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Novel Plasmid and Its Variant Harboring both a bla_{NDM-1} Gene and Type IV Secretion System in Clinical Isolates of *Acinetobacter lwoffii*

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The spread of the bla_{NDM-1} gene is gaining worldwide attentions. This gene is usually carried by large plasmids and has been discovered in diverse bacteria since it was originally found in *Klebsiella pneumoniae*. Here we report the complete sequences of a bla_{NDM-1}-bearing plasmid, pNDM-BJ01, and its variant, pNDM-BJ02, isolated from clinical *Acinetobacter lwoffii* strains. The plasmid pNDM-BJ01 is 47.3 kb in size and cannot be classified into any known plasmid incompatibility group, thus representing a novel plasmid with an unknown maintenance mechanism. This plasmid contains both a bla_{NDM-1} gene and a type IV secretion system (T4SS) gene cluster. The T4SS is assigned to the P-type T4SS group, which usually encode a short, rigid pilus, and the bla_{NDM-1} gene is located within a composite transposon flanked by two insertion elements of IS*Aba125*. Plasmid pNDM-BJ02 is nearly identical to pNDM-BJ01 except that one copy of the IS*Aba125* element is missing, and it is therefore regarded as a variant of pNDM-BJ01. Sequence alignment indicated that this bla_{NDM-1}-containing composite transposon, which can also be captured by other mobile elements, was probably a product of multiple recombination events and can move as a whole by transposition.

The rapid growth of antibiotic resistance has been recognized as a clinical and epidemiological problem for human health (14). The new emergence of bacteria harboring the bla_{NDM-1} gene, encoding the metallo-β-lactamase (MBL) NDM-1, has once again aroused public concern worldwide (1, 18, 24). NDM-1 displays the ability to hydrolyze a wide range of β-lactam antibiotics, including carbapenems, which are a mainstay for the treatment of antibiotic-resistant bacterial infections (11).

The bla_{NDM-1} gene was first discovered in a *Klebsiella pneumoniae* isolate from a Swedish patient of Indian origin (30). Bacteria carrying the bla_{NDM-1} gene have now been reported in many countries worldwide, such as the United States, China, Australia, France, and the Nordic countries (3, 4, 20, 22, 23). The bla_{NDM-1} gene was originally found in a 180-kb plasmid in *K. pneumoniae* (30), and later it was reported to be carried by other plasmids of from 50 to 500 kb size in various Gram-negative species (11). In China, the bla_{NDM-1} gene has been reported in plasmids with sizes of from ~30 to 50 kb in *Acinetobacter baumannii* (4).

Complete sequencing of plasmids harboring the bla_{NDM-1} gene provided important information for the analysis of the genetic environment of the bla_{NDM-1} gene and for a better understanding of the spread of this resistance determinant. So far, three complete sequences of bla_{NDM-1}-bearing plasmids have been reported, i.e., those of pNDM-HK (7), p271A (21), and pNDM_Dok01 (25), isolated from *Escherichia coli*. Sequence analysis indicated that the bla_{NDM-1} gene was adjacent to various insertion elements in these plasmids (for example, two IS*26* elements in pNDM-HK, the IS*EC33* and IS*Sen4* elements in p271A, and two IS*903* elements in pNDM_Dok01). Here we report the complete sequences of bla_{NDM-1}-bearing plasmids isolated from two clinical *Acinetobacter lwoffii* strains and a comparative analysis of available bla_{NDM-1}-related sequences.

CASE REPORTS

On 23 November 2010, a 62-year-old female patient was admitted to our hospital suffering from a urinary tract infection after chemotherapy treatment for pancreatic cancer. A multidrug-resistant Gram-negative bacillus, *A. lwoffii* (strain 10621), was found dominating her urine cultures (8). The production of a metallo-β-lactamase (MBL) by A. *lwoffii* 10621 was confirmed by combined disk synergy testing. According to the drug resistance pattern, the patient was treated with amikacin via intravenous drip (0.2 g, 2 times/day). After 1 week of treatment, her temperature returned to normal and the urine was negative for *A. lwoffii*.

