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The Clinical Isolate *Pseudomonas aeruginosa* MMA83 Carries Two Copies of the bla_{NDM-1} Gene in a Novel Genetic Context

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The genetic context of the bla_{NDM-1} gene in the genome of *Pseudomonas aeruginosa* MMA83 was investigated. Sequencing of the cosmid selected for the bla_{NDM-1} gene revealed the presence of two bla_{NDM-1} copies in the genome of *P. aeruginosa* MMA83 in a unique genetic environment. Additionally, mating assays, DNA-DNA hybridization, and an S1 nuclease assay strongly suggest that the bla_{NDM-1} gene in *P. aeruginosa* MMA83 is chromosome borne.

The genetic context of the bla_{NDM-1} gene has been extensively investigated, mostly among members of the family *Enterobacteriaceae* (1, 2, 3, 4, 5, 6, 7, 8). From a genetic perspective, the key finding is that the bla_{NDM-1} gene can be located on plasmids belonging to different incompatibility groups. Although bla_{NDM-1} genes have the same flanking regions, in a broader sequence context, they reside in diverse genetic environments (6). Although the bla_{NDM-1} gene is typically found in a plasmid, in certain *Escherichia coli* and *Providencia stuartii* isolates, it was chromosome borne (6). The bla_{NDM-1} gene is considered endemic to the Balkan region, and this gene has been found not only in *Enterobacteriaceae* (9) but also in *Pseudomonas aeruginosa* (10). Considering the role of *P. aeruginosa* in the development of nosocomial infections worldwide and the subsequent complicated clinical management of patients infected with this pathogen, the emergence of NDM-1-positive strains is alarming. This is why the analysis of the genetic context of the bla_{NDM-1} gene, not only in *Enterobacteriaceae* but also in all other NDM-1-positive isolates, is crucial and could reveal associations with other genes that confer antimicrobial resistance and potential routes of dissemination. The objective of our study was to identify the genetic location and genetic context of the bla_{NDM-1} gene in *P. aeruginosa* MMA83, one of the NDM-1-positive clinical isolates from Serbia.

To determine the genetic location of the bla_{NDM-1} gene in *P. aeruginosa* MMA83, transformation, mating assays, and DNA-DNA hybridization were performed. Heat shock transformation of *E. coli* DH5α was performed with total DNA isolated from *P. aeruginosa* MMA83 (selection based on 100 μg/ml ampicillin). Triparental mating assays with *P. aeruginosa* MMA83 as a donor, *E. coli*(pRK2013) as a helper, and two recipients, *E. coli* DH5α (selection based on 100 μg/ml ampicillin) and an imipenem-sensitive isolate of *Pseudomonas mendocina* (selection based on 32 μg/ml imipenem), did not result in any transconjugant (11). Counterselection for recipients was based on glycerol for *P. mendocina* and sucrose for *E. coli* DH5α as the sole carbon sources. This result suggested that the gene(s) conferring resistance to β-lactams was not present on a conjugative plasmid(s) in MMA83. To find out if MMA83 is a plasmid-free strain, total DNA was isolated in agarose blocks and treated with S1 nuclease, which is used to visualize large, supercoiled plasmids (12). The results obtained confirmed that MMA83 does not carry any plasmid (see Fig. S1 in the supplemental material). Also, pulsed-field gel electrophoresis and subsequent DNA-DNA hybridization of a bla_{NDM-1} gene probe (1,059-bp EcoRI fragment) (11) with native (not digested) and S1 nuclease-treated total MMA83 DNA revealed the location of the signal in the region where chromosomal DNA migrates (see Fig. S1). These results strongly suggest that the bla_{NDM-1} gene is chromosome borne rather than plasmid located in MMA83. This is an unusual but not unique hallmark, since chromosomally located bla_{NDM-1} genes have been described previously (6). To uncover the genetic context of the bla_{NDM-1} gene, two cosmid libraries of MMA83 were constructed with Gigapack III Gold packaging extract (Stratagene, Amsterdam, Netherlands). Total DNA was partially digested with either BamHI or EcoRI and cloned into the pLAFR3 cosmid (11). Cosmid libraries were screened for the presence of β-lactamases by plating on tetracycline (20 μg/ml, selection for cosmid) and ampicillin (100 μg/ml). The cosmid selected from the BamHI cosmid library conferring resistance to β-lactams was designated pLAFRBamNDM, and the cosmid selected from the EcoRI library was designated pLAFREcoNDM. pLAFRBamNDM encompasses only one large BamHI fragment, so the order of genes in pLAFRBamNDM is the same as in the genome of strain MMA83, from which it originated. Afterwards, pLAFRBamNDM was digested with BamHI and XbaI, in which it produced three fragments of 8,292 bp (BamHI-XbaI fragment), 6,451 bp (XbaI-XbaI fragment), and 4,541 bp (XbaI-BamHI fragment). These fragments were cloned into a pBluescript vector (Kan^r) digested with the same enzymes. The clones obtained were confirmed by restriction enzyme analysis and subsequently sequenced by primer walking. Cosmid pLAFREcoNDM was digested with EcoRI and PstI, and plasmid libraries were constructed in pBluescript (Kan^r). The resulting constructs were sequenced and used as a control in the process of aligning and stacking sequences derived from BamHI-XbaI, XbaI-XbaI, and XbaI-BamHI fragments of pLAFRBamNDM.

