The Role of \textit{nmcR}, \textit{ampR}, and \textit{ampD} in the Regulation of the Class A Carbapenemase NmcA in \textit{Enterobacter ludwigii}

Ryuichi Nakano\textsuperscript{1*}, Yuki Yamada\textsuperscript{2}, Akiyo Nakano\textsuperscript{1}, Yuki Suzuki\textsuperscript{1}, Kai Saito\textsuperscript{1}, Ryuji Sakata\textsuperscript{1}, Miho Ogawa\textsuperscript{3}, Kazuya Narita\textsuperscript{1}, Akio Kuga\textsuperscript{4}, Akira Suwabe\textsuperscript{5} and Hisakazu Yano\textsuperscript{1}

\textsuperscript{1} Department of Microbiology and Infectious Diseases, Nara Medical University, Kashihara, Japan, \textsuperscript{2} Division of Central Clinical Laboratory, Iwate Medical University Hospital, Yahaba, Japan, \textsuperscript{3} Department of Bacteriology, BML Inc., Kawagoe, Japan, \textsuperscript{4} Hamamatsu Pharmaceutical Association, Hamamatsu, Japan, \textsuperscript{5} Department of Laboratory Medicine, Iwate Medical University School of Medicine, Yahaba, Japan

Various carbapenemases have been identified in the Enterobacteriaceae. However, the induction and corresponding regulator genes of carbapenemase NmcA have rarely been detected in the \textit{Enterobacter cloacae} complex (ECC). The NmcA-positive isolate ECC NR1491 was first detected in Japan in 2013. It was characterized and its induction system elucidated by evaluating its associated regulator genes \textit{nmcR}, \textit{ampD}, and \textit{ampR}. The isolate was highly resistant to all \(\beta\)-lactams except for third generation cephalosporins (3GC). Whole-genome analysis revealed that \textit{bla}_{NmcA} was located on a novel 29-kb putatively mobile element called EludIMEX-1 inserted into the chromosome. The inducibility of \(\beta\)-lactamase activity by various agents was evaluated. Cefoxitin was confirmed as a strong concentration-independent \(\beta\)-lactamase inducer. In contrast, carbapenems induced \(\beta\)-lactamase in a concentration-dependent manner. All selected 3GC-mutants harboring substitutions on \textit{ampD} (as \textit{ampR} and \textit{nmcR} were unchanged) were highly resistant to 3GC. The \textit{ampD} mutant strain NR3901 presented with a 700 \(\times\) increase in \(\beta\)-lactamase activity with or without induction. Similar upregulation was also observed for \textit{ampC} and \textit{nmcA}. NR1491 (pKU412) was obtained by transforming the \textit{ampR} mutant (135Asn) clone plasmid whose expression increased by \(\sim 100\times\). Like NR3901, it was highly resistant to 3GC. Overexpression of \textit{ampC}, rather than \textit{nmcA}, may have accounted for the higher MIC in NR1491. The \textit{ampR} mutant repressed \textit{nmcA} despite induction and it remains unclear how it stimulates \textit{nmcA} transcription via induction. Future experiments should analyze the roles of \textit{nmcR} mutant strains.

Keywords: carbapenemase, NmcA, AmpC \(\beta\)-lactamase, \textit{Enterobacter cloacae} complex, induction, regulator genes

INTRODUCTION

The \textit{Enterobacter cloacae} complex (ECC) have become clinically significant opportunistic bacteria and are now common nosocomial pathogens causing pneumonia, urinary tract infections, and septicemia (Davin-Regli and Pages, 2015). Six \textit{Enterobacter} species are assigned to the ECC: \textit{E. cloacae}, \textit{E. asburiae}, \textit{E. hormaechei}, \textit{E. kobei}, \textit{E. ludwigii}, and \textit{E. nimipressuralis} (Mezzatesta et al., 2012).
Multidrug resistance (MDR) has been observed for the last-resort carbapenems and has led to an increased global interest in Enterobacteriaceae in general and carbapenem-resistant ECC, in particular (Annavajhala et al., 2019). ECC are innately resistant to penicillins, first- and second-generation cephalosporins, and cephamycin due to the chromosomally encoded AmpC β-lactamase genes (serine β-lactamase, Ambler class C). AmpC β-lactamase expression is low but inducible in response to β-lactam exposure and is closely linked to a peptidoglycan recycling system, with the β-lactams imipenem, cefoxitin, and clavulanic acid strong ampC inducers (Jacoby, 2009). Regulation of AmpC β-lactamase expression is complex and involves AmpR (a transcriptional regulator of the LysR family), AmpD (a cytosolic amidase), and AmpG (a transmembrane permease) (Guerin et al., 2015). AmpR usually represses ampC in the absence of β-lactam inducers, whereas mutations at specific sites in AmpR derepresses AmpC synthesis and results in constitutive AmpC β-lactamase overexpression. Asp135Asn AmpR substitution is correlated with substantial increases in β-lactamase activity in several Gram-negative organisms including ECC, Citrobacter freundii, and Pseudomonas aeruginosa (Kuga et al., 2000; Caille et al., 2014; Nakano et al., 2017). Mutations that inactivate AmpD permanently induce and increase muropeptide concentrations in the cytoplasm and change the conformation of AmpR so that it becomes a transcriptional activator (Kuga et al., 2000). Specifically, AmpR mutations require site-specific substitution to induce AmpC β-lactamase overexpression whereas AmpD mutations need loss-of-function point mutations (missense mutation) or disruption of the protein carboxy terminus, nonsense mutations, frameshifts, and truncations (Schmidtke and Hanson, 2006). Among the ECC clinical isolates, high-level resistance to third generation cephalosporins (3GC) is caused by constitutive ampC overexpression mainly from ampD mutations and, more rarely, from ampR mutations (Kaneko et al., 2005; Guerin et al., 2015).

