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Instability of the IncFII-Type Plasmid Carrying bla_{NDM-5} in a *Klebsiella pneumoniae* Isolate

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In this study, we characterized the bla_{NDM-5}-bearing plasmid in a *Klebsiella pneumoniae* isolate that had lost the plasmid during serial passage. We determined the complete sequences of the plasmid pCC1410-2, which was extracted from a *K. pneumoniae* ST709 isolate collected at a Korean hospital from which two NDM-5-producing *K. pneumoniae* isolates were subsequently isolated. As a result, the pCC1410-2 plasmid had a backbone structure that was similar to those of two plasmids previously reported from the same hospital, but lacked some antibiotic resistance genes (bla_{TEM-1}, *rmtB*, *mphR*(*A*), *mrx*(*A*), and *mph*(*A*)). A 9-bp repeating unit encoding three amino acids (Gln-Gln-Pro) was inserted in TraD in pCC1410-2. Thus, the pCC1410-2 plasmid might be transferred from the previously identified carbapenem-resistant *K. pneumoniae*, but some delections and inversions might have occurred during the process. We compared the transfer frequency and stability of the plasmids. The relative frequency of conjugative transfer and stability in the host were significantly lower in pCC1410-2 than in previously reported bla_{NDM-5}-bearing plasmids in Korea. A low transfer frequency and instability in the host may cause underestimation of carbapenemase-producing Enterobacteriaceae in the clinical setting and in surveillance studies.

Keywords: NDM-5, plasmid transfer, plasmid stability, *Klebsiella pneumoniae*

Introduction

Since *Klebsiella pneumoniae* isolates have become resistant to first-line antibiotics such as cephaloporins, fluoroquinolones, and aminoglycosides, carbapenems have been used to treat *K. pneumoniae* infections. However, carbapenem-resistant *K. pneumoniae* isolates have recently increased worldwide [1]. In particular, carbapenemases such as *Klebsiella pneumoniae* carbapenemases (KPC) and New Delhi metallo-β-lactamase (NDM) became one of the main mechanisms of carbapenem resistance. Since the first report in 2009 [2], NDM-1-producing Enterobacteriaceae isolates have been found and are considered a threat to human health [1, 3]. In Korea, NDM-1-producing *K. pneumoniae* was first isolated in 2010 [4].

In our previous study, we reported that two plasmids belonging to the incompatibility group IncFIIA carried the bla_{NDM-5} gene in *K. pneumoniae* ST (sequence type) 147 isolates [5]. NDM-5 differs from NDM-1 by two amino acid substitutions at positions 88 (Val→Leu) and 154 (Met→Leu). Based on several pieces of evidence, including the whole sequences of plasmids, we concluded that these plasmids were transmitted by crossborder transfer of a patient from the United Arab Emirates (UAE) to Korea [5, 6]. In the present study, we report an additional *K. pneumoniae* isolate producing NDM-5, which was isolated 1 month after the second isolate in the same hospital in Korea. Although the previously reported carbapenemase-producing *K. pneumoniae* isolates co-produced NDM-5 and OXA-181, the third *K. pneumoniae* isolate, CC1410-2, was positive only for bla_{NDM-5}, but lacked $bla_{OXA-181}$. The third *K. pneumoniae* isolate had been shown to be carbapenem-resistant and

positive for bla_{NDM-5}, but became carbapenem-susceptible and bla_{NDM-5}-negative after serial cultures without antibiotic selective pressure and was thus reported as carbapenem-susceptible to the Korean Centers for Disease Control and Prevention (KCDC). For this reason, we did not report the third isolate in our previous study. In this study, we determined the complete sequences of the third isolate and compared them with those of the previous NDM-5-producing *K. pneumoniae* isolates. In addition, we compared the transfer frequencies and stability of the plasmids in their hosts.

Materials and Methods

Bacterial Isolate

The carbapenem-resistant *K. pneumoniae* isolate CC1410-2 was isolated from a patient at the medical intensive care unit in a tertiary hospital in Korea, where two carbapenem-resistant *K. pneumoniae* ST147 isolates carrying bla_{NDM-5} were identified previously [6]. Multilocus sequence typing analysis indicated that this isolate belonged to ST709, which was different from that of the previously isolated NDM-5-producing *K. pneumoniae*. The plasmid pCC1410-2, harboring bla_{NDM-5}, was isolated from the isolate CC1410-2. The plasmid was conjugated into *Escherichia coli* J53, and plasmid DNA was purified as previously described [5]. In addition, transconjugants with the previously reported plasmids pCC1409-1 and pCC1410-1 were included in the subsequent analysis.

