The Journal of Antibiotics (2022) 75:559–566
https://doi.org/10.1038/s41429-022-00553-3

ARTICLE

# Landscape of *bla*$_{NDM}$ genes in *Enterobacteriaceae*

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Received: 8 July 2022 / Revised: 18 July 2022 / Accepted: 20 July 2022 / Published online: 19 August 2022
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## Abstract

The *bla*NDM-1 gene encodes a carbapenemase, New Delhi metallo-β-lactamase (NDM-1), and the ability to produce NDM-1 is spread among *Enterobacteriaceae* via horizontal gene transfer of plasmids. It has been widely accepted that *bla*$_{NDM-1}$ is regulated by a hybrid promoter (P$_{ISAba125}$) consisting of a –10 box from the original *bla*$_{NDM-1}$ and a –35 box from IS*Aba125*. However, the conservation of this promoter and the vertical transmission of *bla*$_{NDM}$ genes by chromosomal integration have not been comprehensively analyzed. We retrieved the region containing the ORF of *bla*$_{NDM-1}$ (>95% translated protein identity) and a region 120 bp upstream of the *bla*$_{NDM-1}$ start codon from the complete sequence data of *Enterobacteriaceae* plasmids ($n = 10{,}914$) and chromosomes ($n = 4908$) deposited in GenBank, and the 310 extracted *bla*$_{NDM}$ genes were analyzed by an in-silico approach. The results showed that most *bla*$_{NDM}$ genes (99.0%) utilized the promoter, P$_{ISAba125}$. Interestingly, two *bla*$_{NDM-1}$ genes from the genus *Citrobacter* utilized the IS*CR1*-derived outward-oriented promoters P$_{OUT}$ (P$_{ISCR1}$). Furthermore, the insertion of IS*Aba125* and IS*CR1* occurred upstream of the CCATATTT sequence, which is located upstream of the –10 box. We also confirmed that most of the *bla*$_{NDM}$ genes were disseminated by horizontal gene transfer of the plasmid, but 10 cases of the *bla*$_{NDM}$ genes were integrated into the chromosome via mobile genetic elements such as IS*26*, IS*150*, IS*CR1*, ICE, and Tn*7*-like elements. Thus, plasmid-mediated transmission of the P$_{ISAba125}$-*bla*$_{NDM}$ genes is predominant in *Enterobacteriaceae*. However, the spread of *bla*$_{NDM}$ genes with new promoters and vertical dissemination via chromosomal integrations may pose additional serious clinical problems.

## Introduction

In 2008, *Klebsiella pneumoniae*, which produces carbapenemase, was detected in a Swedish patient of Indian origin who suffered a urinary tract infection while traveling in New Delhi, India [1]. The new type of carbapenemase was named New Delhi metallo-β-lactamase (NDM-1), and the *bla*$_{NDM-1}$ gene encoding NDM-1 was present on a large plasmid that can be introduced into *Escherichia coli* J53 [1] by conjugation. Indeed, NDM-1-producing bacteria, mainly *Klebsiella pneumoniae* and *E. coli*, are spread throughout the world via horizontal gene transfer of the plasmid as carbapenem-resistant *Enterobacteriaceae* (CRE) [2]. Plasmids are one of the types of mobile genetic elements (MGEs), and bacteria acquire multiple drug resistance (MDR) by incorporating multi-drug-resistance plasmids from other bacteria via horizontal gene transfer. For example, the *bla*$_{NDM-1}$ and tetracycline-resistance genes of *Citrobacter freundii* ZY198 are located on different plasmids, i.e., pZY-NDM1 and pZY-1, respectively (GenBank: CP055250; CP055248). In addition, the plasmids themselves integrate multidrug-resistance genes by incorporating MGEs such as insertion sequences (ISs) and/or transposons [3].

DNA transposons generally contain accessory genes in the cargo region between the two IS elements that are composed of relatively short DNAs, with a short imperfect terminal inverted repeat sequence (IR) on both sides of the DNA ends, and the *tnp* gene, which encodes transposase [4]. Transposase is involved in target DNA excision and the strand-transfer reaction. Accessory genes within the transposon are involved in drug resistance, heavy metal resistance, metabolism, efflux functions, etc., and the transposition of the transposon into plasmids or chromosomes confers accessory functions on their hosts [5]. The clinical problem is the acquisition of drug-resistance genes via transposon. When transposon is inserted

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**Table 1** Strain data used for initial BLAST search

| Bacterial strain | Plasmid | Accession number |
|---|---|---|
| *Klebsiella pneumoniae* Kp7 | pNDM-KN | JN157804 |
| *Klebsiella pneumoniae* 05-506 | pKpANDM-1 | FN396876 |
| *Klebsiella pneumoniae* NH25 | pNH25.5 | CP024879 |
| *Klebsiella pneumoniae* 601 | pNDM-OM | JX988621 |
| *Escherichia coli* ST410 | p2189-NDM | CP029631 |
| *Escherichia coli* M109 | pM109_FII | AP018139 |
| *Escherichia coli* GN 568 | pNDM-EcoGN568 | KJ802404 |

into a chromosome, it may be accompanied by a gene disruption. However, this is not always disadvantageous; indeed, gene disruption may be advantageous in some cases. For example, the loss of the OmpK36 porin gene reduces the permeability of the outer membrane, and this is known to contribute to drug resistance by reducing the uptake of antimicrobial agents into the bacterial cell [6].