Carbapenem resistance in ECC is conferred either through constitutive AmpC β-lactamase overexpression combined with defective outer membrane (porin) permeability or via the acquisition of carbapenemase genes (Annavajhala et al., 2019), with the latter scenario being more common. Carbapenemases hydrolyze most β-lactams including carbapenems and are classified as serine β-lactamases (Ambler class A; KPC type and D; OXA-48 type) or metallo-β-lactamases (Ambler class B; IMP type, VIM type, and NDM type) (Diene and Rolain, 2014; Nakano et al., 2014; Ando et al., 2018). The distributions of these enzymes differ with geographical location: the KPC type occurs in the United States, NDM in the Indian subcontinent, and IMP in Japan (Chavda et al., 2016; Aoki et al., 2018; Peirano et al., 2018). Chromosomally encoded carbapenemase NmcA (Ambler class A) has been sporadically detected in ECC (Walther-Rasmussen and Hoiby, 2007).

NmcA was originally detected in the carbapenem-resistant E. cloacae strain NOR-1 isolated in France in 1990 (Nordmann et al., 1993). NmcA has occasionally been detected in E. cloacae, E. asburiae, and E. ludwigii from Europe, North America, and South America (Pottumarthy et al., 2003; Radice et al., 2004; Antonelli et al., 2015; Boyd et al., 2017). A recent study revealed that blaNmcA is associated with a novel 29-kb putative Xer-dependent integrative mobile element (EludIMEX-1) inserted into the ECC chromosome (Antonelli et al., 2015). This enzyme hydrolyses different β-lactam agents except for 3GC and has particularly high hydrolytic activity against carbapenems (Nordmann et al., 1993; Mariotte-Boyer et al., 1996). The inducibility of NmcA is similar to AmpC β-lactamase (Pottumarthy et al., 2003), where the LysR family transcriptional regulator gene nmcR upstream of blaNmcA regulates nmcA in the same manner as the ampR–ampC regulatory system does for AmpC. Additionally, AmpD co-regulates nmcA (Naas et al., 2001).

Here, we describe the characteristics of an nmcA-positive ECC isolate first observed in Japan. We also elucidate the nmcA induction system by evaluating nmcA expression in ampD and ampR mutant strains.

**MATERIALS AND METHODS**

**Bacterial Strains and Antimicrobial Susceptibility Testing**

The carbapenem-resistant ECC strain NR1491 was isolated from the urine of a patient in a Japanese hospital in 2013. The species was identified as E. cloacae by MicroScan WalkAway plus (Beckman Coulter, Inc., Brea, CA, United States). To evaluate the effects of ampR mutation on antimicrobial susceptibility and β-lactamase expression, ampR clone plasmids were constructed and used to transform NR1491. The ampR clone plasmids (pKU411 and pKU412) used in this study were already previously constructed (Kuga et al., 2000). An *in vitro* ceftazidime-resistant mutant strain NR3901 was isolated from NR1491. Characteristics of the bacterial strains and plasmids used in the present study are listed in Table 1.