On 25 December 2010, a 37-year-old female patient (Baoding, Hebei Province, China) with urinary tract infection was admitted. According to her description, before admission to our hospital she had suffered from frequent and urgent micturition and urodynia for 3 days, and the symptoms were not alleviated after treatments in the local hospital. Results of urine culture in this case showed that *A. lwoffii* was also the dominant bacterium. The repetitive extragenic palindromic PCR (REP-PCR) binding patterns of this isolate (no. 10659) were not the same as those of *A. lwoffii* 10621 in the first case (see Fig. S1 in the supplemental material), suggesting

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that they were not the same strain and that the two infections were not caused by clonal spread. Antibiotic susceptibility testing results showed that this isolate was resistant to ampicillin, cefazolin, cefotaxime, ceftazidime, cefpirome, ceftriaxone, imipenem, and meropenem but susceptible to amikacin, tigecycline, and colistin. PCR testing indicated that the isolate was bla_{NDM-1} gene positive. The patient was then treated with amikacin by intravenous drip as described above, and she recovered thereafter.

MATERIALS AND METHODS

Microbiology methods. Bacteria were identified with the Vitek 2 system (bioMérieux Vitek Systems Inc., Hazelwood, MO). Antimicrobial susceptibility testing and interpretation were conducted by the standard disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines (5). For metallo-β-lactamase (MBL) detection, combined Disk synergy testing was performed as described by Yong et al. (29). The method involves the use of an imipenem (10 μg) disk with and without EDTA (1.86 mg). REP-PCR was used for strain typing as described by Vila et al. (28).

Filter mating experiments. Filter mating conjugation using *E. coli* J53 Azi^r as the recipient strain was performed as described previously with minor modifications (15, 16). After 24 h of incubation of donor-recipient mixtures at 37°C, cells were recovered by washing the filters in 1 ml of LB medium. Transconjugants were selected on LB agar plates supplemented with sodium azide (100 μg/ml) and ampicillin (100 μg/ml). To confirm the occurrence of transfer, 5 transconjugants from each mating were randomly selected for testing the presence of the plasmid by PCR analysis using 12 pairs of primers with products covering the whole plasmid. Conjugative transfer frequencies were calculated as the number of transconjugant cells per donor.

Sequencing and annotation. The whole genome of *A. lwoffii* 10621 was sequenced using a shotgun strategy with an Illumina genome analyzer (8). In this study, a total of 12 pairs of PCR primers with overlap ends of product were designed for the confirmation of the pNDM-BJ01 structure (see Table S1 in the supplemental material) and then used for plasmid pNDM-BJ02 sequencing. PCR products generated using these 12 pairs of primers were sequenced with an ABI3730 sequencer and assembled by using the SeqMan program within the Lasergene suite version 7 (DNAStar Inc., Madison, WI). The procedures for annotation were performed as described previously (8).

Bioinformatics analysis. A phylogenetic tree of the type IV secretion system (T4SS) family was constructed using the neighbor-joining method with 1,000 bootstrap replicates in Molecular Evolutionary Genetics Analysis software (MEGA version 4) (26). Amino acid sequences for *traB/virB10* and *traC/virB4* were retrieved from GenBank, and their concatenated sequences were used to generate the alignment and then for constructing the phylogenetic tree. A multiple-sequence alignment was constructed using ClustalX version 1.8 (27). Promoter searches were performed by using Softberry's BPROM (Softberry Inc., Mt. Kisco, NY), PPP-Prokaryotic Promoter Prediction (Groningen Biomolecular Sciences and Biotechnology Institute, Haren, The Netherlands [http://bioinformatics.biol.rug.nl/websoftware/ppp/ppp_start.php]), and the Neural Network Promoter Prediction program (http://www.fruitfly.org/seq_tools/promoter.html). The NCBI Basic Local Alignment Search Tool (BLAST) (17) was used repeatedly for sequence comparison and analysis.

Nucleotide sequence accession numbers. The pNDM-BJ01 and pNDM-BJ02 plasmid sequences have been submitted to the GenBank database with accession numbers JQ001791 and JQ060896, respectively.

RESULTS

Whole-genome sequencing of *A. lwoffii* 10621. The genomic draft sequence of *A. lwoffii* 10621 includes a ~3.42-Mb chromosome and a ~45-kb plasmid. Two gaps in the plasmid were closed by PCR and sequencing, and a final complete plasmid (named pNDM-BJ01) with length of 47,274 bp was obtained. The structure of pNDM-BJ01 was further confirmed by PCR using 12 pairs of primers with overlap ends of the product (see Table S1 in the supplemental material).