Plasmid Sequencing

A PacBio RS II sequencer system (Pacific Biosciences of California Inc., USA) was used for complete sequencing of the plasmid pCC1410-2. Sequencing reads were assembled into consensus de novo assembly contigs using GS De Novo Assembler software (ver. 3.0). Polymerase chain reaction combinations and sequencing were used to close gaps between the contigs and confirm the positions of contigs. Open reading frames were predicted and annotated using the Glimmer 3.0 System (http://ccb.jhu.edu/software/glimmer/index.shtml) and confirmed with DNAMAN 5.2.10 software (LynnonBioSoft, Lynnon Corporation; http://www.lynnon.com). Each predicted protein was compared against an all-protein database using BlastP (http://blast.ncbi.nlm.nih.gov/Blast.cgi) with a minimum cut-off of 30% identity and 80% length coverage. The annotated sequence of pCC1410-2 has been submitted to the GenBank nucleotide sequence database, with the GenBank accession number KY288024.

Transfer Frequency and Stability

Transfer frequencies in vitro between plasmid-containing strains and plasmid-free recipients (*E. coli* J53) for the liquid method were determined by incubation of growing bacteria in Luria-Bertani (LB) broth at 37°C with shaking for 5 h, followed by dilution and plating on selective LB plates containing 10 mg/l cefotaxime (for plasmid selection) and 100 mg/l sodium azide (for counter-selection of the donor). Independent plasmid transfer experiments were performed three times, and the mean values are presented [7]. Plasmid stability was tested as described previously with modifications [8]. Briefly, the transconjugant *E. coli* J53 containing the plasmid was grown overnight in LB broth with selection at 37°C and shaken at 180 rpm. Overnight cultures were diluted 1/100,000 into fresh LB broth without antibiotics and grown at 37°C with shaking (180 rpm). At every 12 h, the cultures were diluted 1/100,000 into fresh LB broth without antibiotics and grown at 37°C with shaking (200 rpm). Aliquots were removed at 20, 40, 60, 80, and 100 min (as indicated in the graphs), serially diluted, and plated on LB plates containing 2 mg/l meropenem or on LB plates without antibiotics. The percentages of plasmid-containing colonies were plotted against time. The stability test was performed three times, and the average values are presented.

Results and Discussion

The plasmid pCC1410-2 from the isolate CC1410-2 was identified to be 82,841 bp in size and to contain 90 protein coding sequences. This plasmid belonged to the IncFIIA incompatibility group and had a G+C content of 52.4%. As expected, the plasmid pCC1410-2 was highly similar to pCC1409-1 and pCC1410-1 (GenBank Accession No. KT725789.1 and KT725788.1, respectively), which were from the previously reported NDM-5-producing *K. pneumoniae* isolates from the same Korean hospital [5, 6]. The plasmid was also similar to a plasmid from a carbapenem-resistant OXA-181-producing *K. pneumoniae* isolate, SKGH01, from the UAE (GenBank Accession No. CP015500.1; Fig. 1A). Whereas both previously identified NDM-5-producing *K. pneumoniae* isolates belonged to ST147, the third isolate belonged to ST709. Although carbapenem-resistant *K. pneumoniae* isolates belonging to ST709 have been reported in Saudi Arabia and China since 2014, they produce other carbapenemases, such as OXA-48 or KPC-2 [9]. ST709 is a single locus variant of ST15, which has been reported to be predominant in Korea [10]. Thus, it is more plausible that the plasmid bearing bla_{NDM-5} had transferred from a strain of ST147 originating from the UAE to a strain of ST709.

Although pCC1410-2 had a backbone structure similar to those of pCC1409-1 and pCC1410-1, the regions with antibiotic resistance genes were different. Compared with the previously reported plasmids, the pCC1410-2 plasmid lacked genes associated with resistance to β-lactams (bla_{TEM-1}), aminoglycosides (*rmtB*), and macrolides (*mphR*(*A*), *mrx*(*A*), and *mph*(*A*); Fig. 1A). Thus, the plasmid with some genetic events, such as deletions and inversions, may have transferred [11]. Although 24 *tra* and eight *trb* genes contributing to plasmid transferability showed 100% and