In addition, IR-insertion into the plasmid or chromosome is sometime involved in the gene activation. As reported for IS*CR1* (Insertion Sequence Common Region 1), the IR of the IS element contains an outward-oriented promoter ($P_{OUT}$), and the short transcripts from $P_{OUT}$ repress transposition by forming base-pairs with inwardly transcribed *tnp* mRNAs [7]. It has been reported that IS*CR1* $P_{OUT}$ contains two promoters, $P_{CR1-1}$ and $P_{CR1-2}$, and both are active and involved in the expression of the *bla*$_{CTX-M-9}$ gene [8]. It is widely accepted that the *bla*$_{NDM-1}$ promoter is a hybrid promoter generated by the insertion of a right-hand inverted repeat (IRR) of IS*Aba125* upstream of the *bla*$_{NDM-1}$ –10 box [9]. However, the detailed mechanism by which *bla*$_{NDM-1}$ and its derivatives with this hybrid promoter spread among *Enterobacteriaceae* have remained unclear. Furthermore, the spread of the *bla*$_{NDM}$ family with different promoters by different IS insertions in *Enterobacteriaceae* has not been elucidated in detail. Here, we retrieved the *bla*$_{NDM}$ genes from the database containing the complete chromosome and plasmid sequences of *Enterobacteriaceae* deposited in the GenBank database and performed comprehensive analyses of the *bla*$_{NDM}$ promoter and chromosome insertion by certain ISs.

## Materials and methods

### Bacterial strains and growth media

*Escherichia coli* DH10B was used for plasmid preparation. An *E. coli* NDM-1 strain (ATCC BAA-2469) was used for the *bla*$_{NDM-1}$ cloning. *E. coli* strains were inoculated in lysogeny broth (LB) and incubated at 37 °C for 16 h with shaking.

### Collection of the *bla*$_{NDM}$ gene family from the *Enterobacteriaceae* database

The bacterial databases listed in Table 1 were used for the initial *bla*$_{NDM-1}$ promoter search. The *bla*$_{NDM-1}$ and *bla*$_{NDM-1}$ variants (NDM-1 protein with >95% identity, 100% query coverage) containing the region 120 bp upstream of the start codon were retrieved by BLAST and SeqKit [10] from the complete plasmid and genome sequences of *Enterobacteriaceae* deposited in the GenBank database. Under these conditions, we obtained 300 data points for *bla*$_{NDM-1}$ and its variants from a database of 10,914 plasmids and 10 data points from a database of 4908 chromosomes. Data retrieval was done on November 19,2021.

### Detection of *bla*$_{NDM}$ genes integrated into a bacterial chromosome

To investigate the integration of *bla*$_{NDM}$ into the bacterial chromosome, a BLAST search was carried out using the complete genomic database that consisted of 4908 chromosome data of *Enterobacteriaceae*; the insertion site of the gene cassette containing *bla*$_{NDM}$ genes was identified by comparison with the genome sequence of the respective reference strains; and the IS element of the flanking regions of the gene cassettes integrated into the chromosome were detected by searching the ISfinder database (www-is.biotoul.fr). If no IS was detected, we performed an ORF analysis using FramePlot [11] to predict the elements involved in the chromosome integration. The reference strains used for the genome comparison were as follows: *K. pneumoniae* Kp52.145 (GenBank: FO834906), *E. coli* K-12 substr. W3110 (GenBank: FO834906), and *Enterobacter cloacae* GGT036 (GenBank: CP009756).

### Construction of plasmids for the *bla*$_{NDM-1}$ promoter mapping

A 1.0-kbp fragment of the *bla*$_{NDM-1}$ gene containing the entire ORF and its 194-bp upstream region was amplified by PCR with the 5IF-FL and 3IF-FL primers using genomic DNA of the *E. coli* NDM-1 strain (ATCC BAA-2469) as a template, and the resulting DNA fragment was inserted into the BglII-XhoI sites of plasmid pET-28a (+) using an In-Fusion Cloning system (Takara Bio) to obtain pET-NDM-1_FL. Using the same experimental procedure, pET-NDM-1_166, pET-NDM-1_120, pET-NDM-1_96, pET-NDM-1_71, and pET-NDM-1_42 were also constructed using the primer sets, 5IF-166 and 3IF-FL, 5IF-120 and 3IF-FL, 5IF-96 and 3IF-FL, 5IF-71 and 3IF-FL, and 5IF-42 and 3IF-FL,

**Table 2** Primers used in this study

| Primer | Sequence |
|---|---|
| 5IF-FL | 5′-TAGAGGATCGAGATCTAGAAAGGCGTTAGATTGGCTTACACC-3′ |
| 5IF-166 | 5′-TAGAGGATCGAGATCTTTAGAGAAATTTGCTCAGCTTGTTGATTATC-3′ |
| 5IF-120 | 5′-TAGAGGATCGAGATCTCTGTCGCACCTCATGTTTGAATTC-3′ |
| 5IF-96 | 5′-TAGAGGATCGAGATCTGCCCCATATTTTTGCTACAGTGAAC-3′ |
| 5IF-71 | 5′-TAGAGGATCGAGATCTCAAATTAAGATCATCTATTTACTAGGCCTCG-3′ |
| 5IF-42 | 5′-TAGAGGATCGAGATCTCGCATTTGCGGGGTTTTTAATG-3′ |
| 3IF-FL | 5′-GGTGGTGGTGCTCGAGCTCAGCGCAGCTTGTCGGC-3′ |

respectively. The primers used in this study are listed in Table 2.

### Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) of meropenem were determined by the microdilution method according to the CLSI guidelines [12] using the Dry Plate Eiken. In brief, bacteria ($5 \times 10^4$) were inoculated in 100 µl of Mueller–Hinton broth (Becton, Dickinson and Company, Franklin Lakes, NJ) containing meropenem. The tested plates were then incubated at 35 °C for 18–20 h. The MIC value was determined as the lowest concentration of meropenem where no bacterial growth was visibly observed.

## Results

### Characterization of the minimal promoter region of $bla_{NDM-1}$

To investigate the $bla_{NDM-1}$ promoter, we first performed a BLAST search of the 500 bp upstream region of the $bla_{NDM-1}$ start codon using seven NDM-1-producing strains of *E. coli* and *K. pneumoniae* of the GenBank database (Table 1). The results showed that the 194 bp region upstream of the start codon is highly conserved (Fig. 1b), suggesting that the promoter is located within 194 bp upstream of the $bla_{NDM-1}$ start codon. To analyze the $bla_{NDM-1}$ promoter, we generated four deletion derivatives using pET-NDM-1_FL, which has a 194 bp upstream region of the $bla_{NDM-1}$ start codon (Fig. 1a). These plasmids were introduced into *E. coli* DH10B and grown on LB agar plates containing 100 µg $ml^{-1}$ of ampicillin. *E. coli* harboring pET-NDM-1_42 and pET-NDM-1_71 could not grow on the LB agar plates (100 µg $ml^{-1}$ ampicillin), while *E. coli* harboring pET-NDM-1_FL, pET-NDM-1_166, pET-NDM-1_120, and pET-NDM-1_96 showed ampicillin-resistant colonies. In order to further validate the promoter activity, the MIC of the plasmid-transfected *E. coli* DH10B against meropenem was evaluated according to the CLSI guidelines [12]. The MIC of the bacteria harboring pET-NDM-1_FL, pET-NDM-1_166, and pET-NDM-1_120 was 64 µg $ml^{-1}$. In contrast, the MIC of *E. coli* harboring pET-NDM-1_96 was 1 µg/ml (Fig. 1). These results suggest that the $bla_{NDM-1}$ promoter with sufficient activity is located within a 120 bp region upstream of the $bla_{NDM-1}$ start codon. Furthermore, *E. coli* harboring pET-NDM-1_96 lacking the –35 box showed reduced resistance to meropenem (Fig. 1). It has been reported that $bla_{NDM-1}$ is a hybrid promoter consisting of a –35 box derived from the IRR of IS*Aba125* (Fig. 1b, green highlighted line) and a –10 box derived from the original $bla_{NDM-1}$ (Fig. 1b, c) [9]. Collectively, our deletion analyses demonstrated that the IS*Aba125*-derived –35 box is indeed essential for full activity of the $bla_{NDM-1}$ gene.

### The promoter of the $bla_{NDM}$ gene family was highly conserved among *Enterobacteriaceae*

In the above experiment, we showed that the promoter located within 120 bp is necessary and sufficient for the $bla_{NDM-1}$ expression. We next analyzed whether this promoter (hereafter called $P_{ISAba125}$) is highly conserved among *Enterobacteriaceae* of NDM-1 and NDM-1 variants using an in-silico approach as described in the Materials and Methods. We retrieved the 310 full-length $bla_{NDM}$ genes from the *Enterobacteriaceae* plasmid ($n = 10{,}914$) and chromosome ($n = 4908$) databases. NDM-1 and NDM-1 variants with amino acid substitutions were included, of which NDM-1 and NDM-5 were the most common (Table 3). To investigate the conservation of the $bla_{NDM-1}$ promoter, we performed multiple sequence alignment by MAFFT (https://mafft.cbrc.jp/alignment/server/) using 310 retrieved $bla_{NDM}$ sequences including the region 120 bp upstream of the start codon. Among them, the promoter $P_{ISAba125}$ (Fig. 1c) was highly conserved in 307 (99.0%) of the 310 $bla_{NDM}$ gene family members (Fig. 2). Although we found one $bla_{NDM-5}$ with a single base substitution (A to G) within $P_{ISAba125}$, the –35 and –10 boxes were conserved. Therefore, this promoter was also categorized as $P_{ISAba125}$. In contrast, two $bla_{NDM-1}$ genes were under the control of a different promoter that was generated by insertion of IS*CR1 ori*IS, as described later. A plasmid found in NDM-5 *E. coli* (GenBank: CP083875) had two $bla_{NDM-5}$ genes, and the

