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Frequent Tn*2* Misannotation in the Genetic Background of *rmtB*

Willames M. B. S. Martins, Ana C. Gales

Laboratório Alerta, Division of Infectious Diseases, Department of Internal Medicine, Escola Paulista de Medicina/Universidade Federal de São Paulo, São Paulo, Brazil

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RmtB is a 16S rRNA methyltransferase that confers a high level of resistance to both old and new aminoglycoside agents (1). *rmtB* was first reported in a *Serratia marcescens* clinical isolate from Japan in 2002 (2). Since then, it has been widely reported in isolates recovered from environmental and hospital settings. Usually, IncFII plasmids harboring *rmtB* also carry carbapenemase-encoding genes that make its spread easier (3, 4). The *rmtB* genetic background is frequently related to $bla_{\text{TEM-1}}$, which is usually located upstream of *rmtB* and is recognized as a Tn*3* transposon passenger gene (*tnpA*, *tnpR*, and $bla_{\text{TEM-1}}$) (2–6). However, this β-lactamase-encoding gene has been encountered in diverse transposons, like Tn*1*, Tn*2*, and Tn*3*, which were the first transposons characterized as related to antimicrobial resistance. Although Tn*2* and Tn*3* show high similarity, sharing the same mechanisms of transposition, they possess distinct passenger genes, like $bla_{\text{TEM-1a}}$ in Tn*3* and $bla_{\text{TEM-1b}}$ in Tn*2* (7). In addition, other nucleotide differences can be found in transposase- and resolvase-encoding genes and at the resolution site (*res*). The main mutations are usually found in a region located between nucleotides 94 and 150 at the *res* site (7). In this manner, the four Tn*2* sequences regions (*tnpA*, *res* site, *tnpR*, and bla_{TEM}) contained genetic characteristics capable of differentiating the other related transposons. Based on that, we analyzed the sequences of 19 whole-plasmid sequences carrying *rmtB*. We also studied 15 partial sequences of *rmtB*-associated transposons, which were deposited in GenBank to investigate the *rmtB* genetic background and its association with the $bla_{\text{TEM-1}}$ variants.

Three conserved *rmtB* genetic backgrounds (*tnpA*, *tnpR*, $bla_{\text{TEM-1b}}$, and *rmtB* or *tnpA*, Δ*tnpR*, $bla_{\text{TEM-1b}}$, and *rmtB* or Δ*tnpA*, *tnpR*, $bla_{\text{TEM-1b}}$, and *rmtB*) were observed in all 34 analyzed sequences (Fig. 1A). In all structures, $bla_{\text{TEM-1b}}$ was detected. In this manner, we conclude that *rmtB* is in fact related to Tn*2* and not Tn*3*, as suggested by many authors (2, 3, 5, 6). $bla_{\text{TEM-1a}}$ and $bla_{\text{TEM-1b}}$ show three nucleotide differences ($_{18}$C→T, $_{228}$C→T, $_{396}$G→T) but encode identical TEM-1 enzymes. Although silent, these mutations may be helpful in identifying and suggesting the evolution of mobile genetic elements like Tn*2* and Tn*3*. According to Partridge and Hall (7), the similarity between Tn*2* and Tn*3* has corroborated the misannotation of Tn*2* as Tn*3*. In fact, a Tn*2* or ΔTn*2* transposon located upstream of *rmtB* has been recognized by only a few authors (8–12). Two factors have contributed to the misannotation of Tn*2* as Tn*3*: the high similarity found between these two transposons and an annotation error in the databases. When a Tn*2* nucleotide BLAST analysis is performed using the ISfinder, Tn*3* is the first obtained hit (E value, 0.0; score, 8,387; 97% identity). UniProt and NCBI BLAST analyses also indicate the Tn*3* transposon as the main result, with rare entries for Tn*2*. To make easier the proper automatic annotation, the Tn*2* sequence was submitted to the ISfinder database. We also confirmed the presence of Tn*2* by *in silico* MsII digestion using SnapGene version 3.3 (8). This analysis was able to differentiate Tn*2* from its correlated variant

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Address correspondence to Willames M. B. S. Martins, willamesbrasileiro@hotmail.com.