The minimum inhibitory concentrations (MICs) of the various antimicrobial agents were determined for each

| TABLE 1 | Bacterial strains and plasmids used in the present study. |
| Strain or plasmid | Relevant characteristics | Source or references |
|-------------------|-------------------------|---------------------|
| **Strains**       |                         |                     |
| E. ludwigii NR1491| Clinical isolate from Japan, resistance to carbapenems | This study |
| pKU411/NR1491     | *E. ludwigii* transformed with pKU411 | This study |
| pKU412/NR1491     | *E. ludwigii* transformed with pKU412 | This study |
| NR3901            | Ceftazidime-resistant mutant of *E. ludwigii* NR1491, AmpD mutant (69delG) | This study |
| **Plasmids**      |                         |                     |
| pKU411            | Wild type ampR (135Asp) of *E. cloacae* GN7471 cloned into pMW218 | Kuga et al., 2000 |
| pKU412            | Mutant ampR (135Asn) of *E. cloacae* GN7471 cloned into pMW218 | Kuga et al., 2000 |
strains by the agar dilution method according to CLSI guidelines (CLSI, 2018).

Whole-Genome Sequencing and Analysis
The genomic DNA of NR1491 was prepared with a Qiagen Genomic-tip 500/G kit (Qiagen, Hilden, Germany) and subjected to whole-genome sequencing on the MiSeq X10 platform (llumina, San Diego, CA, United States). Reads were trimmed in Trimmomatic and assembled to contigs with the SPAdes v. 3.8.1 genome assembler in cautious mode (Bankevich et al., 2012).

Species were precisely identified based on their average nucleotide identity (ANI) and in silico DNA-DNA hybridization between strain NR1491 (GenBank accession no. BKZ00000000.1), the E. cloacae type strain ATCC 13047 (GenBank accession no. MTFV00000000.1), the E. ludwigii type strain EN-119 (GenBank accession no. JTLO00000000.1), and the E. ludwigii type strain AOUC-8/14 (GenBank accession no. LGIV00000000.1). Earlier studies recommended ANI of ~95–96% as a species demarcation cutoff (Goris et al., 2007; Chun and Rainey, 2014).

Antimicrobial resistance genes were identified in the genome sequence with the ResFinder database using thresholds of 90% identity and 60% minimum length. β-lactamase genes including carbapenemases and extended-spectrum β-lactamases (ESBLs) were also assessed by PCR. PCR detection of carbapenemases (blaIMP, blaVIM, blaKPC, blaOXA–48–like, blaNDM, blaGES, blaIMI/NmcA, and blaSHV) (Poirel et al., 2011; Hong et al., 2012; Nakano et al., 2018) and ESBLs (blaTEM, blaCMY, and blaCTX-M) (Dallenne et al., 2010) were performed as previously described. The sequence surrounding blaNmcA, a carbapenemase-encoding gene, was elucidated by PCR and Sanger sequencing to close the gaps between the contigs. Sequence alignment and analysis were performed with BLAST at NCBI (National Centre for Biotechnology Information, Bethesda, MD, United States). Multilocus sequence typing (MLST) of the E. cloacae isolates was performed as previously described (Miyoshi-Akiyama et al., 2013). Sequence types were assigned at the PubMLST database. The presence of mobile genetic elements was investigated using the MobileElementFinder (Johansson et al., 2021) and INTEGRALL (Moura et al., 2009). The plasmid content was assessed using PlasmidFinder (Carattoli et al., 2014).

Selection of Third-Generation Cephalosporin-Resistant Mutants and Detection of Sequence Alterations
Third-generation cephalosporin-resistant mutants were obtained by plating ~10^9 CFU mL^{-1} late-logarithmic-phase NR1491 grown in Luria-Bertani (LB) broth and on LB agar plates containing cefazidime or cefotaxime at 2x, 4x, 8x, 16x, and 32x MIC. The mutation frequencies were determined by dividing the colony density in CFU mL^{-1} on LB agar plates containing the antibiotic agents by the total colony density in CFU mL^{-1}.

The DNA sequences of the selected mutants were determined by Sanger sequencing of nmcR, ampR, and ampD amplicons. The primers used are listed in Supplementary Table 1 (Radice et al., 2004). The nucleotides and amino acids of the selected mutants were compared with those of E. ludwigii NR1491 and EN-119.

β-Lactamase Induction Assays
β-lactamase activity was measured in terms of the protein content in the extract and compared among cultures in 50 mM phosphate buffer (pH 7.0) at 30°C by spectrophotometry as previously described (Nakano et al., 2004). The protein concentrations were determined by the Bradford assay (Bradford, 1976). One unit of β-lactamase activity was defined as the amount of β-lactamase hydrolyzing 1 μmol cephalothin in 1 min at 30°C. The β-lactamase induction assays were performed by subjecting mid-logarithmic phase bacteria in Mueller-Hinton broth to β-lactams at 1/16x, 1/8x, 1/4x, 1/2x, and 1x MICs for 2 h (Kuga et al., 2000). The antibiotics cefpodoxime, clavulanic acid, cefoxitin, imipenem, and meropenem were used as inducers. The induction ratios were calculated in terms of the ratio of β-lactamase activity mg^{-1} protein in induced cells to the β-lactamase activity per mg^{-1} protein in uninduced cells.

