Identification of a stable chromosomal tandem multicopy of *bla*$_{VIM-63}$, a new *bla*$_{VIM-2}$ carbapenemase.

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Keywords

VIM-63, VIM-2 variant, carbapenemase, multimer, WGS

Abstract

This study characterizes a new genetic structure containing a multicopy of a *bla*$_{VIM-2}$ variant with an A676C substitution, *bla* $_{VIM-63.}$ This gene was detected on the chromosome of two carbapenem-resistant clinical strains of *Citrobacter freundii* ST22 recovered from two patients, separated by a 6-month period, and previously in *Pseudomonas aeruginosa* ST2242 from the same hospital unit. Short-read-sequencing was used to characterize the new variant in both species, and long- read-sequencing to characterize the genome of *C. freundii*. On the *P. aeruginosa* chromosome, the *bla* $_{VIM-63}$ gene was inserted between *ISPsy* 42-type sequences, flanked by an *intI1* sequence, nearby *aph(3´)-VI* and *sul1*. On the *C. freundii* chromosome, the *bla* $_{VIM-63}$ gene was inserted into a Tn6230-like transposon as a stable five-tandem repeat multimer, flanked by the same *intI1* as in *P. aeruginosa*. This structure was stable across subcultures and did not change in the presence of carbapenems. The *bla* $_{VIM-63}$ gene was cloned into the pCR-Blunt plasmid to study antimicrobial susceptibility patterns, and into pET29a for kinetic activity analysis. VIM-63 showed higher *Km* values than VIM-2 for ceftazidime and cefepime and higher *kcat* values for cefotaxime, ceftazidime, imipenem and ertapenem, without differences in MIC values. This is the first study to describe this new variant, VIM-63, in two different species with a chromosomal location integrated into different mobile elements, and the first time to describe a stable multimer of a metallo-betalactamase. Despite the amino acid substitution, the susceptibility pattern of the new variant was similar to VIM-2.

IMPORTANCE VIM group metallo-betalactamases are usually captured by *IntI1* integrases. This work describes the detection for the first time of a novel, previously unknown variant of VIM-2, VIM-63. This carbapenemase has been found on the chromosome of two different species, *Citrobacter freundii* and *Pseudomonas aeruginosa*, from the same hospital. The adjacent genetic environment of the *bla*$_{VIM-63}$ gene would indicate that the capture of this gene by *IntI1*

has occurred in two different genetic events in each of the species and in one there has been a stable integration of tandem copies of this gene.

Introduction.

In recent years, the emergence and prevalence of Gram-negative bacteria expressing metallo-β-lactamases (MBLs) has become a major health problem worldwide, since they confer broad-spectrum β-lactam resistance, including resistance to carbapenems and new antibiotics active against other types of carbapenemases (1). In Andalusia, a region in the south of Spain, a significant temporal change in the epidemiology of MBL was recently observed, with a clear upward trend associated with several outbreaks (2). One of the main groups of MBLs is the Verona-integron-encoded metallo-β–lactamase (VIM) (3). More than 70 variants of VIM have been described over a 20-year period (https://www.ncbi.nlm.nih.gov/pathogens/isolates#/refgene/gene_family:(blaVIM)).

VIM-type enzymes were widespread in *Pseudomonas aeruginosa* isolates in Mediterranean countries in the late twentieth century (4). bla_{VIM} genes are typically inserted into class I integrons (5) and can be mobilized by different plasmid lineages (*Inc*N, *IncI*1, *Inc*X) (6). Enterobacterales such as *Escherichia coli*, *Klebsiella pneumoniae*, *Citrobacter freundii* and others can acquire VIM-containing plasmids by horizontal transfer of highly conjugative, broad-host-range plasmids (7).

One VIM-producing *P. aeruginosa* and two *C. freundii* isolates were recently detected in 3 patients in the same unit in a hospital in the south of Spain (Andalusia). This study describes a new variant of bla_{VIM-2}, named bla_{VIM-63}, detected for the first time on the chromosome of those isolates, as well as its genetic environment.

Methods

Bacterial isolates.

