Letter to the Editor

First report of a Tn*402*-like class 1 integron carrying *bla*$_{VIM-2}$ in *Pseudomonas putida* from Argentina

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Pseudomonas putida colonizes several niches including soil, fresh water, and animal surfaces. Although rarely isolated from human infections, metallo-beta-lactamase (MBL)-containing *P. putida* clinical strains resistant to most beta-lactams) have recently been described and proposed to act as likely reservoirs of MBL genes [1,2]. This, in addition to the lack of an effective clinical inhibitor of these metallo-enzymes, poses a serious challenge to antimicrobial therapy [3]. *bla*$_{VIM-2}$ represents the most widely distributed MBL gene worldwide, and is most generally present in class 1 integrons [4,5, Pasteran F *et al.* (2005) 45th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC) Abstract C2 108/151]. These elements may be contained within transposons, and the whole arrangement subjected in turn to rapid spread among different bacterial species by the aid of wide-range plasmids [5]. It follows that the elucidation of the genetic platform(s) in which *bla*$_{VIM-2}$ genes are contained enables us to rationally speculate on how they evolve and disseminate. We report here the identification for the first time of an unusual class 1 integron carrying *bla*$_{VIM-2}$ and *aacA4* aminoglycoside acetyl transferase gene cassettes embedded in a complete Tn*402*-like transposon, all elements carried in turn by a self-transferable plasmid present in a carbapenem-resistant *P. putida* clinical strain.

A *P. putida* strain, hereafter referred to as LD209, was isolated in February 2009 from a bronchoalveolar lavage of a newborn affected by a congenital cardiopathy hospitalized in a public health care centre of Rosario, Santa Fe province, Argentina. LD209 identification as *P. putida* and susceptibility testing (VITEK 2 System, bioMérieux, Marcy l'Etoile, France) indicated resistance to imipenem with minimal inhibitory concentration (MIC $\geq$ 16 mg/L), ceftazidime (MIC $\geq$ 64 mg/L), cefepime (MIC $\geq$ 64 mg/L), aztreonam (MIC $\geq$ 64 mg/L), gentamicin (MIC $\geq$16 mg/L), ciprofloxacin (MIC $\geq$ 4 mg/L), levofloxacin (MIC $\geq$ 8 mg/L); intermediate resistance to piperacillin-tazobactam (MIC of 64 mg/L); and susceptibility to amikacin (MIC $\leq$ 2 mg/L).

MBL activity in LD209 bacterial extracts was revealed by the EDTA-imipenem microbiological assay (EIM) [6]. Identification of *bla*$_{VIM}$, *bla*$_{IMP}$, and *bla*$_{SPM}$ by PCR with specific primers (Table 1) followed by sequencing revealed only *bla*$_{VIM-2}$. Further characterization of the genomic context of *bla*$_{VIM-2}$ using primers designed after the 5' and 3' conserved segments of class 1 integrons (5'-CS and 3'-CS, respectively; Table 1) systematically failed to produce amplification bands. Conversely, the use of 5'-CS (forward) and TniC-R (reverse) primers (Table 1) followed by sequencing analyses showed that *bla*$_{VIM-2}$ is present in an unusual class 1 integron which lacks the 3'-CS region (Figure 1A). As shown in the figure, *bla*$_{VIM-2}$ is located immediately adjacent to the 5'-CS region of the integron followed by an *aacA4* gene encoding an AAC(6')-Ib type aminoglycoside acetyl transferase [7], preceding in

Table 1. PCR primers used in this study.

DNA target	Primer sequence (5' to 3')	Reference
$bla_{\text{IMP-like}}$	IMP1-F GAGCAAGTTATYTGTATTCT	12
	IMP19-R TCYMAHGTAMGYTTCAAGAG	
$bla_{\text{VIM-like}}$	VIM-F ATTGGTCTATTTGACCGCGT	12
	VIM-R CTACTCAACGACTGAGCGAT	
$bla_{\text{SPM-1}}$	SPM-F GGCGCGTTTTGTTTGTTG	12
	SPM-R CTACAGTCTCATTTCGCCAACG	
5'-CS	Int1-F GGCATCCAAGCAGCAAG	12
3'-CS	Int1-R AAGCAGACTTGACCTGA	12
aacA4	AacA4-F ATGACTGAGCATGACCTTG	This study
*tni*C	[a]TniC-R CGATCTCTGCGAAGAACTCG	5
	TniC-F TCGAGCTTCCACACGATCAG	This study
	TniC-F1 GCACTGAACAGCGCCCCGTTC	This study
*tni*B	TniB-R GACGATTGCTGCTCACTGCT	This study
	TniB-F CAGAGCCAGTTGCTCCATTT	15
	TniB-F1 CAGTGAGCAGCAATCGTCGT	This study
*tni*A	TniA-R CTTTCACCGCGAAGTCACTC	15
attI1	attI1-R GTAACATCGTTGCTGCTCCATAAC	This study
IntI1	IntI1-F GATCCATCAGGCAACGACGGGCT	This study