Measurement of ampC and nmcA mRNA Levels by qRT-PCR
The mRNA expression levels of ampC and nmcA with and without induction were determined by qRT-PCR as previously described (Nakano et al., 2017). Total RNA was extracted with the RNeasy protect bacteria mini kit and the RNase-free DNase set (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The qRT-PCR was performed in a StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, United States) with a Power SYBR Green DNA-to-CT 1-Step kit (Thermo Fisher Scientific, Waltham, MA, United States) and 100 ng total RNA in a 20-μL reaction, according to the manufacturer’s instructions. The primers used are listed in Supplementary Table 1 (Doumith et al., 2009). The relative gene expression levels were calculated by the 2−ΔΔCT method. The mRNA of the housekeeping gene rpoB was chosen as the endogenous reference for relative quantification. The results are presented as the mRNA expression level compared with that of NR1491. The experiment was performed in triplicate. The final relative expression levels of ampC and nmcA were determined by calculating the averages for their transcripts. The coefficient of variation (SD/mean) among experiments was < 10%.

Nucleotide Sequence Accession Numbers
The nucleotide sequences of the genetic regions surrounding blaNmcA and the whole-genome DNA sequences of NR1491 were deposited in the GenBank database under accession numbers LC482123 and BKZ00000000.1, respectively.
RESULTS

Identification of bla<sub>NmcA</sub>-Harboring Enterobacter ludwigii NR1491

The draft NR1491 genome (GenBank accession no. BKZ00000000.1) was obtained by MiSeq (Illumina, Sa, Diego, CA, United States), and average nucleotide identity (ANI) analysis using <em>E. cloacae</em> strain ATCC13047, <em>E. ludwigii</em> type strain EN-119, and <em>E. ludwigii</em> AOUC-8/14 as reference genomes. Their respective ANI values were 87.82, 98.96, and 98.97%.<sup>4</sup> NR1491 was identified as <em>E. ludwigii</em> belonging to ST258.

Antimicrobial Susceptibility and Resistance Genes

Antimicrobial susceptibility assays showed that <em>E. ludwigii</em> NR1491 was highly resistant to cephalothin, cefmetazole, carbapenems, and fosfomycin (> 512 µg mL<sup>−1</sup>) but susceptible to 3GC, piperacillin–tazobactam, cefepime, aztreonam, levofloxacin (<0.06 µg mL<sup>−1</sup>), and gentamicin (0.5 µg mL<sup>−1</sup>) (Table 2). However, the MIC of cefotaxime increased when the agent was combined with clavulanic acid.

Whole-genome analysis with ResFinder revealed the following resistance-encoding genes: <em>bla</em><sub>NmcA</sub> (carbapenemase), ACT-12 (<em>AmpC</em> β-lactamase), and <em>fosA2</em> (glutathione transferase; fosfomycin resistance). It also disclosed that the regulator genes <em>nmcR</em> and <em>ampR</em> were upstream of <em>bla</em><sub>NmcA</sub> and <em>ampC</em>, respectively. The entire nucleotide sequences of <em>bla</em><sub>NmcA</sub> and <em>nmcR</em> and the intercistronic region were determined by Sanger sequencing. The sequences were identical to that of <em>E. cloacae</em> NOR-1 (accession no. Z21956). PCR demonstrated that no other acquired β-lactamase gene was harbored. The regulatory gene sequences of <em>bla</em><sub>NmcA</sub> (<em>nmcR</em>, <em>ampR</em>, and <em>ampD</em>) were compared with that of the reference strains of ECC (NOR-1, EN-119, and AOUC-8/14); there are no mutations in these genes. Whole-genome analysis indicated that the insertion sequence (IS) elements and an integron were not encoded on the chromosome; the strain did not harbor a plasmid.

