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*bla*_{NDM-1} Is a Chimera Likely Constructed in *Acinetobacter baumannii*

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Alignment of DNA sequences found upstream of *aphA6* and all *bla*_{NDM-1} genes displays 100% identity. This identity continues 19 bp into the *bla*_{NDM-1} gene such that the first 6 amino acids of *aphA6* and *bla*_{NDM-1} are the same. Furthermore, the percent GC content (GC%) of *aphA6* is considerably lower than that of *bla*_{NDM-1} and the GC% within the *bla*_{NDM-1} structural gene changes dramatically after the first 19 bp. This is unequivocal evidence that *bla*_{NDM-1} is a chimera.

Carbapenems are potent broad-spectrum antibiotics that are reserved for life-threatening bacterial infections (7). However, their effectiveness is increasingly compromised by resistance due to carbapenem-hydrolyzing β -lactamases (11). One such enzyme, the New Delhi metallo- β -lactamase (NDM-1), was unknown before 2008, and yet media interest has enabled it to become a focus of world attention (18). We have previously shown that NDM-1 is widely disseminated in the United Kingdom and South Asia (5). We have also identified *bla*_{NDM-1} genes in a broad range of Gram-negative bacteria isolated from the New Delhi environment, including the serious pathogens *Shigella boydii* and *Vibrio cholerae* (19). Possession of *bla*_{NDM-1} confers resistance to all classes of β -lactam antibiotics (penicillins, cephalosporins, and carbapenems), with the sole exception of the monobactam aztreonam (20). In this, NDM-1 resembles previously characterized MBLs but differs from those in its ability to associate with the bacterial outer membrane and in its wide species distribution (4, 19). It is often associated with other antibiotic resistance genes on broad-host-range plasmids that provide transferable cross-class, and occasionally pan-antibiotic, resistance (5). In the continuing absence of new antibacterials effective against Gram-negative pathogens, the spread of multiply resistant NDM-1-producing strains is a cause for significant concern, as evidenced by the recent declaration by the World Health Organization of 7 April 2011 as World Antibiotic Resistance Day. However, the factors responsible for the unprecedented speed of dissemination of *bla*_{NDM-1} remain to be established. Here we use sequence comparisons to show that *bla*_{NDM-1} is a chimeric gene that has arisen by the in-frame fusion of a preexisting MBL gene with the aminoglycoside resistance gene *aphA6*. We propose that the resulting changes in NDM-1 expression, and the properties and processing of the expressed protein, partially explain the greater success with which NDM-1 has disseminated compared to other MBLs that confer a similar resistance phenotype.

Alignment of several sequences surrounding the *bla*_{NDM-1} gene has recently shown that fragments of the insertion sequence *ISAbal25* are often found upstream of the start of the *bla*_{NDM-1} structural gene and provide part of its promoter (10, 13; L. Poirel, R. Bonnin, L. Villa, A. Carattoli, and P. Nordmann, presented at the 21st European Congress of Clinical Microbiology and Infectious Diseases [ECCMID] and 27th International Congress of Chemotherapy [ICC], Milan, Italy, 2011). We used Geneious Pro 5.5.3 software (Biomatters Ltd., New Zealand) to construct an alignment of all available structures found upstream of the *bla*_{NDM-1} gene and measured the percent GC content (GC%) at