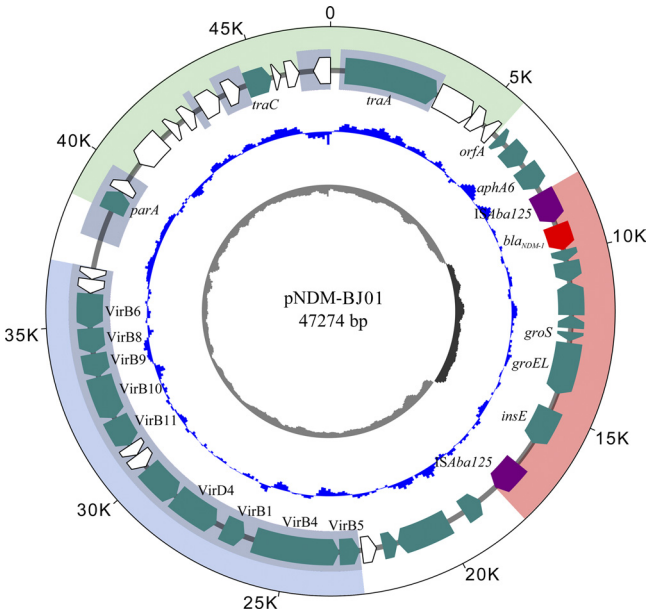

FIG 1 Circular map of plasmid pNDM-BJ01. The circles display (from the outside) (i) coordinates in kilobase pairs, (ii) predicted coding sequences, (iii) GC skew ([G+C]/[G-C]) in a 500-bp window, and (iv) GC content plotted against 50% GC content, with light gray indicating <50% and dark gray indicating >50%. The bla_{NDM-1} gene and insertion element IS*Aba125* are colored with red and purple, respectively. ORFs in white indicate hypothetical proteins. Three major parts corresponding to the bla_{NDM-1}-carrying composite transposon, the type IV secretion system gene cluster, and the putative transfer and replication region are shaded with light red, light blue, and light green, respectively. Homologous regions with *Acinetobacter radioresistens* SK82 contig 00019 are shaded with gray.

General features of plasmid pNDM-BJ01. The plasmid pNDM-BJ01 contains 46 open reading frames (ORFs) with an average GC content of 40.8% (Fig. 1; see Table S2 in the supplemental material). Three different functional regions were predicted (Fig. 1): a putative transfer and replication region containing plasmid transfer genes *traA* and *traC* and plasmid-partitioning gene *parA*, a type IV secretion system (T4SS) gene cluster region containing the quintessential T4SS conjugative transfer genes *traB* and *traC*, and a bla_{NDM-1}-carrying a composite transposon region flanked by two copies of IS*Aba125*. A BLAST search showed that approximately 20 kb of pNDM-BJ01 displayed more than 70% identity with the draft genomic sequence of *Acinetobacter radioresistens* SK82 contig 00019 (GenBank Genome Project ID 55855). However, pNDM-BJ01 cannot be classified by the PCR-based replicon typing method (2), and no plasmid backbone sequence homologous with it can be found, indicating that it may be a novel plasmid with an unknown maintenance mechanism. The plasmid showed a relatively high transfer frequency, ranging from 9.1×10^{-3} to 1.3×10^{-2} per donor cell, to *E. coli* J53 Azi^r, suggesting a high horizontal transfer ability.

Ten genes in the predicted T4SS region show high homology with genes for putative type IV secretion system proteins in *A.*