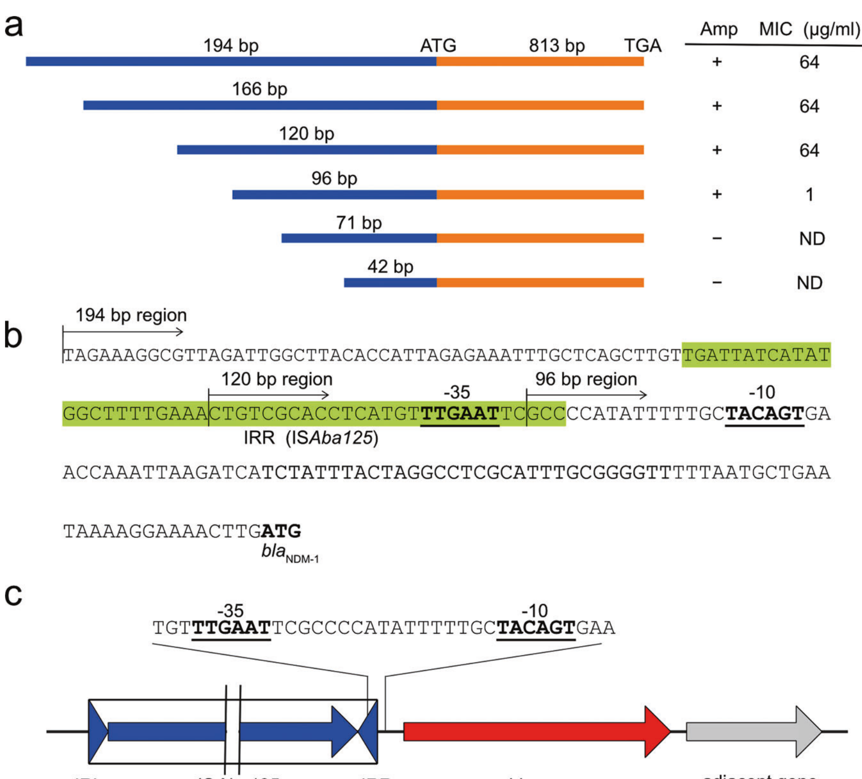



**Fig. 1** The IS*Aba125*-derived –35 box in $bla_{\text{NDM-1}}$ is required for sufficient promoter activity. **a** Domain mapping of the $bla_{\text{NDM-1}}$ promoter. Orange lines indicate an 813 bp ORF encoding NDM-1 carbapenemase. Blue lines indicate the upstream region of the $bla_{\text{NDM-1}}$ start codon. The $bla_{\text{NDM-1}}$ promoter activity was confirmed by growth on LB agar plates containing ampicillin (100 μg $ml^{-1}$) and MIC against meropenem after introduction of the pET-NDM-1 derivatives into *E. coli* DH10B. **b** 194 bp upstream region of the $bla_{\text{NDM-1}}$ start codon. The sequence highlighted in green indicates the IRR of IS*Aba125*. pET-NDM1_96 lacking the –35 box showed significantly reduced meropenem resistance. **c** Schematic model of the $bla_{\text{NDM-1}}$ promoter. The $bla_{\text{NDM-1}}$ promoter is thought to be a hybrid promoter consisting of a –10 box from the original $bla_{\text{NDM-1}}$ and a –35 box from IS*Aba125*. Red and blue arrows indicate the $bla_{\text{NDM-1}}$ gene and truncated IS*Aba125*, respectively. IRL left inverted repeat, IRR right inverted repeat

**Table 3** Promoter type and distribution of $bla_{\text{NDM}}$ genes in the *Enterobacteriaceae* database

| Promoter | NDM type | Number of $bla_{\text{NDM}}$ | Origin |
|---|---|---|---|
| $P_{\text{IS}Aba125}$ | NDM-1 | 144 | Plasmid |
| $P_{\text{IS}Aba125}$ | NDM-5 | 119 | Plasmid |
| $P_{\text{IS}Aba125\text{ (A to G)}}$ | NDM-5 | 1 | Plasmid |
| $P_{\text{IS}Aba125}$ | NDM-7 | 14 | Plasmid |
| $P_{\text{IS}Aba125}$ | NDM-4 | 6 | Plasmid |
| $P_{\text{IS}Aba125}$ | NDM-9 | 6 | Plasmid |
| $P_{\text{IS}Aba125}$ | NDM-6 | 2 | Plasmid |
| $P_{\text{IS}Aba125}$ | NDM-16b | 2 | Plasmid |
| $P_{\text{IS}Aba125}$ | NDM-19 | 1 | Plasmid |
| $P_{\text{IS}Aba125}$ | NDM-21 | 1 | Plasmid |
| $P_{\text{IS}Aba125}$ | NDM-29 | 1 | Plasmid |
| $P_{\text{IS}CR1}$ | NDM-1 | 2 | Plasmid |
| $P_{\text{NF}}$ | NDM-5 | 1 | Plasmid |
| $P_{\text{IS}Aba125}$ | NDM-1 | 7 | Chromosome |
| $P_{\text{IS}Aba125}$ | NDM-3 | 2 | Chromosome |
| $P_{\text{IS}Aba125}$ | NDM-5 | 1 | Chromosome |

promoter types were $P_{\text{IS}Aba125}$ and $P_{\text{NF}}$, respectively. $P_{\text{NF}}$ does not have a conserved –35 box. Therefore, the phenotype of NDM-5 may be due to $bla_{\text{NDM-5}}$ being under the control of $P_{\text{IS}Aba125}$.