Genetic Environment Analysis of <em>bla</em><sub>NmcA</sub>

The genetic environment of <em>bla</em><sub>NmcA</sub> was determined to be a 48,089-bp nucleotide fragment characterized by whole-genome and Sanger sequencing and deposited into GenBank under accession no. LC482123. A BLASTn analysis showed that the fragment was highly similar to <em>E. ludwigii</em> AOUC-8/14 (accession no. KR919803) (44,766/44,874 nucleotides; 99.76% identity). The <em>bla</em><sub>NmcA</sub> was located on a novel putatively mobile 29-kb element designated EludIMEX-1 inserted into the same chromosome location as that in <em>E. ludwigii</em> AOUC-8/14. Two imperfect 29-bp inverted repeat XerC/XerD binding sites associated with EludIMEX were identified at the chromosome–EludIMEX-1 junctions. The genetic regions were compared with the corresponding regions of <em>E. ludwigii</em> P101 (GenBank accession no. CP006580); the schematic representations are depicted in Figure 1. There were highly homologous regions (> 99% identity).
 Regulation System of Carbapenemase NmcA

FIGURE 1 | Schematic representations and nucleotide sequences of the genetic elements surrounding EludIMEX-1 of E. ludwigii NR1491 (GenBank accession no. LC482123) and the corresponding region of E. ludwigii P101 (GenBank accession no. CP006580). (A) Schematic representation of the genetic region of E. ludwigii NR1491 (black arrow) and E. ludwigii P101 (blue arrow). The gray regions between the NR1491 and P101 indicate > 99% nucleotide sequence identity. Insertion of the EludIMEX-1 element (double-headed arrow) in E. ludwigii NR1491 was observed. (B) Nucleotide sequences at the junctions of EludIMEX-1 (a part of left and right of the junctions) of E. ludwigii NR1491 and the corresponding region of E. ludwigii P101. The XerC/XerD binding sites are boxed, the conserved regions are boldfaced, and the consensus repeat sequences in XerC binding site of E. ludwigii NR1491 and corresponding sequences of E. ludwigii P101 are indicated using underlined letters.

The antibiotic inducibility of β-lactamase was analyzed in NR1491 (Figure 2). Cefpodoxime and clavulanic acid were slightly inducer in a concentration-dependent manner. They yielded only a maximum $3.3 \times$ induction of the MIC. Conversely, cefoxitin, imipenem, and meropenem were strong β-lactamase inducers. The carbapenems imipenem and meropenem induced β-lactamase in a concentration-dependent manner to $159 \times$ and $202 \times$, respectively, at half their MIC. In contrast, the cefoxitin induction rate was concentration-independent and remained virtually unchanged (98–113 ×) across the tested concentrations (1/16–1 × MIC).

Properties of the Selected Third Generation Cephalosporins-Resistant Mutants

The 3GC-resistant mutants were selected with cefotaxime and ceftazidime at 2×, 4×, 8×, 16×, and 32× MIC. The
antibiotic-resistant mutants were consistently obtained at a mutation frequency of $\sim 10^{-6}$–$10^{-7}$ both for cefotaxime and ceftazidime (Table 3).

To investigate the molecular mechanism of 3GC resistance in these mutants, 48 clones were randomly selected from each condition. The regulator genes ampR, nmcR, and ampD were sequenced and compared to those of the parent E. ludwigii NR1491 and wild type EN-119 strains. Sequence data revealed that only ampD was altered in all cases whereas neither ampR nor nmcR was changed. Of the 48 3GC-resistant mutants, 34 had possible loss-of-function caused by missense mutations including 18 amino acid substitutions at 16 positions in ampD (Table 3). Premature termination of the AmpD protein was found in 14 mutants. Seven had nonsense mutation at six positions, five had frameshift mutations (three deletions and two insertions), and two had missense mutations in which the start codon (ATG) was changed to Ile (ATA) at position 1. Its effect was transcriptional decay. These mutations were scattered throughout the entire ampD sequence. Moreover, the nucleotide substitutions and mutation types and locations did not differ among selective agents and concentrations. However, certain mutants had the same missense mutation positions. Eight mutations were I78N, seven were T123P, and four were S100L. These mutation positions may have been influential to AmpD activity. These mutants were resistant to 3GC presumably as a consequence of loss of AmpD function via the introduction of substitution mutations or decay of the transcript containing the premature stop codon.

For the mutants, the MICs were determined for the selected β-lactams (Table 3). Compared with the MICs for the parent strain NR1491, the MICs of 3GC (cefotaxime and ceftazidime) and aztreonam for the mutants had increased by $\geq 16 \times$. Thus, these strains were reclassified from susceptible to resistant. The MICs of cefepime increased by only 1–4 $\times$ and they were reclassified as highly resistant. The β-lactamase activity of NR3901 increased by $\sim 706.5$ and $\sim 704.5 \times$ with and without induction, respectively, compared with the NR1491 basal condition. The ampC and nmcA expression levels in NR3901 both increased by $\sim 700 \times$ in the presence and absence of induction. The ampC and nmcA expression levels in NR1491 both increased by $\sim 15 \times$ in response to induction. Hence, ampD equally induced ampC and nmcA expression. NR3901 was highly drug-resistant because it acquired the ampD mutation which derepressed ampC and nmcA expression.