each position with a sliding window of 45 nucleotides. This revealed that *ISAbal25* sequences are indeed universally found 100 bp upstream of all *bla*_{NDM-1} genes for which sequence information is currently available. The sequences include three examples of the complete *ISAbal25*, two of complete *ISAbal25* containing internal insertions of either *IS1* or *ISEc33*, and several 5' truncated versions that range in length from 157 to 179 nucleotides (Fig. 1). The alignment also indicates that the sequences found between the *bla*_{NDM-1} gene and *ISAbal25*, or fragments thereof, are identical. The GC content of *ISAbal25*, and of the 100-bp region that separates the 3' end of *ISAbal25* from the 5' end of *bla*_{NDM-1}, is 37%. However, the *bla*_{NDM-1} gene has a considerably higher GC content of 61.5%. Of considerable interest is the fact that the GC% graph (Fig. 2A) changes dramatically within *bla*_{NDM-1} itself. Specifically, the GC% curve changes from below 50% to above 50% 19 to 20 bp inside the structural gene. Surprisingly, the first 6 amino acids of NDM-1 are identical to those of the aminoglycoside phosphotransferase *AphA6*, an occurrence that is highly unlikely to have happened by chance. To investigate this further, we searched the databases for information on the sequence found at the start of *aphA6* and performed a second alignment of *aphA6* and *bla*_{NDM-1} sequences (Fig. 2). This included the first 240 nucleotides of the *bla*_{NDM-1} and *aphA6* genes and 260 bp of sequence found upstream of both genes. That alignment indicated that *ISAbal25* is inserted at a point upstream of *aphA6* identical to that of *bla*_{NDM-1} and that the intervening DNAs between the two genes are identical. The alignment breaks 19 bp within the *bla*_{NDM-1} gene, the exact point where the GC% graph changes (Fig. 2). This indicates that the first 19 bp of *bla*_{NDM-1}, encoding the first 6 amino acids of the translated protein, are derived from *aphA6* and that *bla*_{NDM-1} is a chimeric gene produced by an in-frame fusion of *aphA6* with a preexisting MBL gene. Several facts strongly suggest that this fusion event happened in *Acinetobacter baumannii*. (i) The *aphA6* gene is strongly associated with *A. baumannii*. *aphA6* was first reported in 1988 in *A. baumannii* (8), and of the eight sequences in the genetic databases, four are from *A. baumannii* (1, 3) and a

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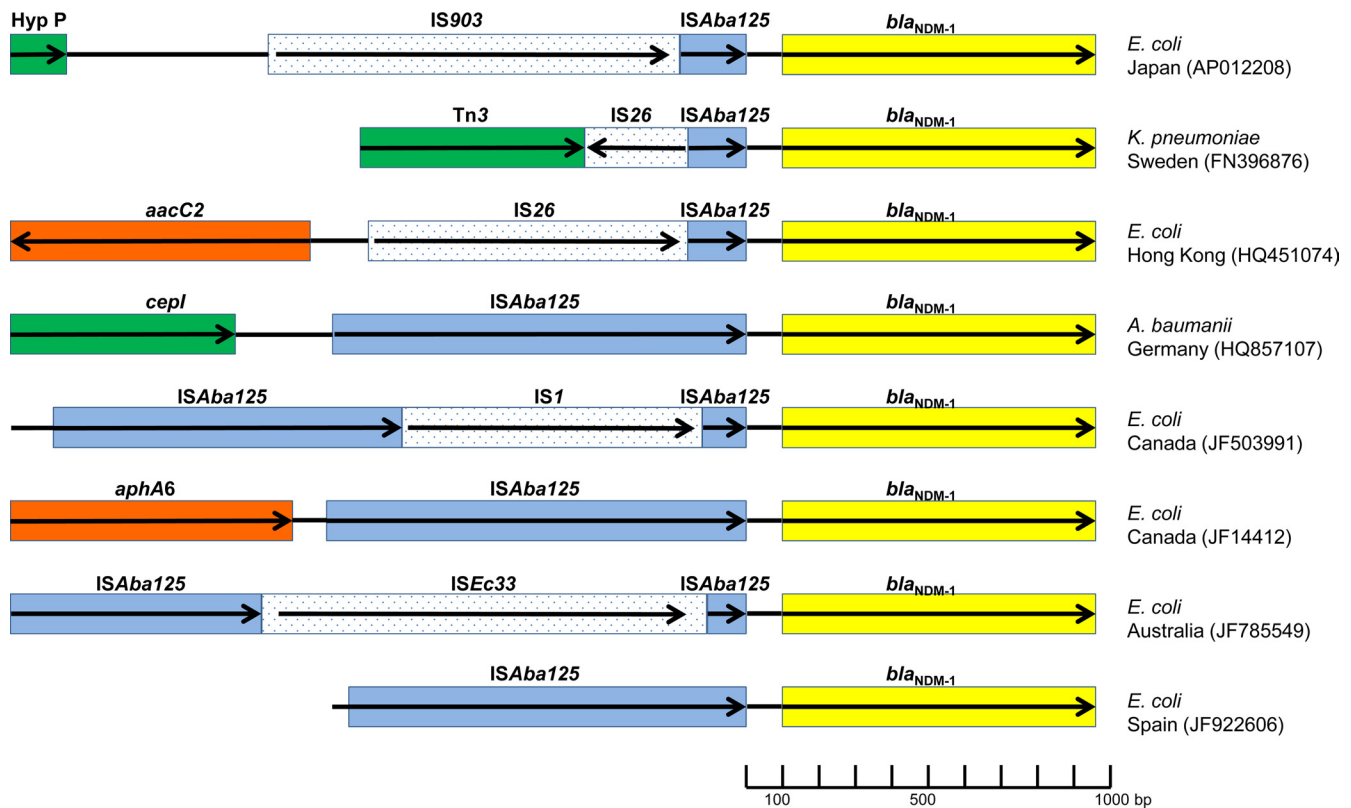