## $P_{\text{IS}Aba125}$-dependent $bla_{\text{NDM}}$ family members are expanded among *Enterobacteriaceae*

The above analysis revealed that the $bla_{\text{NDM}}$ gene family utilizes the highly conserved promoter, $P_{\text{IS}Aba125}$. Using the 297 data points for the $P_{\text{IS}Aba125}$-dependent $bla_{\text{NDM}}$ genes from a database of 10,914 plasmids, we investigated how the $P_{\text{IS}Aba125}$-$bla_{\text{NDM}}$ spread to *Enterobacteriaceae*. As shown in Fig. 3, $P_{\text{IS}Aba125}$-$bla_{\text{NDM-1}}$ and its variant were mostly found in *E. coli* and *Klebsiella* spp., but were also detected in the genera *Enterobacter*, *Raoulltera*, *Salmonella*, and *Citrobacter*. Thus, we confirmed that the $P_{\text{IS}Aba125}$-$bla_{\text{NDM}}$ genes spread to various *Enterobacteriaceae* via horizontal gene transfer with a plasmid and had the ability to confer carbapenem resistance.

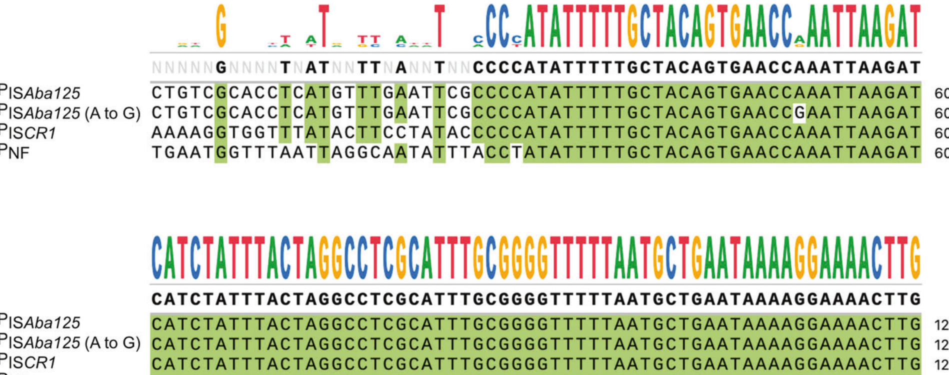



**Fig. 2** Multiple sequence alignment of the $bla_{NDM}$ promoter region. 310 data points (see Table 3) of the 120 bp region upstream of the $bla_{NDM}$ start codon were aligned by MAFFT. Green shaded boxes indicate conserved regions. $P_{ISAba125}$, hybrid promoter derived from IS*Aba125* insertion; $P_{ISCR1}$, $P_{OUT}$ promoter derived from IS*CR1* insertion; $P_{NF}$, non-functional promoter

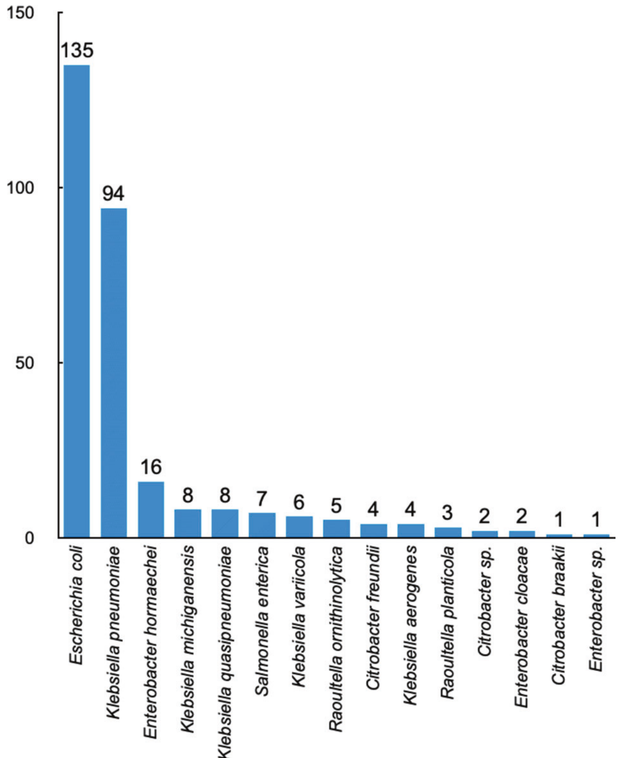



**Fig. 3** Distribution of the $P_{ISAba125}$-$bla_{NDM}$ family members in *Enterobacteriaceae*. The horizontal gene transfer of $bla_{NDM}$-containing plasmids was investigated using 297 data points of $P_{ISAba125}$-$bla_{NDM}$ retrieved from the *Enterobacteriaceae* plasmid database ($n = 10,914$). Although some plasmids contain two $bla_{NDM}$ genes or multiple plasmids each contain a $bla_{NDM}$ gene, the bar graph represents the distribution of 297 data points of $P_{ISAba125}$-$bla_{NDM}$ genes