A VIM-producing *P. aeruginosa* isolate (named 20190031), was recovered in 2019 from a rectal swab of a hematological patient admitted to University Hospital Virgen de las Nieves (Granada, Spain). The bla_{VIM} was identified as a new variant of VIM-2. This new variant was identified on

two subsequent occasions in two *Citrobacter freundii* isolates detected in the same ward: isolate 20200334, recovered 18 months later from a urine sample in a different patient, and isolate 20200723, recovered 6 months after that from the blood sample of another patient. For comparative analysis of sensitivity and enzyme activity, a clinical isolate of VIM-2-producing *P. aeruginosa* (20181090) was also selected.

Sequencing and genome assembly

To characterize the genome of the isolates, genomic DNA from *P. aeruginosa* (isolates 20190031 and 20181090) and *C. freundii* (isolates 20200334 and 20200723) was analyzed using the Nextera XT DNA sample preparation kit (Illumina San Diego, CA, USA), then sequenced using Illumina MiSeq 300-bp paired-end sequencing technology and assembled using the CLC Workbench 9.01.1 (Qiagen). Genomic DNA from isolate 20200334 (*C. freundii*) was also obtained with the DNeasy Blood & Tissue Kit (Qiagen) and sequenced with the PacBio Sequel system. A genome assembly was obtained with Canu v2.1.1 (option --genomeSize=4.5MB) (8) and further polished with Pilon v.1.24 (default parameters) (9). The representative species genome of the FDAARGOS_549 strain (accession number GCA_003812345.1) was used as a reference to manually sort the chromosome contigs according to origin of replication (defined by *dnaA* gene position), using the Artemis Comparison Tool (ACT) v18.1.0 (10). The sorted PacBio genome assembly was used as a reference to re-assemble the Illumina reads from *C. freundii* isolates 20200334 and 20200723, using the SPAdes genome assembler v3.13.0 (option --trusted-contigs) (11, 12) and Pilon. Finally, QUAST v5.0.2 was used for quality control of all genome assemblies (13). Assembly statistics are added as a supplementary file (Table S1).

Assembled genomes were annotated using the Rapid Annotations Subsystems Technology (RAST) server (http://rast.nmpdr.org/), together with ISfinder (14), Resfinder (15), Comprehensive Antibiotic Resistance Database (CARD) (16) and PlasmidFinder databases (17)

for resistance and plasmid replicon genes, and the MLSTfinder (18) for ST assignment. *P. aeruginosa* sequences involved in the regulation of *AmpC* and efflux pumps were compared with the wild-type *P. aeruginosa* reference strain PAO1 (www.pseudomonas.com).

Genomic islands were identified in *P. aeruginosa* 20190031 and *C. freundii* 20200334 isolates with the IslandViewer4 web server (19). In the case of isolate 20190031, the reference genome (*P. aeruginosa* strain PAO1) was required, due to the high fragmentation of our draft genome. Sequence homology searches (percentage BLAST identity > 95%) were performed against the positive reference dataset (i.e. confirmed genomic islands) in the integrated IslandPick database (20) to identify the origin of the predicted islands and their encoded genes.

Burrows-Wheeler Aligner (BWA) software v0.7.17-r1188 (21, 22) and Samtools v1.11 (23) were used to map sequencing reads to the assembled genomes and to evaluate coverage across the assemblies.

All nucleotide-based comparisons between assemblies were performed with BLAST+ v2.10.1 and visualized with Artemis v18.1.0 (10) and EasyFig v2.2.5 (24).

Finally, 3D protein structures were obtained using the Swiss-Model platform (25).

SNP-based phylogenetic trees

Genetic distance between the two *C. freundii* isolates was assessed by identifying the number of single nucleotide polymorphisms (SNPs) between them. Snippy software v4.6.0 (26) was used to map the reads of each isolate to the assembled genome of the other. An assembly of strain CF8_ST22 belonging to clone ST22 (accession number GCA_001880825.1) was used as reference (with the snippy-multi-input option --ref) to produce both a core SNP alignment and a whole genome SNP alignment. Each alignment was used to build a phylogeny with IQ-Tree v2.0.3 (with default options) (27) showing the relationship and genetic distances between the isolates and the reference strain CF8_ST22, which were calculated with snp-dists software v0.8.2 (https://github.com/tseemann/snp-dists).