FIG 1 Sequence information found upstream of *bla*_{NDM-1} in various bacterial species isolated in different geographical regions. Colored blocks indicate regions of identity to various open reading frames or IS elements. The arrows indicate the direction of transcription and length of coding sections. Sections of *ISABa125* are colored blue.

further two are from *bla*_{NDM-1}-harboring plasmids. (ii) *ISABa125* is also generally found in *A. baumannii*. Of 16 reported sequences, 12 are from *A. baumannii*, and a further 3 sequences are associated with *bla*_{NDM-1} plasmids. (iii) The first European isolate of *A. baumannii* containing *bla*_{NDM-1} (collected in Germany in 2007) contains both *bla*_{NDM-1} and an *ISABa125* composite transposon with *aphA6* (12). (iv) The sequence used for the alignment described above was taken from a composite transposon composed of direct repeats of *ISABa125* found in *A. baumannii* (9). However, it is also possible that the fusion occurred in an environmental acinetobacter, since *aphA6* is also found widespread in other *Acinetobacter* spp. (6).

The in-frame fusion of *aphA6* with an MBL gene to form *bla*_{NDM-1} is likely to have several consequences for the expression and function of the NDM-1 enzyme. First, the success of NDM-1, in terms of its expression, and the ability to confer resistance in diverse bacterial species are likely linked to possession of a new promoter arising from the natural genetic engineering described here. It has recently been shown that control by the endogenous *bla*_{NDM-1} promoter gives carbapenem resistance in *Escherichia coli* higher than that seen when *bla*_{NDM-1} is controlled by either the *plac* or pBAD promoter (2). Second, the gene fusion event may exert functional changes linked to the altered N terminus. The fusion adds six new amino acids to the previous MBL, likely changing the N-terminal sequence from MHPVAKL to MELP NIM. A putative type I lipidation signal peptide has been identified that appears to be involved in directing the mature protein to

the bacterial outer membrane (3). If the methionine at position M7 is in fact the initiation methionine codon of the previous MBL, then the previous MBL has a more impressive lipidation signal score of 9.87 (<http://www.cbs.dtu.dk/services/LipoP/>) and the change in lipidation signal may have consequences for functionality.

The fusion of a *bla*_{NDM-1} to the aminoglycoside gene, *aphA6*, forming a chimera can be postulated to have arisen by one of two routes. A deletion event occurring between an upstream *aphA6* composite transposon and the *bla*_{NDM-1} gene would remove the intervening *ISABa125* gene and the majority of *aphA6* together with all sequence found upstream of the ancestral MBL gene (Fig. 3a). Alternatively, an ISCR element could have accumulated these genes by a rolling-circle mechanism (Fig. 3b) (14–17). When found in a favorable position adjacent to *bla*_{NDM-1}, a movement event would move the ancestral gene without its upstream sequence to a new location within the *aphA6* gene (Fig. 3b). Comparison of the genetic environments of *bla*_{NDM-1} in different bacterial species, including *A. baumannii*, has identified a common downstream structure consisting of an ISCR16-like ISCR element here named ISCR27 (Fig. 3) adjacent to the *groEL* and *groES* genes (12). We suggest that this element is responsible for both the original acquisition of the preexisting MBL gene and the construction of the *aphA6*-*bla*_{NDM-1} chimera.

