichia coli* M719 possessing both chromosomes- and plasmid-located *bla*$_{NDM}$ genes was used as a wild-type strain (WT) for the construction of mutants, Δp*bla*$_{NDM}$ and Δc*bla*$_{NDM}$, wherein chromosome- or plasmid-located *bla*$_{NDM}$, was knocked out, respectively. The mutant Δp*bla*$_{NDM}$ showed lower hydrolyzing activity against imipenem and gene expression than the WT or Δc*bla*$_{NDM}$ mutant. The MICs of both mutant strains were still above the breakpoint of imipenem and meropenem. Moreover, the chromosome-located *bla*$_{NDM}$ gene was stable for at least 30 days in the absence of antimicrobial pressure, whereas the Δc*bla*$_{NDM}$ mutant lost *bla*$_{NDM}$ to 87% at 30 days compared to that of the initial inoculum. Organisms harboring the plasmid-located carbapenemase genes were found to provide a higher level of carbapenem resistance than those with chromosome-located genes. However, the latter organisms with chromosomal carbapenemase genes exhibited more stable carbapenem resistance than did the former ones. In summary, chromosomally located carbapenemase genes require further monitoring and more attention should be paid to them.

IMPORTANCE Carbapenem-resistant *Enterobacterales* (CRE) carrying *bla*$_{NDM}$ have spread worldwide since they were first reported in 2009. Many studies using whole-genome sequencing have identified the genetic structures, plasmid scaffolds of *bla*$_{NDM}$, and mechanisms of spread via horizontal transfer. Chromosome-located *bla*$_{NDM}$ and integration mechanisms from plasmids have rarely been reported, and their significance is not fully understood. Here, we showed that the chromosome-located *bla*$_{NDM}$ was associated with lower levels of carbapenem resistance and carbapenemase activity than the plasmid-located *bla*$_{NDM}$. However, it conferred carbapenem resistance above the breakpoints and the loss of chromosome-located *bla*$_{NDM}$ was not observed in the absence of antibiotic pressure. This study suggests that CRE strains carrying chromosome-located *bla*$_{NDM}$ may persist in clinical and environmental settings for a long period even without antibiotic pressure and need to be monitored along with plasmid-located *bla*$_{NDM}$.

KEYWORDS carbapenemase-producing *Enterobacterales*, *E. coli*, carbapenem, *bla*$_{NDM}$, β-lactamase

Editor Cheryl P. Andam, University at Albany, State University of New York

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The authors declare no conflict of interest.

Received 8 March 2022
Accepted 20 July 2022
Published 2 August 2022

Carbapenem-resistant *Enterobacterales* (CRE) represents a major health concern in clinical settings. Various types of carbapenemases have been identified, including NDM, IMP, VIM, KPC, and OXA-48 type. NDM-type carbapenemase originating from the Indian subcontinent has rapidly spread to different parts of the world, including Southeast Asia (1–5). The NDM gene, bla_{NDM}, is typically located in plasmids that facilitate the spread of carbapenem resistance via horizontal gene transfer. Plasmids act as scaffolds that assemble arrays of antibiotic resistance genes, thereby generating multiple-drug-resistant phenotypes in members of the order *Enterobacterales*. However, few CREs have been reported to carry the bla_{NDM} on their chromosome (6–9). Although the genetic contexts and integration mechanisms of chromosome-located carbapenemase genes have been reported occasionally, the difference in phenotype between isolates with the chromosome- and plasmid-located genes has not been extensively studied. Mathers et al. (10) identified five *K. pneumoniae* clinical isolates showing chromosomal integration of bla_{KPC}. They compared the MICs of meropenem and ertapenem between these isolates and others harboring plasmid with bla_{KPC}. They found that chromosomal genes exhibited weaker carbapenemase activity and lower expression of bla_{KPC}. However, the genetic background, including sequence types (STs) and porin gene status, is variable among individual isolates, and the prediction of an accurate correlation between carbapenem resistance and location of the carbapenemase gene is challenging. Therefore, phenotypic assays using the same genetic background are required to evaluate the true significance in terms of carbapenem resistance between chromosomal or plasmid-located carbapenemase genes. Here, we isolated an *E. coli* strain possessing bla_{NDM-5} on plasmid as well as chromosome and used it in further experiments to explore the comparative significance of chromosome- and plasmid-located bla_{NDM} in CRE.