Stability of bla$_{VIM-63}$ platform in *C. freundii*

To analyze the stability of the 5 tandem repeats in the genetic organization of bla_{VIM-63}, we used two approaches. First, *C. freundii* isolates were serially subcultured on MHB agar plates without antimicrobial pressure (15 passages). Minimum inhibitory concentrations (MICs) for meropenem, cefepime and piperacillin/tazobactam were monitored every 3 passages, and the strains obtained after all 15 passages were also sequenced using Illumina sequencing technology. Second, to analyze whether exposure to carbapenems affects the copy number of bla_{VIM-63}, the two *C. freundii* isolates were exposed by subculturing for 24 hours on Mueller Hinton agar supplemented with various concentrations of meropenem (0.25, 0.5, 1, 2 and 4 µg/ml) and sequencing the resulting colonies.

In both approaches, bla_{VIM-63} gene coverage was analyzed as described above.

Cloning experiments

In order to express bla_{VIM} genes in an identical background, bacterial DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen), following the manufacturer´s instructions. The bla_{VIM} genes were cloned into *E. coli* TOP10 cells using pCR-Blunt. Clones were selected on 0.06, 0.12, 0.25 and 0.5 µg/ml meropenem agar plates. The corresponding recombinant strains were used for MIC determination.

Susceptibility testing

The MICs of ampicillin, cefotaxime, ceftazidime, cefepime, aztreonam, piperacillin/tazobactam, ertapenem, imipenem and meropenem were determined by gradient strip method (Liofilchem, Spain) and disk diffusion assays according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. (https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_10.0_Breakpoint_Tables.pdf).

Protein purification

The bla_{VIM} genes were cloned into the pET29a vector. The recombinants, pET29a-bla_{VIM-2} or pET29a- $bla_{VIM-63,}$ were used to transform electrocompetent *E. coli* BL21(DES) bacteria. Transformed cells were grown overnight in LB agar supplemented with 50 µg/ml kanamycin at 37°C. Colonies were used to sequence plasmids and confirm transformation. Bacteria were grown in 60 ml LB containing 50 µg/ml kanamycin to OD_{600nm} = 0.6. VIM expression was then induced with 1 mM IPTG for 3 hours. The bacterial cells were pelleted and VIM proteins were purified using the *Ni-NTA Spin Kit* (Qiagen), following the manufacturer´s instructions. Purified proteins were dialyzed against 100 mM phosphate buffer (pH 7.2). The BCA assay was used to determine protein concentration. The purity of VIM protein extracts was determined by SDS-polyacrylamide gel electrophoresis.

Carbapenemase activity was evaluated in 100 mM phosphate buffer (pH 7.2) supplemented with 50 µM $ZnCl_2$ and different antimicrobials as substrates. Kinetic measurements (*kcat* and *Km*) of purified carbapenemases were performed spectrophotometrically, as described previously (28, 29).

For inhibition assays, the half-maximal inhibitory concentration (IC50) was determined using EDTA. Briefly, enzymes were pre-incubated with different concentrations of EDTA for 10 min at 25°C and hydrolysis was quantified spectrophotometrically with 100 mM imipenem as the reporter substrate (30).

Results and discussion.

Characterization of the genetic environment of *bla_{VIM-63}*

Whole genome sequencing of the *P. aeruginosa* 20190031 isolate revealed that this strain had a novel bla_{VIM} variant, designated $bla_{VIM-63,}$ which differed from bla_{VIM-2} by an Ile226Leu substitution due to an A676C mutation. The predicted amino acid sequence of bla_{VIM-63} showed a single amino acid substitution (Iso226Leu) as compared to bla_{VIM-2}. The isolate was assigned to ST2242, a single locus variant of ST762, both low frequency STs. Analysis of the genetic

environment of *bla*$_{VIM-63}$ in *P. aeruginosa* showed that it was located on the chromosome close to *ISPsy42* and a *ISPsy*30, both belonging to the Tn3 family. Next to the *bla*$_{VIM-63}$ gene, an *intl1* sequence was found, followed by resistance genes such as *aph(3´)-VI* and *sul1,* and a *VapC* toxin-antitoxin system (Figure 1, A). Some studies have reported that other members of the *bla*$_{VIM}$ family are located in integrons with a similar structure to the sequence described above, while other studies have found that these integrons are flanked by two insertion sequences (ISs*)* (31–34), although, in this case. only one complete IS was found.