Finally, the 100% sequence identity found between the *aphA6* transposon and the *bla*_{NDM-1} upstream sequence indicates that the

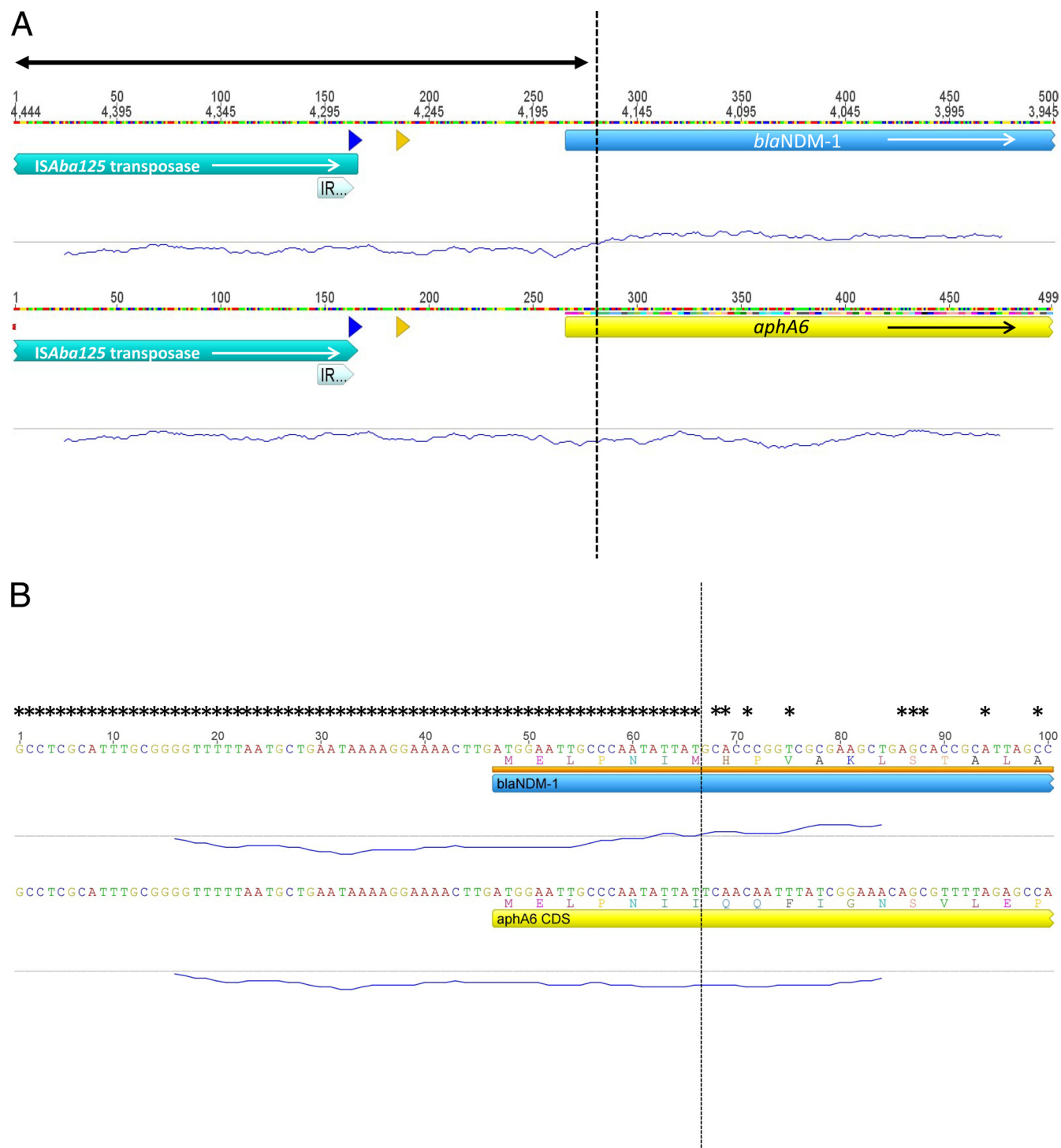


FIG 2 (A) Alignment of 500-bp sections of DNA, including the 5' ends of *bla*_{NDM-1} and *aphA6* genes and 260 bp of upstream sequence. Open reading frames (ORFs) are depicted as colored blocks with arrows indicating the direction of transcription. The double-ended arrow indicates the region of sequences that are 100% identical. The right inverted repeat of *ISAbal25* is depicted as a thick arrow at the end of the transposase ORFs. Promoter sequences are depicted as a blue arrow at -35 and a yellow arrow at -10 . The trace underneath each sequence represents the GC%, which was calculated using a sliding window of 45 bp, and the dashed vertical line indicates the point at which the GC% changes from below 50% to above 50% within the *bla*_{NDM-1} sequence. (B) Amplification of 100-bp section of alignment A, indicating the shared first six amino acids of NDM-1 and AphA6 and the exact nucleotide sequence of each region. Asterisks indicate nucleotides that are identical in the two loci; the dashed vertical line indicates the exact site of the gene fusion for all other annotations as described above.

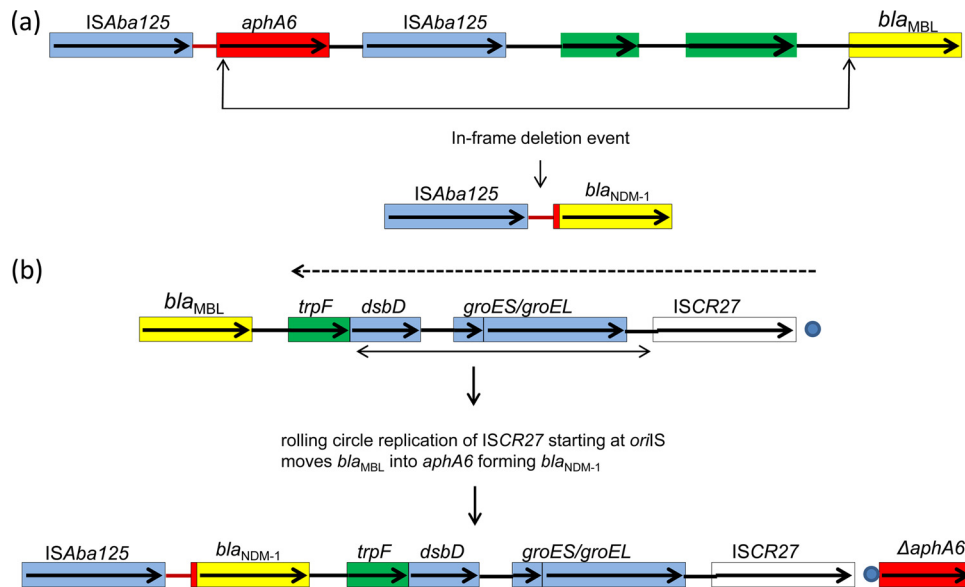


