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Characterization of Tn*6238* with a New Allele of *aac(6′)-Ib-cr*

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Here, we report that the genetic structure of Tn*1331* remained conserved in Argentina from 1989 to 2013 (72/73), except for the plasmid-borne Tn*1331*-like transposon Tn*6238* containing a new *aac(6′)-Ib-cr* allele recovered from a colistin-resistant *Klebsiella pneumoniae* clinical isolate. A bioinformatic analysis of *aac(6′)-Ib*-like gene cassettes suggests that this new *aac(6′)-Ib-cr* allele emerged through mutation or homologous recombination in the Tn*1331* genetic platform. Tn*6238* is a novel platform for the dissemination of aminoglycoside and fluoroquinolone resistance determinants.

AQ: A

The Tn*1331* transposon was the first member of the Tn*3* subfamily reported to contain a structure of an array of integron gene cassettes, using an *attI1*-aac(6′)-Ib-attC*$_{aac(6')-Ib}$*-aadA1-attI1*-bla*$_{OXA-9}$*-attC*$_{blaOXA-9}$ array (1–5). The basic structure of a gene cassette consists of a gene and a recombination site (called *attC*), which can be targeted by integrases encoded by genes carried on integrons. The *attC* recombination sites are 57 to 141 bp long and are composed of 2 short regions of sequence similarity at their boundaries (1R to 2R and 1L to 2L) separated by a stretch (20 to 104 bp) of imperfect internal dyad symmetry (6). *attI1** in Tn*1331* shows identity with 8 bp of the *attI1* recombination site from class 1 integrons (2).

It was previously shown that two point mutations (W87R and D164Y) (Fig. 1) within the *aac(6′)-Ib* gene confer the capability of the gene product to acetylate not only aminoglycosides but also the fluoroquinolones norfloxacin and ciprofloxacin (7). This gene, named *aac(6′)-Ib-cr*, (also known as *aacA4-cr*), is the first that encodes an enzyme able to inactivate two families of antibiotics (7). Currently, this gene, together with *qnrB* alleles, are the prevalent plasmid-mediated quinolone resistance genes (8).

F1

Currently, six alleles of *aac(6′)-Ib-cr* have been identified, all as gene cassettes, with 5 of them generating amino acid changes at the protein level (7, 9–12) (Fig. 1). They have been found mainly in classical class 1 integrons (13, 14) and sporadically in gene cassette arrays with IS*26* in the structures IS*26-aac(6′)-Ib-cr1-bla*$_{OXA-30}$*-catB3*-IS*26* (GenBank accession no. AY458016), *aac(3)-II*-IS*26-aac(6′)-Ib-cr2-bla*$_{OXA-1}$ (GenBank accession no. GQ438247), and *aac(6′)-Ib-cr2-bla*$_{OXA-1}$-Δ*catB3*-IS*26-aac(3)-II* (GenBank accession no. GQ438248) (15, 16). In the plasmid pMdT1, the allele is not associated with other gene cassettes or integron-related sequences and appears to have inserted in a secondary site (12).

Taking into account that the first isolation of gene cassettes, including *aac(6′)-Ib* embedded in Tn*3*, was found in Argentina in the 1980s (GenBank accession no. AF479774.1) (1, 2, 4, 17–19), we decided to evaluate the evolution of Tn*1331*, focusing on the nucleotide sequence of *aac(6′)-Ib* to study the emergence of *aac(6′)-Ib-cr* within this genetic structure, due to its clinical importance.