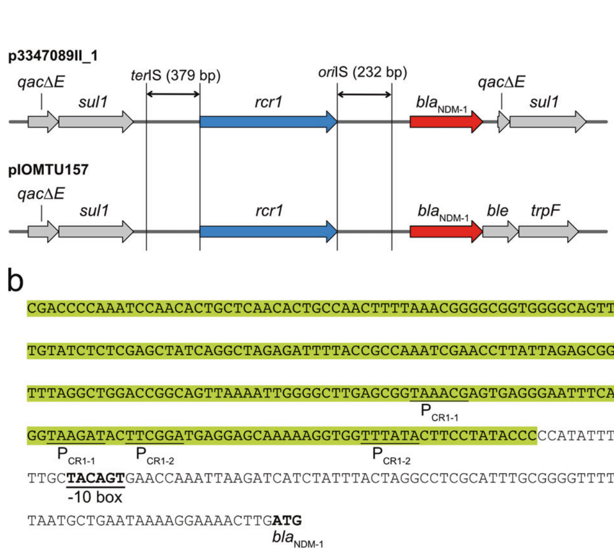



**Fig. 4** Characterization of the $P_{OUT}$ promoter of IS*CR1* in *Citrobacter* spp. **a** $bla_{NDM-1}$ using the $P_{OUT}$ promoter ($P_{ISCR1}$) derived from the IS*CR1* insertion was found in *Citrobacter sedlakii* and *Citrobacter portucalensis* plasmids. Red and blue arrows indicate *bla*NDM-1 and *rcr1*, respectively. Gray arrows indicate neighboring genes surrounding *rcr1* and $bla_{NDM-1}$ genes. *sul1*, sulfonamide resistance gene; *rcr1*, transposase gene; *trpF*, N-(5'-phosphoribosyl) anthranilate isomerase gene; *ble*, bleomycin resistance gene; *qacΔE*, semi-functional derivative of quaternary ammonium compound resistance gene. **b** The 232 bp region of *ori*IS is highlighted in green. Two promoters ($P_{CR1-1}$, $P_{CR1-2}$) were generated by the IS*CR1* *ori*IS insertion

## Emergence of the promoter $P_{ISCR1}$ by insertion of IS*CR1*

Although most of the CRE utilize the $P_{ISAba125}$-$bla_{NDM}$ genes, $bla_{NDM-1}$ utilizing the promoter $P_{OUT}$ derived from the IS*CR1* element was found in plasmids possessed by *Citrobacter sedlakii* [13] and *Citrobacter portucalensis* (Fig. 4). Interestingly, the IS*CR1* element was inserted at the same site in both plasmids, upstream of the –10 box of $bla_{NDM-1}$ via the conserved sequence CTTCCTATACCC located at the 3′ end of the *ori*IS of IS*CR1* (Fig. 4b: the 232 bp *ori*IS is highlighted in green). The insertion of the *ori*IS upstream of the –10 box of the $bla_{NDM-1}$ promoter generated two *ori*IS-derived promoters called $P_{CR1-1}$ (−35 box: TAACG; −10 box: TAAGAT) and $P_{CR1-2}$ (–35 box: TTCGGA; −10 box: TTTATA) [8]. Thus, two $P_{OUT}$ promoters were involved in the expression of $bla_{NDM-1}$, and we designated the *ori*IS-derived $P_{out}$ as $P_{ISCR1}$.

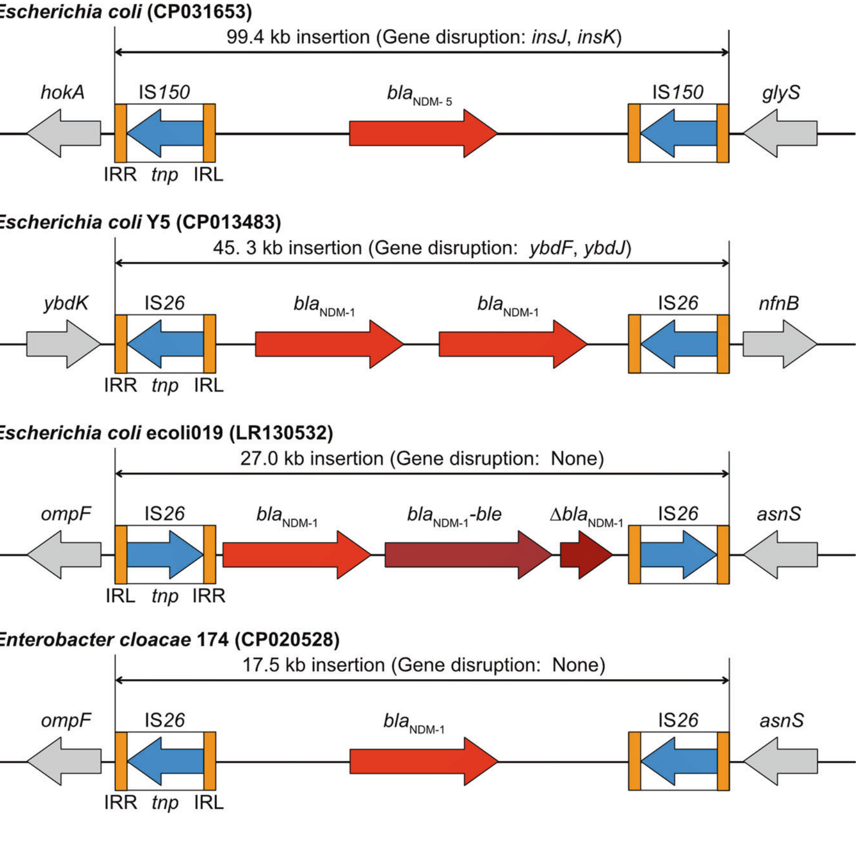
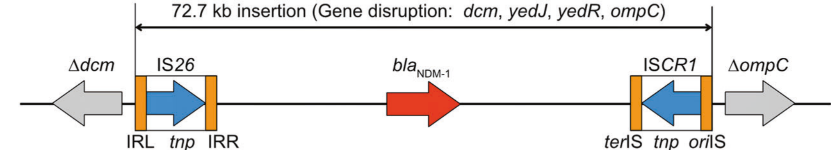
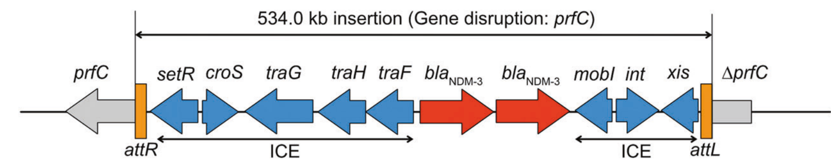