FIG 3 Two possible routes of *bla*_{NDM-1} construction. (a) *bla*_{NDM-1} could have been constructed by a deletion event that occurred between an upstream *aphA6*-*ISAbA125* composite transposon and an MBL *bla*_{MBL} gene. This would remove all sequence between the arrows, including most of *aphA6*, and all sequence upstream of *bla*_{MBL}. (b) A rolling-circle replication event involving the *ISCR27* element starting at the *oriIS* site could mobilize the upstream DNA that includes *bla*_{MBL} into the *aphA6* composite transposon, causing an insertion into the *aphA6* gene and a fusion producing *bla*_{NDM-1}. Genes are depicted as colored boxes with arrows indicating the direction of their transcription and intervening DNA as horizontal lines. The intervening DNA between the *aphA6* gene and the *ISAbA125* element is colored red. The dotted horizontal arrow indicates both the direction of rolling-circle replication of *ISCR27* and the common sequence found in several *bla*_{NDM-1} plasmids and on the chromosome in *A. baumannii*. The filled dot indicates the *oriIS*, and the double-ended arrow indicates the DNA that has been mobilized from a *Xanthomonas*-type organism (14, 16) by *ISCR27*.

fusion event described here was a very recent event in line with what is known about the emergence of *bla*_{NDM-1}.

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