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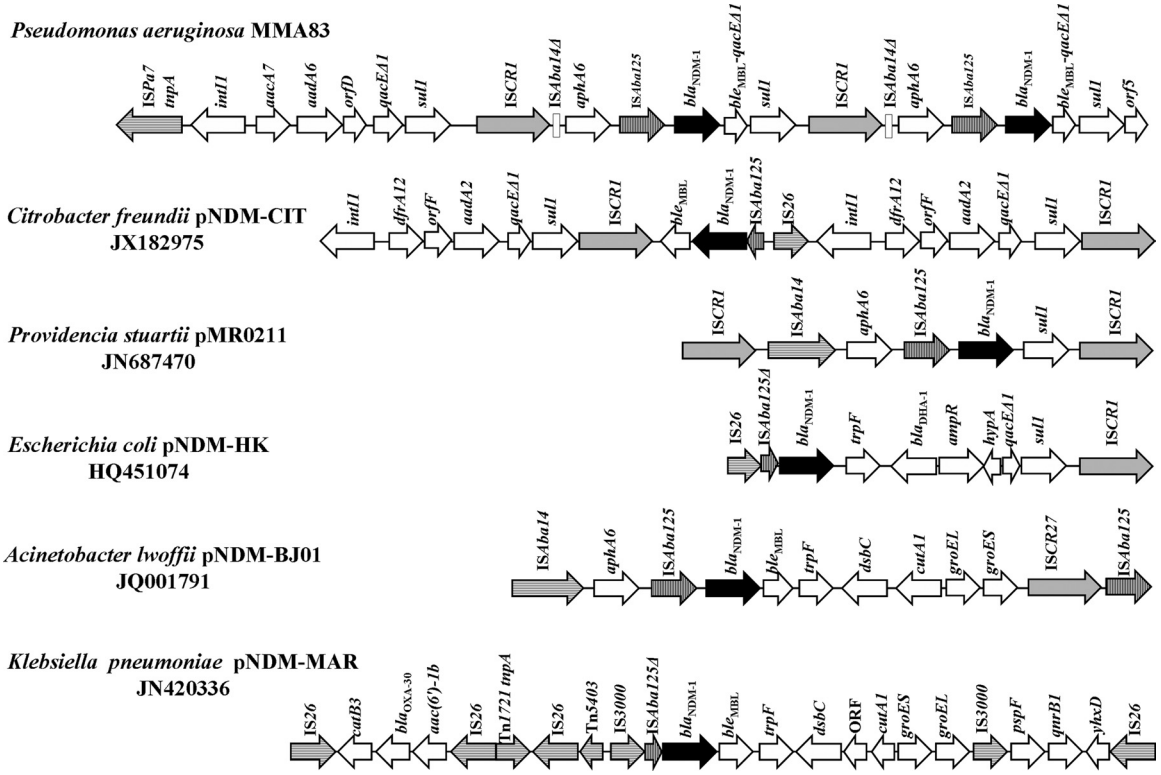