We performed a retrospective study over 24 years in which we included 331 clinical isolates resistant to at least three families of antibiotics. The isolates belonged to 8 species from 5 hospitals in Argentina recovered since 1989. According to PCR mapping (Table 1), Tn*1331* was found in 65% of *Klebsiella pneumoniae* (39/60), 14% of *Serratia marcescens* (4/28), 18% of *Enterobacter cloacae* (2/12), 17% of *Citrobacter freundii* (1/6), 60% of *Proteus mirabilis* (24/40), and 10% of *Escherichia coli* (3/30) isolates, and it was not detected in *Acinetobacter baumannii* (0/80) or in *Pseudomonas aeruginosa* (0/75) isolates. This finding shows that Tn*1331* is frequently found and stably maintained in clinical isolates from Buenos Aires analyzed over the 24 years. Furthermore, this shows a different dissemination of Tn*1331* among fermenting and nonfermenting bacilli. A sequence analysis of the 73 Tn*1331*-like-positive isolates showed that all but one contained the typical gene cassette array of the transposon, as found previously in isolates from Argentina (19, 20). The colistin-resistant *K. pneumoniae* KF7 isolate contained a novel allele of *aac(6′)-Ib-cr*, named here *aac(6′)-Ib-cr7*, instead of *aac(6′)-Ib* (Fig. 1, *aac(6′)-Ib2*). KF7 was isolated in 2008 from a urine sample from a 48-year-old female patient with a nosocomial infection who was at the intensive care unit and treated with different antibiotics but not fluoroquinolones. The multidrug-resistant profile of the isolate (21, 22) is shown in Table 2. This new gene cassette array gives rise to the transposon Tn*6238* (GenBank accession no. KJ511462). This new *aac(6′)-Ib-cr* allele is like variant A, as defined by Partridge (13), plus a new additional point mutation (GAT to GTT at position 548) that encodes a valine as the penultimate amino acid, as found in Tn*1331*, instead of the aspartic acid found in *aac(6′)-Ib-cr* and most of the *aac(6′)-Ib* alleles (30/35) (7, 9–11, 13, 18) (Fig. 1). Like the *aac(6′)-Ib* allele in Tn*1331*, *aac(6′)-Ib-cr7* also contains a unique structure at the 5′ end, consisting of *attI1** and the nucleotides that encode the last six amino acids of *bla*$_{TEM-1}$ (23) instead of the previously reported 5′ *aac(6′)-Ib-cr* gene cassette variations (13). To determine if Tn*6238* was located in a transferable plasmid, we performed a biparental conjugation, as described before