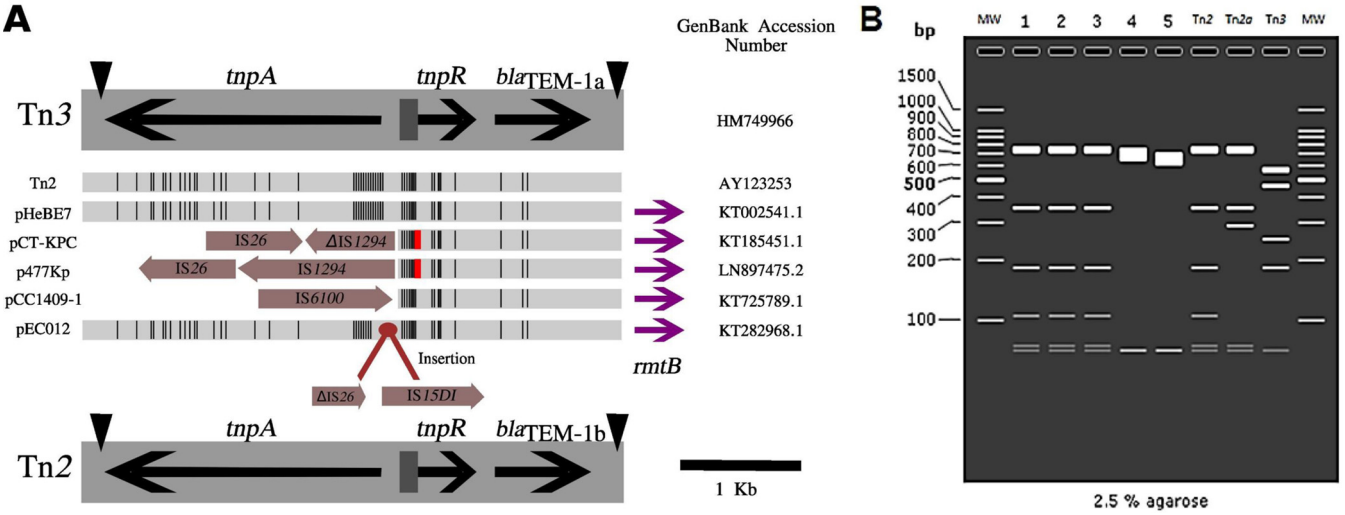

FIG 1 (A) Comparison of the main transposon sequence regions (*tnpA*, *res*, *tnpR*, and bla_{TEM}) with Tn*3*. Genes are shown by labeled arrows, inverted repeats of Tn*3* are shown as black triangles, and the resolution site is a dark-gray box. Inverted repeats are located in the same positions of Tn*2*. Vertical black lines indicate differences in the nucleotide sequences of Tn*3* (reference sequence) (GenBank accession number HM749966) from those of the other analyzed transposons. The red box in pCT-KPC and the p477Kp transposons indicate the presence of truncated *tnpR*. Insertion sequences were identified by ISfinder and are demonstrated by light-purple arrows. The pEC012 plasmid shows an insertion of a partial IS*26* (ΔIS*26*) sequence and an IS*15DI* sequence that show 99% similarity with IS*26*, according to ISfinder. Purple arrows indicate the presence of *rmtB* downstream of Tn*2* transposons in pHeBE7 (Tn*2*), pCT-KPC (ΔTn*2*), p477Kp (ΔTn*2*), pCC1409-1 (ΔTn*2*), and pEC012 (Tn*2*). This figure was compiled from alignments created using SeaView version 4.6.1. (B) *In silico* MslI restriction analysis of Tn*2* and its variants proposed by Bailey and colleagues (8). Lane 1, Tn*2* of pHeBE7; lane 2, Tn*2* of pIP1206; lane 3, Tn*2* of pMC-NDM; lane 4, ΔTn*2* (*tnpR* and bla_{TEM-1b}) of pC06114; lane 5, ΔTn*2* of p397Kp (Δ*tnpR* and bla_{TEM-1b}). In both ΔTn*2* sequences analyzed, *tnpA* was missing. MslI restriction confirmed the presence of Tn*2* in isolates with the complete transposon present (*tnpA*, *tnpR*, and bla_{TEM-1b}). However, this methodology was shown to not be accurate in identifying the ΔTn*2* sequence, since the digested fragment comprises the end of *tnpA* (absent in ΔTn*2*) and the beginning of bla_{TEM-1b}. MW, molecular weight markers.

Tn*2a* (Fig. 1B). Tn*2a* was described in 2011 (8) and differs from Tn*2* by 9 nucleotides located in the resolution site.

The origin of the *rmtB* and Tn*2* association remains unclear, since the right and left inverted repeats (IRR and IRL, respectively) of Tn*2* have been located downstream of *tnpA* and upstream of bla_{TEM-1b} (Fig. 1A), respectively. The absence of these sequences upstream of *rmtB* suggests the nonmobilization of *rmtB* by Tn*2*. For this mobilization to occur, the IRL upstream bla_{TEM-1b} gene would have been inactivated by a new passenger gene (in this case, *rmtB*) and a more distant "surrogate" IR sequence recruited (13). This kind of mobilization had occurred with Tn*4401*, a derivate transposon of Tn*3* that carries bla_{KPC-2} (14). Despite this genetic evidence, transposition *in vitro* experiments are necessary to confirm this hypothesis.

In conclusion, we reinforce the need for a proper annotation of the *rmtB* genetic structure through manual checking of automatic annotation to avoid a misperception about an epidemiological change. To date, *rmtB* has always been associated with Tn*2* and never with Tn*3*.

Accession number(s). The following GenBank accession numbers have been assigned: AM886293.1, KR078259.1, JF927996.1, KP893385.1, NC_016839.1, KT185451.1, KT002541.1, KT725788.1, KT725789.1, KT282968.1, KJ020575.1, KR259132.1, JN232517.1, LN897475.2, LN897474.2, NC_025106.1, NC_020278.2, NZ_CP015725.1, NZ_CP016035.1, EU213261.1, JN315966.1, FJ556899.1, EU491958.1, FJ167861.1, FJ410927.1, KM598665.1, HQ174461.1, KX064436.1, FJ556900.1, FJ539137.1, FJ183463.1, JQ941741.1, FJ744121.1, and HQ665010.1.

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