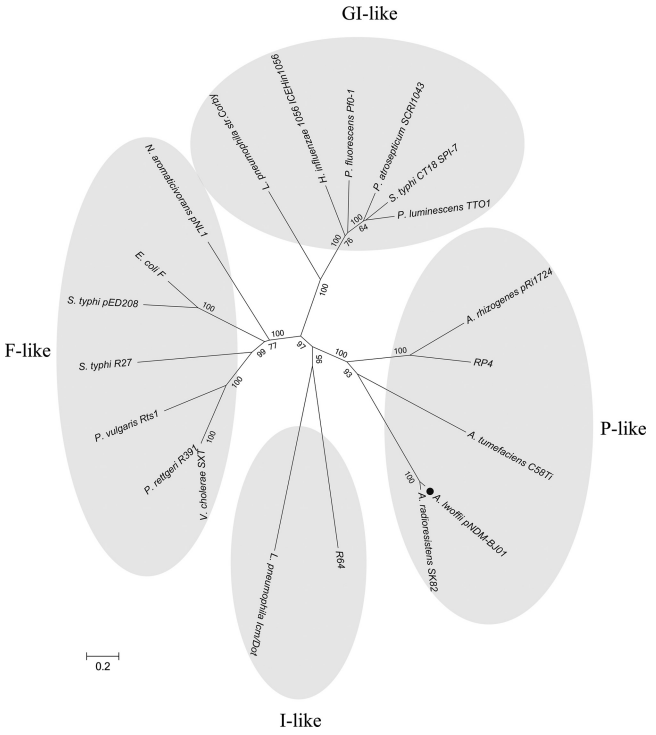

FIG 2 Phylogenetic tree of representative members of the T4SS family. The tree was constructed by the neighbor-joining method using concatenated amino acid sequences of *virB10* and *virB4* of the pNDM-Bj01 T4SS (●) and the previously described T4SSs. Bootstrap values of more than 50% are shown at the respective nodes (1,000 replications). The lengths of the branches indicate the divergence among the amino acid sequences. The scale bar corresponds to 20% estimated sequence divergence.

radioresistens SK82. Previous studies showed that genes *traB*/*virB10* and *traC*/*virB4*, encoding quintessential T4SS proteins, are ideal for comparative amino acid alignment and appear to be sufficient to define membership in a T4SS type (9, 10). A phylogenetic tree was therefore constructed by using concatenated amino acid sequences for *virB10* and *virB4*. The results indicated that the T4SS in pNDM-BJ01 was closely related to that in *A. radioresistens* SK82, and it was classified as a known P-type T4SS, members of which include those in RP4 (*Pseudomonas aeruginosa*), Ti (*Agrobacterium tumefaciens* C58), and pRi1724 (*Agrobacterium rhizogenes*) (Fig. 2).

The $bla_{\text{NDM-1}}$ gene was organized in a composite transposon structure within a ~7-kb abnormal GC region bracketed between two copies of insert sequence IS*Aba125* which differed by 6 nucleotides, one of which resulted in the amino acid substitution Arg-60 → Gln. With both IS*Aba125* insertion elements, this composite transposon was 10,099 bp in length, and the transposition event of this composite transposon was confirmed by (i) a different G+C content compared with the rest of the plasmid and (ii) a 3-bp (GTT) target site duplication at the point of insertion between the *aphA6* gene and its 3′ flanking region, which were continuous in *Alcaligenes faecalis* transposon Tn*5393d* (Fig. 3A). It was interesting that a nearly identical composite transposon structure (with a difference of 2 bp in a 10,099-bp length) was found to be inserted in the gene encoding a major facilitator superfamily (MFS) metabolite/H^+ symporter in the chromosome of *A. baumannii* 161/07, which was isolated from a patient who had been repatriated to Germany from Serbia (19). Moreover, the whole component of this composite transposon (excluding IS*Aba125*) was also captured by the IS*903* mobile element in pNDM_Dok01 (Fig. 3A).

Comparative analysis of the genetic environment of $bla_{\text{NDM-1}}$. The $bla_{\text{NDM-1}}$ gene-containing region between two copies of IS*Aba125* in pNDM-BJ01 comprised three major parts (Fig.