FIG 1 Comparative schematic representations of the bla_{NDM-1} gene's genetic context in the genomes of *Enterobacteriaceae* and *Acinetobacter* and the genetic environment of the bla_{NDM-1} gene in *P. aeruginosa* MMA83. Insertion sequence transposase genes are drawn as arrows filled with different patterns. The box in the scheme of the MMA83 bla_{NDM-1} gene's genetic context represents the left end of IS*Aba14*. The selection of the genetic environments represented was based on the presence of IS*CR1* and relationships to different bacterial genera.

The order of the open reading frames in fragments from pLAFREcoNDM was the same as that in pLAFRBamNDM, which confirmed that the order of genes in pLAFRBamNDM was the same as in the genome of strain MMA83. Surprisingly, these results revealed that *P. aeruginosa* MMA83 carries two copies of the bla_{NDM-1} gene (Fig. 1). Moreover, hybridization of total DNA digested with different restriction enzymes with a bla_{NDM-1} gene probe revealed the presence of signals at positions predicted based on the sequence of a cloned BamHI fragment of 19.2 kb (see Fig. S1 in the supplemental material). Studies published thus far describe the presence of only one copy of the bla_{NDM-1} gene in the chromosome or plasmids of bacteria. To our knowledge, ours is the first observation and report of two bla_{NDM-1} gene copies. Amplification of genes conferring resistance to antibiotics, as a common adaptive mechanism in bacteria, could be related to a gene dosage effect (13). The genetic environment of the bla_{NDM-1} gene in the genome of MMA83 showed certain similarities to but, in general, was different from the nucleotide sequences of the other bacterial strains analyzed. IS*Aba125* insertion sequences were identified immediately upstream of the bla_{NDM-1} genes (Fig. 1). As observed in other bla_{NDM-1} gene environments, IS*Aba125* provides the −35 promoter sequence for the bla_{NDM-1} gene (6). The IS*CR1* mobile element lies upstream of both copies of the bla_{NDM-1} gene and is followed by the gene encoding type VI aminoglycoside phosphotransferase. Since the regions downstream of both IS*CR1* elements are identical, it is possible that the two copies of the bla_{NDM-1} gene were the result of genetic duplication via IS*CR1*-mediated gene mobilization. IS*CR1* undergoes rolling-circle transposition, in which a single IS element can mobilize the sequences to which it is attached and subsequent homologous recombination may result in gene duplication (14). The genetic organization downstream of the bla_{NDM-1} genes in MMA83 is the same, encompassing the fusion of a bleomycin resistance gene and the *qacEΔ1* gene, followed by the *sul1* gene. The regions upstream of both bla_{NDM-1} gene copies in MMA83 are also identical and show similarity to the regions upstream of the bla_{NDM-1} gene in pNDM-BJ01 and pMR0211 (4, 5). In addition, regions downstream of the bla_{NDM-1} genes in MMA83 show some similarity to regions of pMR0211 and pNDM-BJ01 downstream from the bla_{NDM-1} gene but are not identical to them (Fig. 1). The first IS*CR1* insertion sequence was preceded by a class 1 integron harboring *aacA7*, *aadA6*, *orfD*, and *qacEΔ1* and *sul1* resistance genes. This is not atypical, since IS*CR1* is commonly associated with class 1 integrons (14). Moreover, although the bla_{NDM-1} gene is associated with other *bla* genes, in the case of *P. aeruginosa* MMA83, no other β-lactamase-encoding gene was found in the vicinity of two bla_{NDM-1} genes.

This study provides new data on the complexity and diversity of genetic features associated with the bla_{NDM-1} gene, which are of great importance for further analyses of dissemination routes and mechanisms, including clonal dispersions of the bla_{NDM-1} gene within clinical isolates.

Nucleotide sequence accession number. The nucleotide sequence of the BamHI fragment (19,272 bp) obtained from the pLAFRBamNDM cosmid is available in GenBank/EMBL under accession number HF546976.

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