NR1491 (pKU412) was obtained by transfecting the ampR mutant (135Asn) clone plasmid pKU412 into NR1491. The MICs of NR1491 (pKU412) were elevated as they were for NR3901. The β-lactamase activity had increased by $\sim 100 \times$ at basal condition. ampC expression also increased by $\sim 100 \times$ whereas that of nmcA did not change. AmpR may induce AmpC β-lactamase but does not affect nmcA expression. NR1491 (pKU412) induction

### Table 3

| Mutation | Selective agents ($\mu$g mL$^{-1}$)$^a$ | No. of selected strains | Amino acid and nucleotide changes detected in AmpD (no. of strains)$^b$ | MIC range ($\mu$g mL$^{-1}$)$^a$ |
|----------|--------------------------------|------------------------|-------------------------------------------------|---------------------------------|
| Misense  | CTX (1, 2, 4, 8, 16) | 36 | M1I (2), N35K (2), L56P, L56Q, T55P, H75Y, I78N (8), I78S, G82V, W95G, G98D, S100L (4), L117R, E118G, T123P (7), T137P, G166A, D170Y | 16–128 8–32 128–256 8–32 16–64 128–256 16–64 0.125–0.5 | 512–512 32–256 16–64 8–16 |
| Misense  | CAZ (2, 4, 8, 16, 32) | 36 | M1I (2), N35K (2), L56P, L56Q, T55P, H75Y, I78N (8), I78S, G82V, W95G, G98D, S100L (4), L117R, E118G, T123P (7), T137P, G166A, D170Y | 16–128 8–32 128–256 8–32 16–64 128–256 16–64 0.125–0.5 | 512–512 32–256 16–64 8–16 |
| Misense  | CAZ (2, 4, 8, 16, 32) | 36 | M1I (2), N35K (2), L56P, L56Q, T55P, H75Y, I78N (8), I78S, G82V, W95G, G98D, S100L (4), L117R, E118G, T123P (7), T137P, G166A, D170Y | 16–128 8–32 128–256 8–32 16–64 128–256 16–64 0.125–0.5 | 512–512 32–256 16–64 8–16 |
| Non-sense | CTX (1, 8, 16) | 7 | W7*, E26*, E83*, Q86*, Y102* (2), Q103* | 128 32 256–512 16 8–16 64 0.25–0.5 | 512 256 16–32 8–16 |
| Non-sense | CAZ (8, 32) | 7 | W7*, E26*, E83*, Q86*, Y102* (2), Q103* | 128 32 256–512 16 8–16 64 0.25–0.5 | 512 256 16–32 8–16 |
| Frameshift | CTX (8, 16) | 5 | 69delG, 129_130insT, 270_271insT, 372delC, 401_404del | 128 16–32 256–512 16–32 8–16 64 0.5 | 512–512 256 32 16 |

$^a$ Antibiotics: PIP, piperacillin; TAZ, tazobactam; CEF, cephalothin; CPD, cepodoxime; CTX, cefotaxime; CLA, clavulanic acid; CAZ, ceftazidime; FEP, ceftipime; CFX, cefoxitin; AZT, aztreonam; IPM, imipenem; MER, meropenem.

$^b$ Nucleotide and deduced amino acid differences in AmpD were compared with E. ludwigii NR1491 and EN-119. *, stop codon; del, deletion; ins, insertion.
resulted in a $1.5 \times$ increase which suggests partial derepression. 
ampC expression increased by $\sim 200 \times$ after induction. However, 
nmcA expression only doubled despite NR1491 expression 
increasing by $\sim 15 \times$. Plasmid pKU411 comprising the wild 
type ampR (I35Asp)-harboring strain NR1491 (pKU411) was 
compared with the ampR mutant strain and used to verify it. The 
MICs and β-lactamase activity of NR1491 (pKU411) were nearly 
the same as those for NR1491.

**DISCUSSION**

The incidence of CPE is increasing globally. However, it has 
seldom (0.34%) been detected in Japan (Ohno et al., 2017). The most 
common carbapenemase genotype detected in Japan is 
IMP. Here, we isolated NmcA carbapenemase-producing ECC. 
NmcA carbapenemase has occasionally been reported for ECC in Europe, North America, and South America (Radice et al., 2004; Antonelli et al., 2015; Boyd et al., 2017). To the best of our knowledge, this is the first reported clinical isolation of an NmcA carbapenemase producer in Japan.