Among CRE isolates previously obtained from CRE surveillance at a tertiary-care hospital in Yangon, Myanmar (3–5), *E. coli* isolate M719 carrying bla_{NDM} on the plasmid and chromosome was found using S1 nuclease pulsed-field gel electrophoresis (S1-PFGE) combined with Southern hybridization targeting bla_{NDM}. This isolate belonged to ST8453 which was originally assigned to a clinical isolate from Yangon (5). Whole-genome sequencing using the MinION system on *E. coli* isolate M719 identified multiple resistance genes on the chromosome and plasmid genomes (Table S1 in Supplemental File 1). The chromosome of M719 was found to include an 11.2 kbp region that contained bla_{NDM-5} and three other antimicrobial resistance genes against aminoglycosides, sulfonamide, and trimethoprim, which were bracketed by IS*26*. This region was integrated between a quinone oxidoreductase gene and the *dna*B gene and shared $>$ 99% sequence identity with that integrated into the IncFII bla_{NDM-5}-encoding plasmid (94.6 kbp) of M719 (Fig. 1). We also found 8 bp direct repeats adjacent to the IS*26* copies, indicating intermolecular transposition from pM71901 to the chromosome (Fig. 1). IS*26* is commonly detected in plasmids in *Enterobacterales* and surrounding regions of the chromosome and bla_{NDM} (7, 11). The chromosomal integration of drug-resistant genes and virulence genes via IS*26* has frequently been reported recently (12, 13). Therefore, chromosomal integration of bla_{NDM} via IS*26*-mediated transposition and/or homologous recombination from plasmids are likely to occur in various CRE organisms.

We used M719 (WT) harboring one copy of the bla_{NDM-5} gene in its chromosome and plasmid for the construction of mutants by deleting the plasmid- or chromosomal-located bla_{NDM-5} gene, yielding a plasmid-located bla_{NDM-5} knockout mutant (Δpbla_{NDM-5}) and chromosomal bla_{NDM-5} knockout mutant (Δcbla_{NDM-5}) (Fig. S1 in Supplemental File 1). The MICs of imipenem and meropenem against mutants Δpbla_{NDM-5} and Δcbla_{NDM-5} were lower than those against the WT strain (Fig. 2A). Imipenem showed a lower MIC value against mutant Δpbla_{NDM-5} than against mutant Δcbla_{NDM-5} ($P < 0.05$), while significant difference was not observed between meropenem MIC values against the two mutants. This observation could be related to the difference in permeability of these two beta-lactams (14), although the detailed mechanism is unclear. Consistent with the above result, the hydrolytic activity of imipenem by Δpbla_{NDM-5} was significantly lower than that of the WT and Δcbla_{NDM-5}