T2

T1

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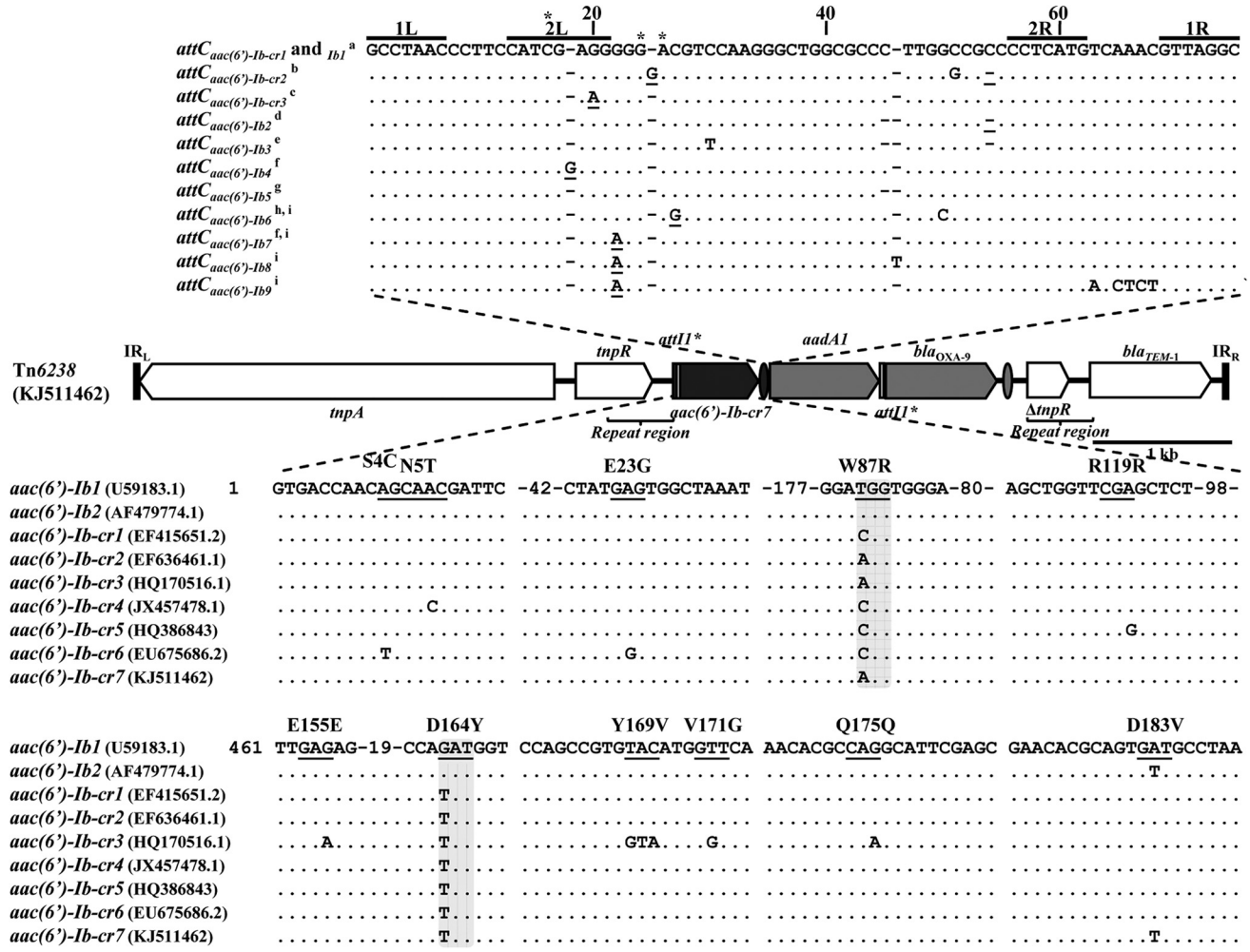