ANI revealed that NR1491 was, in fact, E. ludwigii belonging 
to ST258. A previous study reported that bla\textsubscript{NmcA} 
was highly associated with E. ludwigii. ST258 is a genotype of 
the NmcA carbapenemase producer (Boyd et al., 2017). The 
genetic environment of bla\textsubscript{NmcA} was nearly identical to that of 
E. ludwigii AOUC-8/14. Thus, bla\textsubscript{NmcA} was putatively integrated 
into the chromosome by EludIMEX-1 via a Xer-dependent 
recombination mechanism as previously described for E. ludwigii 
AOUC-8/14 (Antonelli et al., 2015). Interestingly, E. ludwigii 
AOUC-8/14 was isolated from a Japanese tourist in Italy. These 
strains may have been concealed in Japan and unintentionally 
isolated in the present study. Comparative genome analysis 
revealed that there were 10 strains including AOUC-8/14, which 
have high homology regions with NR1491. These EludIMEX-1 
was integrated in the chromosome at the same site as in NR1491. 
The genotypes of these strains were different; the EludIMEX-1 
insertion event has possibility occurred in these STs strains.

As with bla\textsubscript{NmcA}, NR1491 coexists with the regulator gene 
nmcR. A β-lactamase induction assay on NR1491 showed that it 
was weakly induced by clavulanic acid which was already known 
to be an inhibitor of Class A β-lactamases. Thus, it is inhibitory 
against NmcA β-lactamase (Mariotte-Boyer et al., 1996). On the 
other hand, clavulanic acid also induces chromosomally mediated 
AmpC β-lactamases in several Enterobacteriaceae (Drawz and Bonomo, 2010). Here, clavulanic acid induced β-lactamases via 
transcriptional regulator genes and not by inhibiting NmcA. The 
MIC of cefotaxime was increased in combination with clavulanic 
acid while imipenem and meropenem induced β-lactamase in a 
concentration-dependent manner. Previous study of its kinetic 
parameters show that NmcA demonstrated unusually strong 
hydrolytic activity toward imipenem and meropenem (Mariotte- 
Boyer et al., 1996). Therefore, NmcA producers are highly 
resistant to carbapenems as their inducers are upregulated and 
they are potently hydrolytic to carbapenems. Cefoxitin is a strong, 
stable, dose-independent β-lactamase inducer (100 × induction). 
The catalytic efficiency ($k_{\text{cat}}/K_m$) of cefoxitin is lower than 
those of the carbapenems but its MIC is comparatively higher 
(Mariotte-Boyer et al., 1996) possibly because of its high and 
stable inducibility.

To elucidate the mechanism of β-lactamase induction in 
NR1491, 3GC-resistant mutants were selected with cefotaxime 
and ceftazidime. The mutation frequencies were $10^{-9}$–$10^{-7}$ as 
previously described (Naas et al., 2001). Forty-eight randomly 
selected clones had variable susceptibilities to 3GC, piperacillin-
tazobactam, and aztreonam (Table 3). A DNA sequence analysis 
revealed that all mutants presented with nucleotide substitutions 
(frameshift, missense, or nonsense) in ampD alone. In contrast, 
ampR and nmcR were unchanged. The mutants were resistant to 
3GC and probably had loss of AmpD function. Premature AmpD 
termination with a stop codon or frameshift induced strong 3GC 
resistance. The MICs of the ampD mutant strains with missense 
mutation had different 3GC resistance levels. The degree of 3GC 
resistance depended on the position of the substitution at the core 
residues of the active site of AmpD.

In the present study, no mutants of the transcriptional 
regulator genes ampR and nmcR were obtained. We investigated 
the effects of ampR in the presence of a mutant or wild 
type ampR clone plasmid. AmpR is a member of the lysR 
family and regulates the expression of chromosomal AmpC 
β-lactamase. Nevertheless, ampR mutants cause constitutive 
AmpC overproduction (75–470× increase) irrespective of 
induction (Kuga et al., 2000). In the enzyme activity assay, 
NR1491 (pKU412) increased β-lactamase activity by 100 × at 
the basal level compared with NR1491, also upregulating ampC. 
β-lactamase activity in the NR1491 strain with wild type ampR 
was increased by 100 × by induction and ampC and nmcA 
were each upregulated 15×. Whereas NR1491 with wild type 
ampR upregulated nmcA 15×, nmcR expression in NR1491 
(pKU412) only doubled. Mutant ampR may negatively regulate 
nmcR expression. Putative ampR binding sequences in the 
E. cloacae ampR–ampC intergenic region were highly conserved 
with nmcR–bla\textsubscript{NmcA} and cross-reaction may have occurred 
(Naas and Nordmann, 1994). Earlier studies suggested that 
ampR is a global transcriptional regulator affecting the expression of several 
genes as well as ampC (Balasubramanian et al., 2012). NmcR 
was described as a positive regulator both in the absence and 
especially in the presence of a β-lactam inducer. In the absence 
of inducer, ampR is a negative regulator of ampC expression. 
In its presence, it positively regulates ampC expression (Naas 
and Nordmann, 1994). These findings suggest that even with available 
induction, mutant ampR represses the expression of nmcA. We 
believe this is the first study to describe the association between 
ampR and nmcR expression.