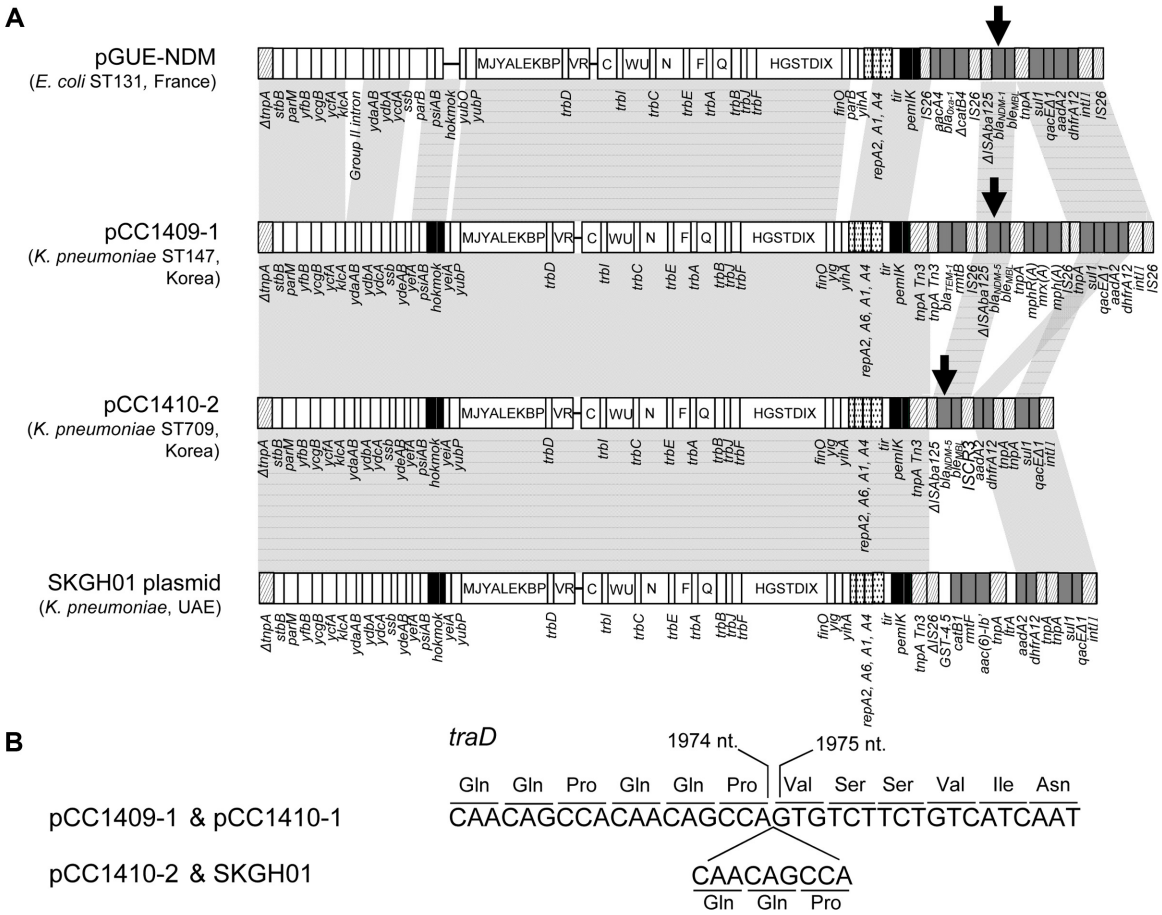

Fig. 1. Characterization of plasmids from three *Klebsiella pneumoniae* isolates from a Korean hospital.
(**A**) Major structural features of pCC1410-2 compared with those of the IncFII-type plasmids pGUE-NDM (GenBank Accession No. JQ364967.1) and pCC1409-1 (KT725789.1) and a plasmid from SKGH01 (CP015503.1). White boxes indicate plasmid scaffold regions that were common among the plasmids. The *tra* locus is indicated within the white boxes with capital letters. Resistance genes are indicated by grey boxes, and transposon-related genes, integrase, and insertion sequences are indicated by diagonally lined boxes. Other genes are indicated by shaded boxes as follows: replicase genes in dots, and addiction system genes in black. Light blue shading denotes shared regions of homology. Arrows indicate $bla_{NDM\text{-}type}$ genes. (**B**) Nucleotide and amino acid sequences of the *traD* gene in plasmids (pCC14109-1, pCC1410-1, pCC1410-2, and a plasmid from SKGH01). In pCC1410-2 and a plasmid from SKGH01, nine nucleotides encoding a repeat unit of three amino acids (Gln-Gln-Pro) were inserted, compared with the sequences of pCC1409-1 and pCC1410-1.