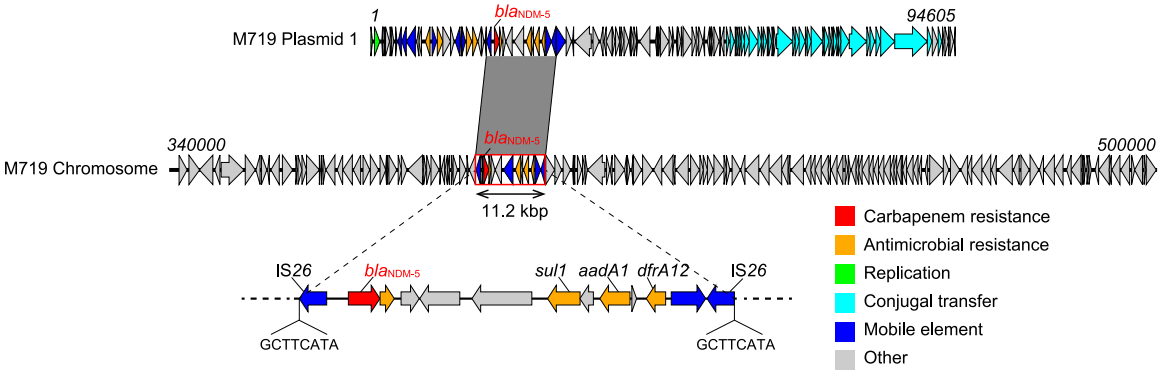

FIG 1 Comparison of the chromosomal region (11.2 kbp) containing bla_{NDM-5} in *E. coli* M719 and the bla_{NDM-5}-located region in the pM71901 genome. The area shaded in dark gray indicates the region possessing the identical DNA sequence (with > 99% similarity) between the two genomes. The 11.2 kbp region containing bla_{NDM-5} is highlighted in a red box. Nucleotide letters below the sequences of the M719 chromosome represent target site duplications.

mutant ($P < 0.05$) (Fig. 2B and C). The hydrolytic activity of Δpbla_{NDM-5} mutant also exhibited a significant decrease, compared to that of the WT. The double-knockout mutant, Δpcbla_{NDM-5} was also constructed, and it was found to be susceptible to both carbapenems and showed no hydrolytic activity against imipenem. A recent study demonstrated that the permeability of meropenem was substantially lower than imipenem in *K. pneumoniae* and *E. cloacae* (14). Therefore, meropenem penetrated periplasm could be hydrolyzed immediately by a certain amount of the NDM enzyme, resulting in a higher MIC value. As a result, our meropenem MIC assay may have not shown a significant difference between the Δpbla_{NDM-5} and Δcbla_{NDM-5} mutants. These results indicated that organisms carrying plasmid-located bla_{NDM} exhibited higher levels of carbapenem resistance and enhanced carbapenemase activity than mutants carrying chromosomal bla_{NDM} only. A similar phenomenon has been reported in clinical isolates carrying chromosomal bla_{KPC}, which exhibited lower MIC and tested negative or weakly positive in phenotypic tests probably due to a single chromosomal copy number with a lower mRNA level (10). We then investigated the correlation between the copy number of the carbapenemase gene and the mRNA level of the carbapenemase gene and the MICs against carbapenems (15–17). The WT containing two copies of bla_{NDM-5} (chromosome- and plasmid-located) showed the highest level of carbapenem resistance and bla_{NDM-5} mRNA level. The transcription levels of bla_{NDM-5} in mutant Δpbla_{NDM-5} and Δcbla_{NDM-5} were 0.4-fold and 0.7-fold compared to those of the WT, respectively (Fig. 2D). These results were consistent with those of MICs of carbapenems and hydrolytic activity against imipenem (Fig. 2C), showing that the mRNA level of bla_{NDM-5} and carbapenemase activity are positively correlated. We also analyzed the sequences of the promoter region upstream of chromosomal and plasmid-mediated bla_{NDM-5}. We found that excluding promoter sequences involved in the difference in transcription, the 51 bp adjacent sequence of bla_{NDM-5} was completely conserved and identical to the promoter region of bla_{NDM-1} as described previously (Fig. S2 in Supplemental File 1) (18). The bla_{NDM-5} mRNA levels in mutant Δcbla_{NDM-5} were 1.73-fold higher than those of mutant Δpbla_{NDM-5}, suggesting that the former harboring plasmid with bla_{NDM-5} showed higher levels of bla_{NDM-5} transcription and carbapenem resistance than did the latter with chromosomal bla_{NDM-5}. The increase in the mRNA level of Δcbla_{NDM-5} likely reflects the plasmid copy number of pM71901, and we subsequently estimated the plasmid copy number of the bla_{NDM-5} gene of pM71901 in the WT strain. The plasmid copy number of pM71901 was 2.12, which is similar to the copy number of IncFII plasmids, which are recognized as low-copy-number plasmids.