FIG 1 Structure of Tn*6238* (GenBank accession no. KJ511462). The transposon number was assigned by the Tn number registry Web page (http://www.ucl.ac.uk/eastman/research/departments/microbial-diseases/tn). The horizontal bars represent inverted repeats, the arrows represent ORFs, the white bars represent *attI1**, and the gray arrows and ovals represent the ORFs and *attC* sites of the gene cassettes, respectively. The top strands of the 11 different *attC* sites found in the *aac(6′)-Ib-cr* and *aac(6′)-Ib* gene cassettes are shown above the Tn*6238* structure. The dots indicate identities and the dashes indicate insertions. The 1R, 2R, 1L, and 2L sites are highlighted in gray, and the extrahelical bases are marked with an asterisk. The most important mutations related to their potential impact on the *attC* functionality are underlined. [a]Most frequent are $attC_{aac(6')\text{-}Ib\text{-}cr}$ and $attC_{aac(6')\text{-}Ib}$, which are found in all alleles of the *aac(6′)-Ib-cr* gene cassettes other than *aac(6′)-Ib-cr3* (e.g., GenBank accession no. KJ511462, EF636461.1, and EF415651.2). They are also found in the prevalent *aac(6′)-Ib* gene cassette in integrons, the Tn*1331*, Tn*1331.2*, and Tn*1332* transposons, and all the AAC(6′)-Ib protein variants except 3 and 4 (e.g., GenBank accession no. U59183.1 used as reference of *aac(6′)-Ib* gene cassette) (14); [b]$attC_{aac(6')\text{-}Ib\text{-}cr}$ is linked to *aac(6′)-Ib-cr2* (e.g., GenBank accession no. HM998988.1); [c]$attC_{aac(6')\text{-}Ib\text{-}cr}$ is associated with *aac(6′)-Ib-cr3*, found only in GenBank accession no. HQ170516.1; [d]$attC_{aac(6')\text{-}Ib}$ is found in Tn*1331* of pJHCMW1 (GenBank accession no. AF479774.1); [e]$attC_{aac(6')\text{-}Ib}$ is found in gene cassettes, with the constant region of the ORF identical to the *aac(6′)-Ib* of the reference, but whose complete ORF encodes variants 4 and 39 of the AAC(6′)-Ib protein (GenBank accession no. AY370764.1 and X60321.1, respectively) (14); [f]$attC_{aac(6')\text{-}Ib4}$, $attC_{aac(6')\text{-}Ib7}$, and $attC_{aac(6')\text{-}Ib}$ are found in gene cassettes in which the complete ORF encodes variants 8 ($attC_{aac(6')\text{-}Ib4}$, $attC_{aac(6')\text{-}Ib7}$) and 14 ($attC_{aac(6')\text{-}Ib}$) of the AAC(6′)-Ib protein (GenBank accession no. DQ767903.1 and GQ293499.1) (14); [g]$attC_{aac(6')\text{-}Ib}$ is found in the *aac(6′)-Ib* gene cassette in a Tn*1331*-like structure in which the complete ORF encodes variant 3 of the AAC(6′)-Ib protein (GenBank accession no. M23634.1) (14); [h]This is the second $attC_{aac(6')\text{-}Ib}$ in terms of frequency (e.g., GenBank accession no. AF458080.1); [i]$attC_{aac(6')\text{-}Ib6}$, $attC_{aac(6')\text{-}Ib7}$, $attC_{aac(6')\text{-}Ib8}$, $attC_{aac(6')\text{-}Ib9}$, and $attC_{aac(6')\text{-}Ib}$ are found in gene cassettes, with the constant region of the ORF identical to the *aac(6′)-Ib* of the reference, in which the complete ORF encodes variants 13, 14, and 17 of AAC(6′)-Ib protein (e.g., GenBank accession no. AF458080.1, AJ313334.1, JF262177.1, and FO203354.1). The ORFs of the *aac(6′)-Ib-cr* alleles are shown below the Tn*6238* structure. They are compared with the AAC(6′)-Ib protein encoded by *aac(6′)-Ib1* (*aac(6′)-Ib* allele of the reference, GenBank accession no. U59183.1, bases 301 to 859). The dots indicate identities. The numbers between dashes indicate the number of bases not shown. The cvodons with mutations are underlined, and above them are shown the codified amino acids, numbered from the GTG start codon. The *aac(6′)-Ib2* allele found in Tn*1331* is shown. The key mutations that make the protein capable of modifying quinolones are highlighted with gray boxes. All the *aac(6′)-Ib-cr* variants share the mutation GAT to TAT at position 490 of the *aac(6′)-Ib1* ORF (D164Y). Position 259, also responsible for the ciprofloxacin-resistant phenotype, exhibits the mutation TGG to CGG (variant C, called here *aac(6′)-Ib-cr1*) or to AGG (variant A, called here *aac(6′)-Ib-cr2*), both generating W87R (13).

AQ: C

(24), with *E. coli* J53-AZ^r (AZ^r, azide resistant) as the recipient strain. The transconjugants were selected on Mueller-Hinton agar supplemented with sodium azide (100 μg/ml) and ampicillin (25 μg/ml). The transconjugant *E. coli* strain J53 KF7-TC6 showed the aminoglycoside and quinolone resistance profiles for *aac(6′)-Ib-cr* (Table 2), with a difference of 6 mm between the inhibition zones of levofloxacin (LVX) and ciprofloxacin (CIP) in the disk diffusion method ($\Delta_{LVX\text{-}CIP} \geq 5$), as described before (25). Al-