99% sequence similarities among pCC1409-1, pCC1410-1, and pCC1410-2, respectively, nine nucleotides encoding three amino acids (Gln-Gln-Pro) were inserted in the *traD* gene of pCC1410-2 and a plasmid from SKGH01 as compared with those of pCC1409-1 and pCC1410-1 (Fig. 1B). The amino acid sequence Gln-Gln-Pro is a repeating unit in TraD, and alterations in this sequence in TraD may decrease the transfer frequency because the *traD* operon is involved in efficient conjugative transfer [12]. In this study, we found that pCC1410-2 showed a significantly lower transfer frequency than pCC1409-1 and pCC1410-1 (Fig. 2A). However, it is unclear whether addition of repeating sequences in the *traD* gene affects the transferability of the IncFII-type plasmid because we did not perform further experiments.

The stabilities of the plasmids during antibiotic-free serial passage in liquid culture were also compared using their *E. coli* J53 transconjugants. Whereas pCC1409-1 and pCC1410-1 were preserved in 54% and 43% of *E. coli* J53 cells after 100 generations, respectively, pCC1410-2 was identified only in 19% of cells (Fig. 2B). That is, pCC1410-2 was less stable than the previous identified plasmids, despite that the three plasmids used the same addiction systems. The efficacy of addiction systems of plasmids in

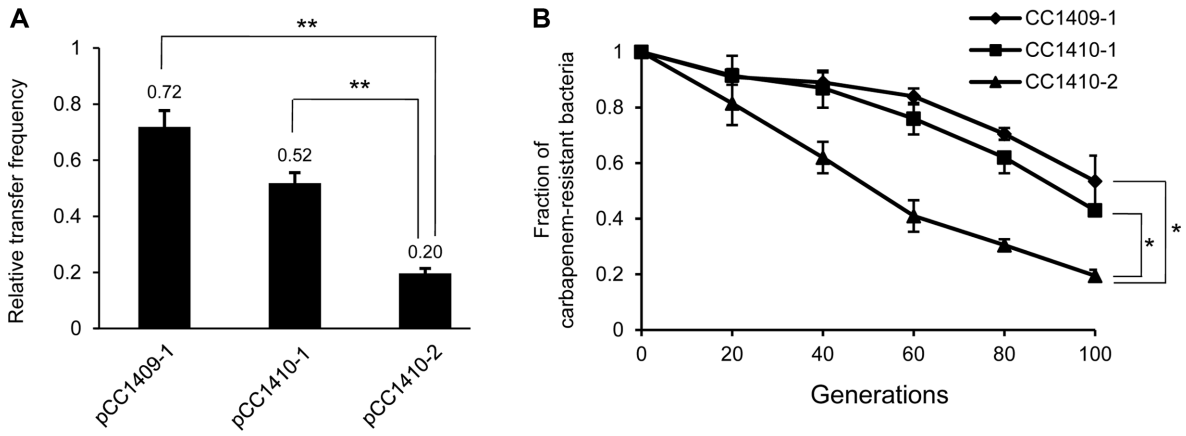

Fig. 2. Transferability and stability of the plasmids from three *Klebsiella pneumoniae* isolates from a Korean hospital.
(**A**) Relative transfer frequencies of pCC1409-1, pCC1410-1, and pCC1410-2 transconjugants. The transfer frequency was defined as the ratio of the number of CFU of the transconjugant surviving in medium with antibiotics versus bacterial cells in medium without antibiotics. (**B**) Stability of plasmids carrying the bla_{NDM-5} gene. Plasmid loss was measured in liquid culture and is plotted as the fraction of plasmid-containing cells per generation. Fisher's exact test was used to determine the significance of differences in plasmid-containing colonies between strains, using SPSS ver. 11.5 (SPSS, USA): **, p <0.001; *, p <0.05.

the host may be different, and several antibiotic resistance genes that are present in pCC1409-1 and pCC1410-1 but absent in pCC1410-2 may affect their stabilities, but they were not verified in this study. Owing to the instability of the plasmid, the isolate CC1410-2 was finally reported as carbapenem-susceptible, in contrast to the first identification as carbapenem-resistant. This may be one of the reasons why carbapenemase-producing Enterobacteriaceae (CPE) isolates are often not identified in the clinical setting or by surveillance systems.

In this study, we reported a third NDM-5-bearing plasmid, identified after two other plasmids found in *K. pneumoniae* isolates transmitted by crossborder transfer of a patient. The plasmid showed a structure similar to those of the previously reported NDM-5-bearing plasmids, but lacked some antibiotic resistance genes. Importantly, the plasmid showed a lower transfer frequency and instability in the host, which may affect underestimation of the CPE frequency in the clinical setting and in surveillance studies.

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