**Fig. 5** Transposons are involved in the chromosome-integration of $bla_{\mathrm{NDM}}$ in *Escherichia coli* and *Enterobacter cloacae*. Red and blue arrows indicate the $bla_{\mathrm{NDM}}$ and transposase genes (*tnp*), respectively. Brown arrows indicate the $bla_{\mathrm{NDM-1}}$-*ble* fusion gene and the truncated derivative of $bla_{\mathrm{NDM-1}}$, respectively, and both genes appear to be non-functional. Orange boxes indicate inverted repeats (IRL, IRR). Gray arrows indicate adjacent genes of chromosomes located in the transposon insertion. Genes within brackets represent chromosomal genes disrupted by the transposon insertion



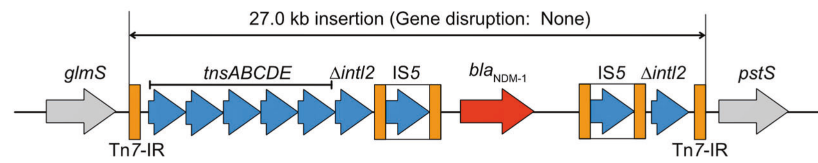




**Fig. 6** $bla_{\mathrm{NDM}}$ integrations into chromosomes are diverse in *Klebsiella pneumoniae*. Red arrows indicate $bla_{\mathrm{NDM}}$ genes. Blue arrows indicate related genes involved in the chromosome integration of the $bla_{\mathrm{NDM}}$ gene: the transposase gene (*tnp*), ICE and Tn7-like transposon. Orange boxes indicate insertion sequences involved in the chromosomal integrations. Gray arrows indicate adjacent chromosomal genes located in the transposon insertion. Genes within brackets represent chromosomal genes disrupted by the transposon insertion

## The $bla_{\mathrm{NDM}}$ genes are integrated into a bacterial chromosome

To investigate the $bla_{\mathrm{NDM}}$ integration into the bacteria chromosome, a BLAST search was carried out using the complete genomic database that consisted of 4908 chromosome data of *Enterobacteriaceae*, as described in the Materials and Methods. The chromosome integration of $bla_{\mathrm{NDM}}$ genes was detected in *E. coli*, *K. pneumoniae*, and *Enterobacter cloacae*. Although the inserted gene cassettes contained various drug-resistance genes as well as the $bla_{\mathrm{NDM}}$ gene, only the $bla_{\mathrm{NDM}}$ and the element involved in the genome integration are illustrated here (Figs. 5, 6). Interestingly, the gene cassette integrated into the

chromosome contains two $bla_{NDM}$ genes in the *E. coli* Y5 [14] and *K. pneumoniae* QD23 [15] strains (Figs. 5, 6). Furthermore, all of the chromosome-integrated $bla_{NDM}$ genes were under the control of $P_{ISAba125}$. In *E. coli* and *Enterobacter* cloacae, gene cassettes ranging from 17.5 kbp to 99.4 kbp were surrounded by IS*150* or IS*26* (Fig. 5). In *E. coli* ecoli019 and *Enterobacter cloacae* 174, the $bla_{NDM-1}$ gene was inserted at the same site on the chromosome, between *ompF* and *asnS*.

In contrast, the manner of the chromosome integration was somewhat complicated in *Klebsiella* species. In the case of *K. pneumoniae* 33476891I, the gene cassette containing the $bla_{NDM-1}$ gene was surrounded by different IS elements, IS*26* and IS*CR1* (Fig. 6). Interestingly, the integrating and conjugative element (ICE) and Tn*7*-like transposon were also involved in chromosome integration of $bla_{NDM}$ genes (Fig. 6). Two copies of $bla_{NDM-3}$ were integrated in a chromosome in *K. pneumoniae* QD23 via ICE. It has been reported that ICE is inserted via a specific sequence in *prfC* on the chromosome [16]. Indeed, we found that the chromosome integration of ICE occurred in *prfC* and was mediated by the typical *attR* and *attL* sequences (Fig. 6). The $bla_{NDM}$ genes of *K. pneumoniae* KP64 and KP67, which are similar in genome composition, were reported to be inserted into the chromosome via IS*5* [17]. We found that the site of IS*5*-insertion into the chromosome was within the Tn*7*-like transposon (Fig. 6). Thus, the *bla*NDM genes were distributed not only by horizontal gene transfer via plasmids but also by vertical gene transfer via the integration of various MGEs into the chromosome.