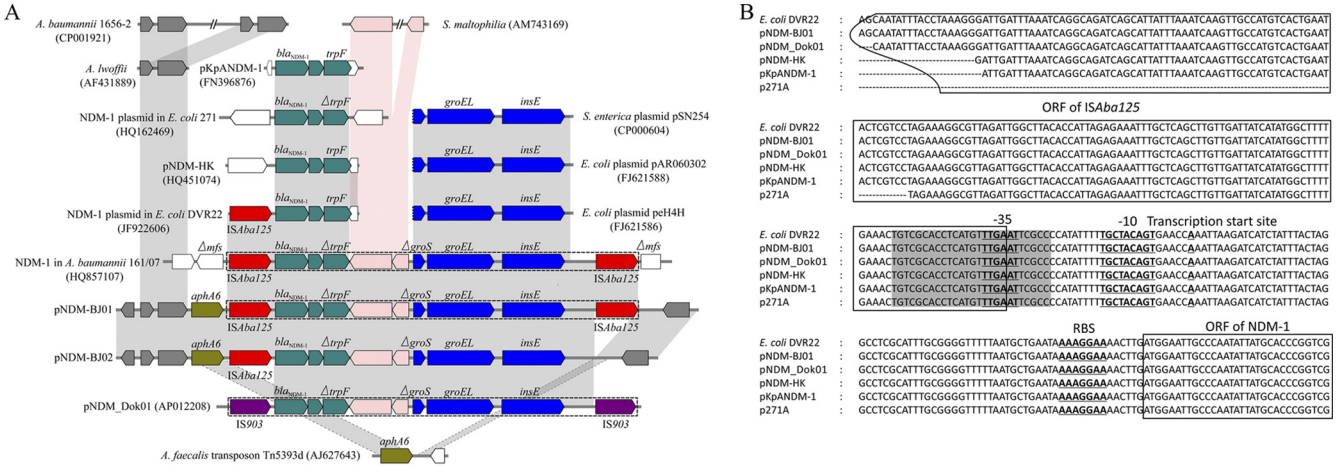

FIG 3 Comparative analysis of $bla_{\text{NDM-1}}$-carrying composite transposon sequences (A), promoter sequences of $bla_{\text{NDM-1}}$ (B), and 3′-end sequences of *trpF* (C) in different genetic environments. (A) Different colors of ORFs denote different sequence origins. Light gray and pink shadings indicate more than 97% and less than 75% nucleotide identity, respectively. IS*Aba125* is colored with red. The dashed line box indicates the complete composite transposon. (B) Gray shading indicates the right inverted repeat (IR-R) of IS*Aba125*.

3A). In the 5′ region, the bla_{NDM-1} gene was adjacent to a bleomycin resistance protein followed by a truncated *trpF* gene. This three-gene combination was frequently found in other genetic backgrounds: *E. coli* plasmids pNDM-HK, p271A, and pNDM_Dok01; the NDM-1 plasmid in *E. coli* DVR22; and *K. pneumoniae* plasmid pKpANDM-1. In the 3′ region, genes encoding the chaperonin subunits GroS (truncated) and GroEL and the transposase InsE constitute a 4-kb element showing high homology to that described in *E. coli* plasmids peH4H and pAR060302 and *Salmonella enterica* plasmid pSN254. The third part consisted of two ORFs between the truncated *trpF* and *groS* genes displaying ~70% nucleotide homology with the genome of *Stenotrophomonas maltophilia* K279a. It seemed that this part was related to insertion events. However, no feature sequences, such as direct repeats or inverted repeats, can be found flanking this region therefore, exactly how the insertions happened needs to be further investigated. These results indicated that the bla_{NDM-1}-containing region in pNDM-BJ01 was probably formed via multiple recombination events by genetic elements of different origins.

Detailed analysis of the sequence spanning IS*Aba125* and bla_{NDM-1} revealed the existence of a promoter of bla_{NDM-1} gene in which the −35 region was located inside the right inverted repeat (IR-R). Sequence alignment showed that all the reported NDM-1-containing sequences have the same promoter region and harbor residual nucleotides of the IS*Aba125* 3′ sequence (including the right inverted repeat of IS*Aba125*) in different lengths (pNDM-HK, 256 bp; pKpANDM-1, 255 bp, p271A, 194 bp; pNDM_Dok01, 273 bp; *E. coli* DVR22, the same copy of IS*Aba125* as in pNDM-BJ01) (Fig. 3B). These truncated IS*Aba125* ORFs had been disrupted by other insertion sequences, such as IS*EC33* in p271A, IS*26* in pNDM-HK and pKpANDM-1, and IS*903* in pNDM_Dok01. Interestingly, available sequence information showed that the 3′ end of the bla_{NDM-1} gene was always adjacent to a bleomycin resistance protein followed by a *trpF* gene. Sequence alignment indicated that in *E. coli* DVR22, pNDM-HK, and pKpANDM-1 there is a complete ORF of *trpF* (660 bp) but that in pNDM-BJ01 and pNDM_Dok01 79 bp of its 3′ end is missing and that in p271A 271 bp of its 3′ end is missing (Fig. 3C). These results suggested that the bla_{NDM-1} gene was probably originally linked to IS*Aba125* and then disseminated in the form of truncated IS*Aba125* and that the sequence in *E. coli* DVR22 may be closely related to the ancestral form, as both IS*Aba125* and *trpF* flanking the bla_{NDM-1} gene were intact.

Upstream of the IS*Aba125* gene was a complete ORF carrying the aminoglycoside resistance gene *aphA6* (12); however, the results of antibiotic susceptibility testing showed that *A. lwoffii* 10621 harboring pNDM-BJ01 was susceptible to amikacin, an aminoglycoside antibiotic. Analysis of the 5′ flanking region of the *aphA6* gene indicated that no obvious promoter sequence could be detected by using different promoter searching programs, and upstream of the *aphA6* gene were two transpose genes which were very similar to those in the genomes of *A. lwoffii* and *A. baumannii* (two copies) (Fig. 3A). Therefore, we suggest that the promoter sequence of the *aphA6* gene was probably disrupted by the transposition event, thus resulting in the failure to resistant amikacin.