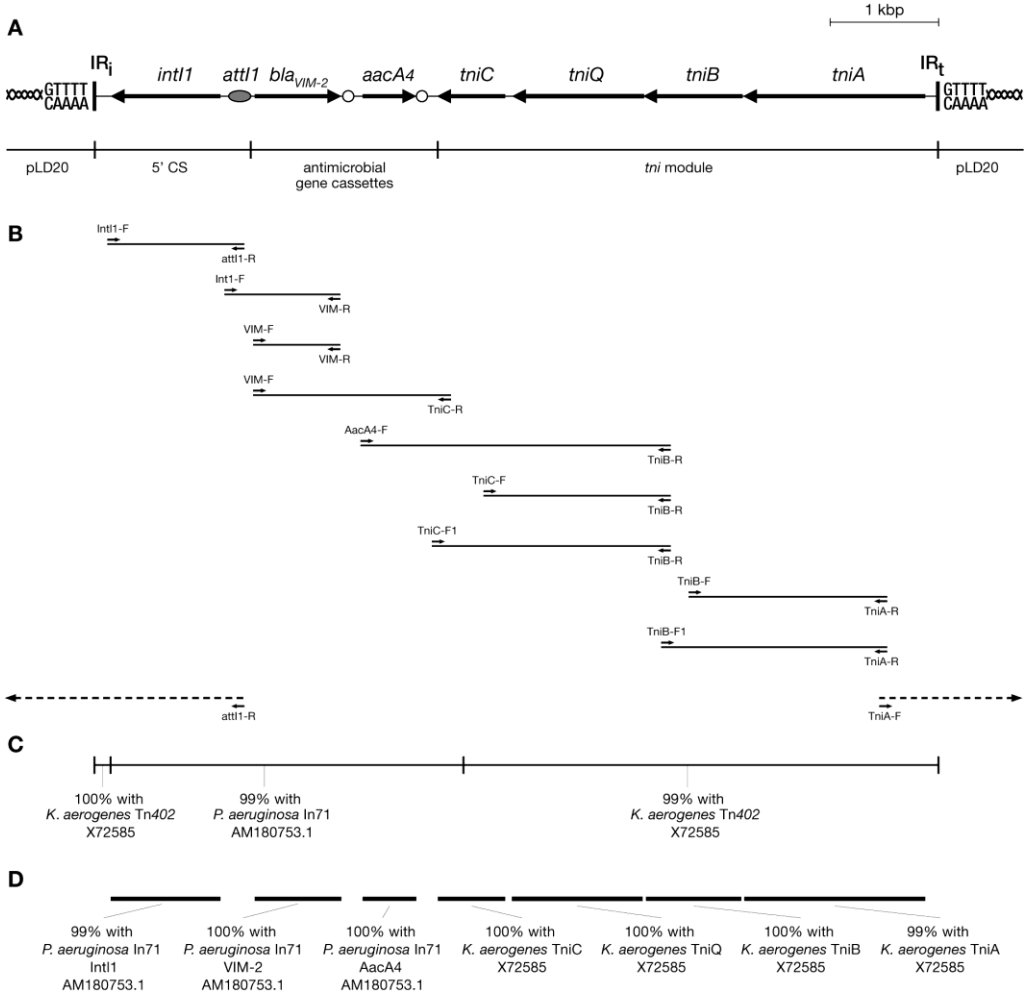
Y: C, T; M: A, C; H: A, T, C
[a]Primer TniC-R is identical to TniCF of ref. 5.

turn the 3´ region of a *tniC* gene encoding the Tn*5090* resolvase [5] (Figure 1A). It is worth noting that a class 1 integron almost identical to that reported here (In71, Figure 1) was detected in a chromosomal location of clonally related carbapenem-resistant *P. aeruginosa* strains responsible for an outbreak occurring in 2000 in Trieste, Italy [8].

All class 1 integrons have been proposed to have originated from multiple excision/rearrangements of a transposon derived from Tn*402*/Tn*5090* originally containing a complete *tni* module [5,7,9]. Characterization of the immediate genomic context of the unusual class 1 integron described here in *P. putida* by PCR using appropriate primer combinations (Figure 1B and Table 1) followed by sequencing and database searching analyses revealed the presence of a complete *tni* module displaying 99% identity at the nucleotide level (between 99-100% identity at the level of the corresponding encoded proteins) with the transposase (*tniA*), transposition auxiliary proteins (*tniB*, *tniQ*) and resolvase (*tniC*) genes of *Klebsiella aerogenes* Tn*402* (Figures 1, C and D). As seen in Figure 1A, the genes composing this *tni* module are located downstream of the *aacA4* gene cassette and transcribed in the opposite direction from the resistance genes.

The finding of a Tn*402*-like class 1 integron containing $bla_{\text{VIM-2}}$ and *aacA4* gene cassettes accompanied by a complete *tni* module constitutes to our knowledge the first report of such an arrangement in *P. putida*. A Tn*402*-like class 1 integron carrying *aacA4* and $bla_{\text{VIM-2}}$ gene cassettes as well as a complete *tni* module has been described recently in *P. putida* clinical strains isolated in the Balearic Islands, Spain [10]. However, several differences exist between this element and the one described here, including an inverted order of $bla_{\text{VIM-2}}$ and *aacA4* genes, significant sequence divergence of the corresponding *tni* modules (only ~68% identical at the nucleotide level between the *tni* region shown in Figure 1 and the equivalent region deposited at GenBank GQ227991) [10], and the absence of a specific polymorphism (Ala108Thr) in the AacA4

Figure 1. Schematic representation of the *P. putida* plasmid-borne Tn*402* derivative described in this work.