## Discussion

In this study, we retrieved 310 $bla_{NDM}$ genes encoding NDM-1 and its variants from the complete plasmid (10,914 data points) and chromosome (4908 data points) databases of *Enterobacteriaceae* and performed a BLAST search for the $bla_{NDM}$ promoter analysis. Most of the $bla_{NDM}$ promoters were a hybrid-type $P_{ISAba125}$ consisting of a –35 box from IS*Aba125* IRR and –10 box from the original $bla_{NDM-1}$. Furthermore, the deletion analysis of $P_{ISAba125}$ revealed that the –35 box was required for the sufficient expression of $bla_{NDM-1}$. Although in previous studies the $P_{ISAba125}$-$bla_{NDM}$ genes were found to constitute the majority of genes in various CRE, the $P_{ISCR1}$-type of $bla_{NDM-1}$ was recently detected in a plasmid in *Citrobacter sedlakii* 3347689II for the first time [13]. This clinical strain was isolated from a Swiss man who had been transferred from a hospital in Macedonia, where he had been hospitalized as a polytraumatized patient, to a hospital in Switzerland. Using an in-silico approach, we detected $P_{ISCR1}$-$bla_{NDM-1}$ in a plasmid of *Citrobacter portucalensis*, which was a clinical isolate from Nepal. As shown in Fig. 3, $P_{ISCR1}$ showed the following characteristic features: 1) IS*CR1* was located downstream of the *qac*Δ*E-sul1* genes; (2) the *rcr1*-encoding transposase was surrounded by the 379 bp *ter*IS and 232 bp *ori*IS; and 3) IS*CR1* was inserted upstream of the –10 box of $bla_{NDM-1}$ via a conserved sequence, CTTCCTATACCC, located at the 3′ end of *ori*IS. The promoter $P_{ISCR1}$ has been reported to be involved in the expression of genes conferring trimethoprim resistance, aminoglycoside resistance, ciprofloxacin resistance, and the abilities to produce class A and C β-lactamase [7]. Among the 310 $bla_{NDM}$ data points, only two *bla*NDM-1 genes were found to utilize the promoter $P_{ISCR1}$. However, because the above *Citrobacter* spp. were isolated from different geographical locations, the clinical spread of $P_{ISCR1}$-$bla_{NDM}$ genes should be carefully monitored. In this study, we retrieved 310 data points for $bla_{NDM-1}$ and its variants (NDM-1 proteins with >95% identity, 100% query coverage) from both plasmid and chromosome databases for promoter analysis. In the process of data extraction, new promoters with different IS-insertions might be detected by reducing the similarity to the NDM-1 protein.

$bla_{NDM}$ genes were generally spread via the horizontal gene transfer of plasmids. We found that a small number of $bla_{NDM}$ genes were integrated into the bacterial chromosome. In *E. coli* and *Enterobacter cloacae*, the gene cassette including $bla_{NDM-1}$ or $bla_{NDM-5}$ surrounded by the same IS was integrated into the chromosome as transposons or IS26-mediated pseudo-compound transposons [18] (Fig. 5). In the case of *K. pneumoniae* strains, the manner of chromosome integration was different. The flanking region of the gene cassette had different ISs, ISCR1 and IS26, in *K. pneumoniae* 3347689I. On the other hand, ICE was involved in the chromosome integration of two copies of $bla_{NDM-3}$ in *K. pneumoniae* QD23 [15]. Like plasmids, ICE is a self-transmissible MGE, but it differs from plasmids in that ICE is inserted into the bacterial chromosomes [19]. In ICE, several proteins are involved in the excision, integration, and self-transfer, and we detected the genes encoding these proteins (Fig. 6). In *K. pneumoniae* KP64 and KP67, $bla_{NDM-1}$ was reported to be integrated into the chromosome via IS*5* [17]. Interestingly, the gene cassette surrounded by IS*5* was located within the Tn*7*-like transposon. The transposon Tn7 is known to recognize the *att*T7 sequence at a specific site downstream of the *glmS* gene in the chromosome [20]. Indeed, the transposition of Tn7 occurred downstream of *glmS* (Fig. 6). Furthermore, *tnsABCDE*, which is involved in the transposition of T7, and IR sequences specific to Tn7 (Tn7-IR) were also detected (Fig. 6). Thus, the Tn*7*-like transposon is also involved in the chromosome integration of $bla_{NDM-1}$ in *K. pneumoniae* KP64 and KP67.

In this study, we demonstrated that the *bla*$_{NDM}$ genes utilized a highly conserved promoter, $P_{ISAba125}$. On the other hand, two cases of *bla*$_{NDM-1}$ using $P_{ISCR1}$ were detected in the *Citrobacter* species. Interestingly, IS*Aba125* and IS*CR1* were inserted upstream of the CCATATTT sequence located upstream of the –10 box of the *bla*$_{NDM}$ gene, suggesting that they function as hotspot sites for promoter acquisition. Furthermore, we found that the *bla*NDM genes spread not only through the horizontal transfer of plasmids but also through the vertical transfer of genes integrated into the chromosome, which may be a clinical problem in the future.

**Funding** This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology and the Japan Society for the Promotion of Science (KAKENHI) nos. 19K07542 (to AA), 20K07485 (to AK) and by AMED under Grant Number JP20nk0101552 (to AA) and JP22nk0101587 (to AA). The funders had no role in the study design, date collection or analysis, the decision to publish, or the preparation of manuscript.

## Compliance with ethical standards

**Conflict of interest** The authors declare no competing interests.

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