TABLE 1 Primers used in the study

Primers by target[a]	Sequence (5′–3′)	Location in Tn*6238* (GenBank accession no. KJ511462)[b]	Reference
Tn*1331* and Tn*6238*			
IRs Tn3 inside	GGGGTCTGACGCTCAGTGG	Fw 1–19 Rev 7977–7995	This study
TnpA F3′	CTCTCCCCGCTTTGCCACG	Rev 139–159	This study
TnpA R	TCTGACTGGCGTAACAAAGC	Fw 946–965	This study
TnpA Rc	TACTGCTCCACCATTTCGTC	Fw 1874–1893	This study
TnpR	AAGTTCATCGGGTTCGC	Fw 2968–2984	29
TnpA F	AGGTTGAGAGTTATGGCAGG	Rev 2992–3011	This study
aac(6′)-F	GAAGAAGCACGCCCGAC	Fw 4100–4116	This study
aac(6′)-R	GTGTTCGCTCGAATGCC	Rev 4516–4532	This study
aadA1	TCGATGACGCCAACTAC	Rev 4661–4677	30
aadA1F	TTGCTGGCCGTACATTTG	Fw 4697–4714	9
aadA1R	TCATTGCGCTGCCATTC	Rev 4946–4962	9
Oxa9-fb	GAACACCAACATATGCA	Rev 5483–5499	29
Oxa9r	GGGACAATAACGGCAAG	Fw 6101–6117	29
blaTEM1F	GCTCACCCAGAAACGCTGGTGAAAG	Fw 7054–7078	This study
blaTEM1R	CACCCAACTGATCTTCAGCATC	Rev 7084–7105	This study
blaTEM F3′	GGGAGTCAGGCAACTATGG	Fw 7774–7792	This study
Class 1 integrons			
Inti1F	CGAGGCATAGACTGTAC		29
Inti1R	TTCGAATGTCGTAACCGC		29
sulpro3	GCCTGACGATGCGTGGA		30
3′CsNew	AAGCAGACTTGACCTGATAG	Rev 6412–6431	31

[a] PCR mapping was performed targeting first a portion of the *tnpA* gene and the gene cassette array of the transposon using the TnpR and 3′CsNew primer pair. Subsequently, we used different combinations of the primers listed to amplify and sequence the complete transposon.
[b] Fw, forward primer; Rev, reverse primer.

though the plasmid incompatibility group was not determined by replicon typing (26), the presence of Tn*6238* in the transconjugant was confirmed by PCR mapping. By sequencing this transconjugant with outward primers targeting the *tnpA* and bla_{TEM-1} genes, we determined that the inverted repeats (IRs) of Tn*6238* were 100% identical to the IRs of Tn*3* and Tn*1331* (4, 27). The In197 class 1 integron, which harbors the *dfrA16c* gene cassette, was also detected in the transconjugant by PCR mapping (Integrall [http://integrall.bio.ua.pt/]). The fact that this plasmid contains a Tn*1331* derivative and a complete class 1 integron potentiates the exchange of gene cassettes between the two genetic platforms.