A. The *attI1* site (grey oval) and the two 59-bp elements (open circles) of the class 1 integron are indicated. *intI1*: integrase gene, bla_{VIM-2}: VIM-2: MBL gene; *aacA4*: aminoglycoside acetyl transferase gene; *tniC*: resolvase gene; *tniQ/tniB*: transposition auxiliary protein genes; *tniA*: transposase gene. The directions of transcription of the corresponding genes are depicted by arrows. IRi and IRt represent the 25-bp inverted repeats [14]. The sequences representing the 5´ CS and the antimicrobial gene cassettes of the integron as well as the Tn*402 tni* module are indicated below the figure. The IRi, IRt, the *attI1* site, and the 59-bp elements are slightly enlarged for clarity, as well as the duplicated GTTTT sequences at the site of insertion in the plasmid.

B. PCR-overlapping assay used for the identification and characterization of the different gene and other components of the integron/transposon element described here. The different primers employed and their corresponding target sites are indicated by small arrows (see Table 1 for sequence details). DNA sequences of IRi, IRt, and corresponding outer boundaries were obtained by primer walking starting with primers attI1-R and TniA-F (5'-CGTAAGGCCACGGTATTGCGC-3') as indicated in the lower part of the figure.

C. Percent identities at the nucleotide sequence level between the indicated regions and the corresponding GenBank [8,14] sequences.

D. Percent identities at the amino acid sequence level between the polypeptides encoded in the indicated regions and the corresponding GenBank sequences. For further details see GenBank accession number GQ857074.

enzyme present in LD209 which is characteristic of the equivalent enzymes produced by *P. putida* strains isolated in Spain. Also, an unusual class 1 integron carrying bla_{VIM-2} immediately downstream of the 5'CS has been recently reported in a *P. putida* clinical strain isolated in Portugal [11]. In this case, however, the bla_{VIM-2} gene was followed by an unknown open reading frame rather which was in turn followed by the 3' region of *tniC*.

We next analyzed whether the above Tn*402* derivative was plasmid-borne. LD209 cells were subjected to alkaline lysis and the obtained DNA was transformed into electrocompetent *Escherichia coli* (*E. coli)* DH5α cells [12] followed by a selection of resistant colonies in LB agar containing 16 μg/ml ampicillin. Analysis of plasmid content by restriction enzyme digestion (EcoRI and HindIII) and agarose gel electrophoresis indicated the same plasmid in all cases, which was designated as pLD20. MBL production by *E. coli* harbouring pLD20 was confirmed by a microbiological assay [6]. Also, a three-fold increment in the MIC values for both imipenem and meropenem was found as compared to non-transformed cells. Concerning expression of the *aacA4* gene present in the integron described here, *E. coli* MIC values for gentamicin and amikacin increased three- and one-fold, respectively, as compared to non-transformed bacteria. Similar

results were observed by other authors using *E. coli* cells transformed with plasmids directing production of AAC(6`)-$Ib_{7/8}$ type enzymes [13]. Agar mating studies [12] confirmed the ability of pLD20 to undergo conjugative mobilization from *P. putida* LD209 (donor) to rifampicin-resistant *E. coli* DH5α. Moreover, agar mating analyses [12] indicated that pLD20 can mobilize from *E. coli* DH5α (donor) to *P. aeruginosa* PAO1 or *E. coli* MC4100 (recipients). Thus pLD20 constitutes a self-mobilisable plasmid capable of spreading the bla_{VIM-2} containing the Tn*402*-like class 1 integron described in this work among a wide range of bacterial species.

The plasmid location of the above Tn*402*-like derivative allowed us to accurately characterize the transposon outer boundaries. Sequencing analyses (Figure 1) revealed the presence of the 25 bp IRi and IRt inverted repeats and all associated 19 bp adjoining repeats characteristic of the Tn*402* transposon family [14]. Moreover, the two GTTTT direct repeats bracketing the IRs (Figure 1) strongly indicate small target duplications associated with a recent transposition event. *In toto*, all the above features indicate that *P. putida* LD209 carries a self-transferable, wide-range plasmid containing a functional Tn*402* transposon carrying a class 1 integron bearing bla_{VIM-2} and *aacA4* gene cassettes.

It remains to be tested whether bla_{VIM-2} genes captured by highly mobile genetic platforms such as those identified here in a likely reservoir of antimicrobial resistance genes such as *P. putida* [1,10] could account for the high levels of incidence of this MBL in *P. aeruginosa* both in our geographic region [4, Pasteran F *et al.* (2005) 45th ICAAC Abstract C2 108/151] and other areas worldwide [2,4,5,8,10-12].

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