In vitro and *in silico* experiments have demonstrated that plasmid-located drug resistance genes are frequently lost from plasmids under antibiotic-free conditions, i.e., deprived selection pressures (19–21). Therefore, we tested the stability of bla_{NDM} in the absence of carbapenems. During the 30 days of daily passaging in the absence of meropenem, mutant Δpbla_{NDM-5} retained chromosomal bla_{NDM-5}, while mutant Δcbla_{NDM-5}

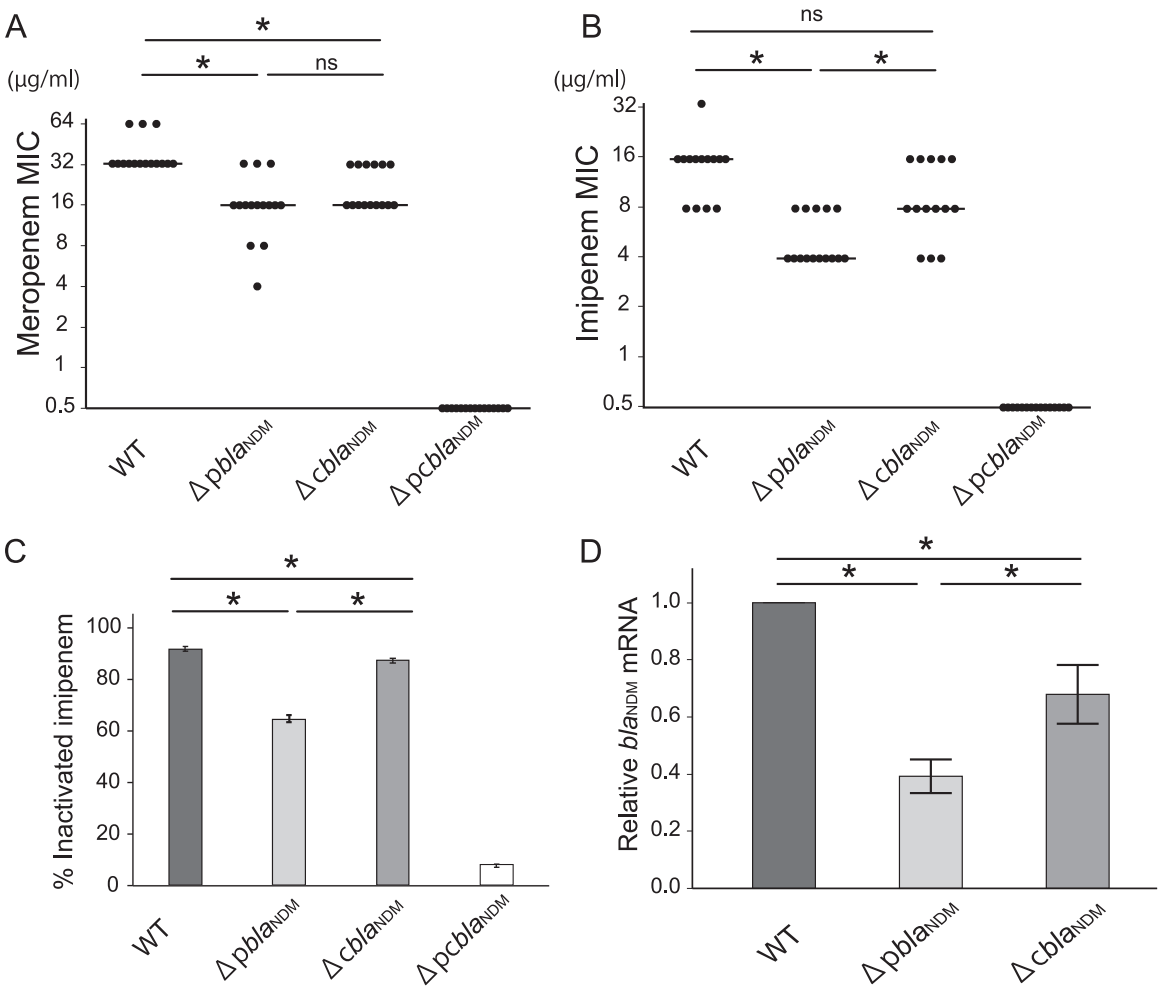

FIG 2 Comparative features of *E. coli* M719 and its bla_{NDM} mutants in terms of MIC to meropenem/imipenem, inactivation of imipenem, and bla_{NDM} mRNA levels in their cultures. MICs of (A) meropenem and (B) imipenem stratified based on the location of bla_{NDM} in *Escherichia coli* M719. Solid lines represent median MICs. The significance of *P* values was determined using the Steel-Dwass test (*, $P < 0.05$). (C) Hydrolysis of imipenem by the WT and knockout mutants ($\Delta pbla_{NDM}$ and $\Delta cbla_{NDM}$) after 30 min incubation. Statistical comparisons were performed using the Steel-Dwass test for multiple comparisons. *, $P < 0.05$; ns, not significant. Error bars represent standard deviation. (D) Relative mRNA levels of bla_{NDM-5} as determined with qPCR. Values were normalized to the mRNA level in the WT. Statistical comparisons were performed using the Steel-Dwass test for multiple comparisons; *, $P < 0.05$. Error bars represent standard deviations.

showed a gradual loss of the plasmid-located bla_{NDM-5} with a 13% reduction in the population (Table S2 in Supplemental File 1). In contrast, the loss of bla_{NDM-5} was not observed in the serially passaged culture in the presence of meropenem. These results suggested that the chromosome-located bla_{NDM-5} was stable in the absence of selective pressure of antibiotics.