NmcR mutant strain has not been identified yet; therefore, 
the effect of nmcR mutations on the expression of nmcA could 
not be assessed. Point mutations in nmcR may be required to 
enhance its efficacy as an activator of nmcA in the same way as 
mutant ampR (such as that with a change in Asp135Asn). 
Alternatively, it may repress nmcA expression in the same way 
as wild type ampR. In a previous study, ampR mutants were 
obtained from the ampD mutant strain at a very low frequency 
(Kuga et al., 2000). The ampD mutant strain NR3901 selected 
nmcR mutants using ceftazidime at double and quadruple the
MIC (128 and 256 μg mL⁻¹). Nevertheless, no nmcR mutants were obtained (data not shown). nmcR mutants may be selected using ampD-mutant E. coli strains carrying blaNmcA and nmcR cloning plasmids. NR1491 co-harbors ampC on the chromosome. The observed differences in β-lactamase substrate specificity may influence selection conditions, and further investigation is needed to clarify whether nmcR mutation increases resistance by upregulating nmcA. This study has certain limitations. The analysis included only one strain and the conclusions were based on the results from this strain. Therefore, further studies are required to clarify the mechanisms of nmcR by selecting the nmcR mutant strains using other NmcA-producing ECC or IMI (closely related carbapenemase NmcA) producing E. coli. Moreover, the ampR mutation in NR1491 resulted in strong 3GC resistance via ampC overexpression. However, it remains unclear as to how mutant ampR stimulates nmcA transcription through induction. Future research should aim to elucidate the function of ampR.

NR3901 with an ampD mutation presented with a 700 × increase in β-lactamase activity as well as upregulated ampC and nmcA. Consequently, the MICs of 3GC were elevated. It indicates that ampD mutation has a similar influence on the expression of both ampC and nmcA, suggesting that structurally unrelated genes could be under the control of an identical regulatory system (Naas et al., 2001). The MICs for NR3901 and NR1491 (pKU412) bearing the ampR mutant plasmid were nearly equal. The upregulated nmcA in NR3901 had no effect on MIC compared with NR1491 (pKU412). Moreover, the MICs of the carbapenem-resistant NR3901 were almost always the same as those of the parent strain despite the ampD mutant constitutively upregulating ampC and nmcA. High-level nmcA expression may elevate the MICs of carbapenems; in fact, the MICs were same as those of the parent strain. NmcA metabolism and its associated physiology may be connected with MICs. However, further experimentation is required to clarify this mechanism.

Here, we detected the NmcA-producing strain NR1491 in a hospital patient. Examination of MIC patterns showed high resistance to carbapenems but susceptibility to 3GC. The ampD mutant strains were identified among clinical isolates of ceftazidime-resistant E. cloacae as previously reported (Kaneko et al., 2005). Therefore, ampD-mutant NmcA producers may occur and interfere with the clinical detection of their wild type counterparts. We characterized NmcA producers that were highly resistant to carbapenems and yet susceptible to cefepime, whether they acquired the ampD mutation. In future works, it would be informative to compare these strains with the Big Five carbapenemases (KPC, IMP, NDM, VIM, and OXA).

CONCLUSION
In the present study, we identified the E. ludwigii isolate NR1491 in Japan that produces NmcA. The blaNmcA was located on a novel 29-kb putatively mobile element designated EludIMEX-1 identical in structure to that previously described in Europe. Induction studies revealed that the ampD mutants equally upregulated β-lactamases nmcA and ampC and were highly resistant to 3GC. However, the observed increase in the MIC value of 3GC was caused mainly by ampC overexpression. The ampR mutants also upregulated ampC, however, that of nmcA seemed to be repressed. Further research is necessary to elucidate the functions of ampR and nmcR.

DATA AVAILABILITY STATEMENT
The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

AUTHOR CONTRIBUTIONS
RN conceived and designed the experiments, performed the experiments, analyzed and interpreted the data, and wrote the manuscript. YY contributed reagents and materials and performed the experiments. AN, YS, KS, RS, MO, and KN performed the experiments, validation, and interpreted the data. AK contributed reagents and materials, conceived, and designed the experiments. AS and HY supervision and project administration. All authors contributed to manuscript, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.794134/full#supplementary-material

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Conflict of Interest: RS and MO were employed by the company BML Inc.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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