Characterization of pNDM-BJ02. In the second case described above, we had the opportunity to isolate another bla_{NDM-1} gene-positive *A. lwoffii* strain harboring a plasmid (designated pNDM-BJ02) very similar in size to pNDM-BJ01. To investigate whether the two plasmids were identical, the whole pNDM-BJ02 was sequenced by primer walking of 12 PCR products; a final assembly with a length of 46,165 bp was obtained. Sequence comparison of pNDM-BJ02 with pNDM-BJ01 revealed that an 1,109-bp fragment adjacent to transposase gene *insE* was missing in pNDM-BJ02 (Fig. 3A). This deletion includes 94 bp of the 5′ flanking region of the IS*Aba125* ORF and 1,015 bp of the ORF itself (total, 1,026 bp), thus leaving 11 nucleotides of the 3′ end of IS*Aba125* gene and its downstream 3-bp transposition target site duplication on pNDM-BJ02. The rest of the sequences of the two plasmids are identical to each other. We therefore suggest that plasmid pNDM-BJ02 is a recent variant of pNDM-BJ01.

DISCUSSION

The New Delhi metallo-β-lactamase-1 (NDM-1) gene, bla_{NDM-1}, has been found in diverse strains since it was first discovered in *K. pneumoniae*. To our knowledge, no NDM-1 gene has been reported in *A. lwoffii* so far, and none of the recently sequenced plasmids (pNDM-HK, p271A, and pNDM_Dok01) share a genetic link with the plasmids pNDM-BJ01 and pNDM-BJ02 described here.

Both plasmids pNDM-BJ01 and pNDM-BJ02 harbored a T4SS. It has been recognized that T4SSs not only mediate horizontal gene transfer, thus contributing to genome plasticity and the evolution of pathogens through dissemination of antibiotic resistance and virulence genes, but also are used for the delivery of bacterial effector proteins to eukaryotic host cells, thus contributing directly to bacterial pathogenicity (6, 15). The T4SS in pNDM-BJ01 was classified as a P-type T4SS, which have been reported to encode short, rigid pili (13). All conjugative plasmids bearing a P-type T4SS have a broad host range (IncP, -W, and -N) (13), which prompted us to consider pNDM-BJ01 to be a broad-host-range plasmid as well; further investigations are necessary to confirm this. Furthermore, the bla_{NDM-1} gene in pNDM-BJ01 is located within a composite transposon, and it might be captured by other mobile vehicles or even integrated into the chromosome, as evidenced in *A. baumannii* 161/07 (19). Therefore, the plasmid pNDM-BJ01, harboring both a T4SS and the bla_{NDM-1} gene, represents a significant threat, especially when it spreads into human pathogens.

It is very interesting that the whole sequence of the composite transposon in pNDM-BJ01 was nearly identical to that of its counterpart in the chromosome of *A. baumannii* 161/07 isolated from Germany, which seems to have been caused by horizontal gene transfer events. Why this happened is puzzling. First, contacts with persons from abroad who were infected with bla_{NDM-1}-bearing bacteria cannot be excluded, as the patient was hospitalized several times before for chemotherapeutic treatment of cancer. Second, there may be a common ancestor which has not been recognized. Third, these two composite transposons may have been formed independently by chance via recombination events with similar components, which is less likely. In any case, we believe that this whole composite transposon or its variants will be found in other genetic environments in the future, captured either by IS*Aba125* as in pNDM-BJ01 or by other mobile elements, for example, by IS*903* as in pNDM_Dok01 (25).

Concluding remarks. We report here the complete sequences of plasmid pNDM-BJ01 and its variant pNDM-BJ02 in clinical isolates of *A. lwoffii*. Plasmid pNDM-BJ01, with an unknown maintenance mechanism, harbors both a bla_{NDM-1} gene and a P-type T4SS. The bla_{NDM-1} gene in pNDM-BJ01 is located inside a

composite transposon structure composed of two copies of insertion sequence IS*Aba125*. This novel plasmid represents a potential threat in the future because of its high horizontal transfer ability, and the force exerted by antibiotic abuse in China might increase this risk greatly.

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