In conclusion, we demonstrated unique features of plasmid/chromosome-located bla_{NDM-5}. The plasmid-carrying bla_{NDM-5} exhibited higher levels of hydrolyzing activity and resistance against imipenem than did the chromosome-located gene because of its higher copy number, and frequent transmission to other bacteria via conjugation. Our results could apply to other antimicrobial resistant determinants that can be found on chromosomes and plasmids. However, it might not apply even to the same gene because the copy number depends on plasmid types and transcriptional activity should be affected by other factors than plasmid copy number. It should not also apply to the case in which a single copy of an antimicrobial-resistant determinant is sufficient to confer resistance on its bacterial host. Meanwhile, it is interesting to explore an upper threshold of β-lactamase genes' copy number at which there is not an increase in the relative degradation of substrates of their encoding enzymes. This could be

plausible because they are usually located in the periplasmic space or, for NDM-type β-lactamases, anchored to the outer membrane (22), which could potentially reach a saturation.

Although chromosomal bla_{NDM-5} showed essentially no conjugation ability and a relatively lower level of carbapenem resistance, it was stably carried in CRE even in the absence of antimicrobial selective pressure. The stability of bla_{NDM} via chromosomal integration in outbreak clones or environmental isolates may pose a serious public health concern. Further studies are required to understand the biological behavior of CREs with chromosomal carbapenemase genes, which may be of epidemiological significance.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.2 MB.

ACKNOWLEDGMENTS

This work was supported by the Japan Initiative for Global Research Network on Infectious Diseases of the Ministry of Education, Culture, Sports, Science, and Technology of Japan and the Japan Agency for Medical Research and Development under grant numbers JP18fm0108003, 21wm0225013h0502, and 21wm0125010h0001. This work was also supported by Grants-in-Aid for Scientific Research for NS (number 19K16642).

We declare no conflict of interest.

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