In order to further analyze the origin of *aac(6′)-Ib-cr7*, we performed a bioinformatic analysis of the *aac(6′)-Ib-cr7* gene cassette and related sequences from GenBank (Fig. 1). To perform the comparison, we selected the conserved region of the open reading frame (ORF) of each *aac(6′)-Ib-cr* allele, discarding the heterogeneous 5′ end that generates N-terminal extensions of the encoded proteins (13), and subsequently compared their *attC* sites. We found 3 $attC_{aac(6')\text{-}Ib\text{-}cr}$ sites among 61 complete *aac(6′)-Ib-cr* gene cassettes (Fig. 1). One of them, $attC_{aac(6')\text{-}Ib\text{-}cr1}$, occurred very frequently. It was present in 57/61 sequences from all the *aac(6′)-Ib-cr* alleles other than *aac(6′)-Ib-cr3*, which showed a unique *attC* site called $attC_{aac(6')\text{-}Ib\text{-}cr3}$ (1/61). The remaining $attC_{aac(6')\text{-}Ib\text{-}cr2}$ sites (3/61) were associated with *aac(6′)-Ib-cr2*. In addition, we identified 9 *attC* sites in the *aac(6′)-Ib* gene cassettes (Fig. 1). The $attC_{aac(6')\text{-}Ib\text{-}cr1}$ site described above was identical to the most common $attC_{aac(6')\text{-}Ib}$ site [$attC_{aac(6')\text{-}Ib1}$] among the *aac(6′)-Ib* gene cassettes (586/634), including those in class 1 integrons, Tn*1331*, Tn*1331.2*, or Tn*1332*, and it was associated with almost all the *aac(6′)-Ib* alleles. Among the *aac(6′)-Ib* gene cassettes from the 15 Tn*1331* transposons found in GenBank, only $attC_{aac(6')\text{-}Ib2}$ in Tn*1331* from pJHCMW1 showed a different and unique *attC* site (Fig. 1). As expected, the $attC_{aac(6')\text{-}Ib\text{-}cr}$ and $attC_{aac(6')\text{-}Ib}$ sites are highly related, and there is one prevalent *attC* site, while the remaining are associated with only a few alleles. Nevertheless, some of the mutations in the *attC* sites may have an effect on the recombination efficiencies, since they disrupt the 2R/2L complementarity or generate an additional extrahelical base, thus affecting their

TABLE 2 Relevant characteristics of the clinical isolate harboring *aac(6′)Ib-cr7* and transconjugants from this study

Isolate or strain	Relevant resistance phenotype[a]	MIC (mg/liter)[b]	
		AMK	CIP
K. pneumoniae KF7	AMP^r CEF^r SXT^r AMK^r NAL^r CIP^r LVX^r TZP^r SAM^r AMC^r CST^r STR^r KAN^r TOB^r NOR^r	6	>32
E. coli J53	AZ^r	0.25	0.01
E. coli J53 KF7-TC6	AMP^r CEF^r SXT^r AMK^r NAL^r CIP^r SAM^r AMC^r STR^r KAN^r TOB^r	2	0.5

[a] Antimicrobial resistance and reduced susceptibility were tested by the disk diffusion method (19). AMP, ampicillin; CEF, cephalothin; SXT; trimethoprim-sulfamethoxazole; AMK, amikacin; NAL, nalidixic acid; CIP, ciprofloxacin; LVX, levofloxacin; TZP, piperacillin-tazobactam; SAM, ampicillin-sulbactam; AMC, amoxicillin-clavulanic acid; CST, colistin; STR, streptomycin; KAN, kanamycin; TOB, tobramycin; NOR, norfloxacin; AZ, azide.
[b] MIC determinations were performed according to CLSI recommendations (19).

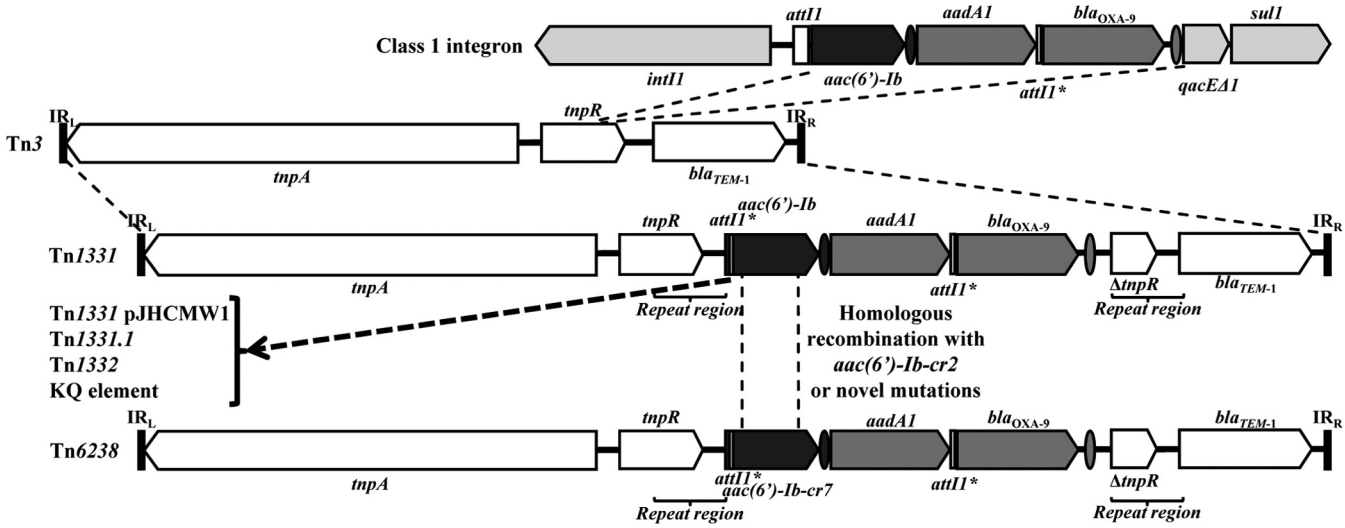

FIG 2 Evolutionary scheme of Tn*6238*. The horizontal bars represent inverted repeats, the arrows represent ORFs, the white bars represent *attI1**, and the gray arrows and ovals represent the ORFs and *attC* sites of gene cassettes, respectively.

dissemination (Fig. 1). In regard to *aac(6′)-Ib-cr7* in Tn*6238* and *aac(6′)-Ib* in the other Tn*1331*-related transposons, the 8-bp AA ACAAAG motif of the *attI1** site located at the 5′ end has a crucial effect in minimizing IntI1-mediated recombination (28).

The *attI1*-aac(6′)-Ib-attC*$_{aac(6')-Ib}$*-aadA1-attI1*-bla*$_{OXA-9}$*-attC*$_{blaOXA-9}$ array in Tn*1331* found in our isolates and others (14, 19, 20) reveals that the event that created Tn*1331* is not very common. Based on this and the analyses showing that (i) the mutation that determines the D183V change in the AAC(6′)-Ib protein encoded in Tn*1331* is not very frequent in *aac(6′)-Ib* found in class 1 integrons from GenBank or in Argentinian isolates (data not shown), (ii) the *attC* site is the same as the most frequent variant found in both Tn*1331* from pMET and in class 1 integrons, (iii) the *aac(6′)-Ib-cr* variant found in Tn*6238* has only the two mutations that lead to fluoroquinolone resistance, (iv) the excision of *aac(6′)-Ib* from Tn*1331* by IntI1 is null or very low (28), and (v) we could not discard homologous recombination with a transient *aac(6′)-Ib-cr* that would include only the region that contains both crucial mutations for the fluoroquinolone resistance phenotype, and our subsequent proposal that the creation of Tn*6238* that requires fewer events of mobilization and/or mutation and hence is the most likely, implies that both mutations leading to amino acid changes D164Y and W87R or even homologous recombination with a transient *aac(6′)-Ib-cr2* have happened in the genetic platform of Tn*1331* (Fig. 2). F2

In conclusion, Tn*1331* is frequent and stably maintained among fermenting bacilli in clinical isolates analyzed from Buenos Aires over the 24 years, but it has the potential to increase its antimicrobial resistance background, as shown by the emergence of Tn*6238*. There are now seven alleles of *aac(6′)-Ib*-cr reported around the world. These alleles are spreading in two successful genetic platforms, class 1 integrons and Tn*3*-derivative transposons, in the clinical bacterial isolates from Argentina (9, 25). The emergence of Tn*6238* shows how bacteria are able to acquire new resistance determinants and novel platforms that enhance the dissemination of antimicrobial resistance.

Nucleotide sequence accession number. The nucleotide sequences determined in this work have been submitted to the GenBank database and assigned accession no. KJ511462. The bioinformatics analysis has been performed using sequences from GenBank, National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). AQ: B

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