An identical *bla*$_{VIM-63}$ gene was found in the two *C. freundii* isolates. To compare the genetic environments of the two species, we performed pairwise nucleotide BLAST comparisons of the *P. aeruginosa* 20190031 and *C. freundii* 20200334 (PacBio assembly) genomes and visualized the genetic region containing the *bla*$_{VIM-63}$ gene (Figure 1, B). The close genetic environments of the *bla*$_{VIM-63}$ gene in both species included the same *intl1* sequence found in *P. aeruginosa*, as well as the *sul1* and *aph(3')-VI* genes, although *C. freundii* also harbored *ant(2'')-Ia* in this location. According to Guerin *et al.* (35), the P2 promoter of the class 1 integron was at 137 bp and contained sequences for binding of transcription factors rpoD17/cpxR.

The main difference between the *P. aeruginosa* and *C. freundii* isolates was that *P. aeruginosa* 20190031 encoded one copy of the *bla*$_{VIM-63}$ gene, whereas the *C. freundii* isolates harbored five tandem copies. This scenario was observed both in the PacBio genome assembly and in the hybrid assemblies. To rule out potential assembly artifacts, we aligned the sequencing reads with the respective assemblies produced (Supplementary figure S2). In the case of the PacBio genome assembly (isolate 20200334), we identified several reads covering the entire chromosomal region encoding the five gene copies (supplementary figure S2, A). Next, we assessed the read coverage of the Illumina assemblies of isolates 20200334 and 20200723 (supplementary figure S2, B and C). A marked increase of coverage in the region encoding only the *bla*$_{VIM-63}$ gene was observed, which was consistent with the number of copies observed in the PacBio assembly (an increase of 4.91 and 5.22 times compared to mean coverage of the

whole genome, respectively). We therefore confirmed the presence of 5 tandem copies of the *bla*$_{VIM-63}$ gene in *C. freundii* isolates. Further genomic analyses were based on the PacBio genome assembly obtained from isolate 20200334, which captured the complete multimer.

It has been reported that tandem amplification of plasmid-borne carbapenemase genes enhances carbapenem resistance, usually after multimerization of plasmids by homologous recombination rather than replicative transposition of insertion sequences (36). By contrast, multimers of the same carbapenemase, as in the *C. freundii* case here, have only been detected on an IncF+IncR plasmid in *Klebsiella pneumoniae* (37). This plasmid originally contained 3 copies of NDM-1 flanked by a Tn3-like element and increased to 7 copies after *in vitro* exposure to imipenem. In our case, the carbapenemase genes were in a chromosomal location but also integrated into a transposon. The presence of 5 copies of *attC* sites joining the *bla*$_{VIM-63}$ copies may indicate the multimer was the result of multiple inclusion mediated by the integrase. We tested to see whether exposure to carbapenems would also modify the copy number of the resistance genes, but detected no increase in read coverage on the gene relative to the total genome following exposure to an increased concentration of meropenem. The stability of this structure was also analyzed by performing serial subcultures of the two *C. freundii* isolates. After 15 passages, the MIC values remained the same. The two isolates obtained after 15 passages were also sequenced, yielding an identical 5-fold increase in *bla*$_{VIM-63}$ gene coverage compared to the total genome. Both results suggest that this multimer is stable.

The other difference between the genetic environments of *P. aeruginosa* and *C. freundii* was that the *bla*$_{VIM-63}$ genes encoded in the two *C. freundii* strains were integrated into a Tn6230-like transposon (positions 233431 to 263680). This structure encodes more than 30 genes, and together with *bla*$_{VIM-63}$, it also encodes the *tetA* and *tetR genes*, an operon for mercury transport and several transposition-related genes (among others). All these genes are flanked by *tnsA, tnsB, tnsC* and *tnsD/tniQ* genes, which code for transposase proteins. In addition, this

structure is inserted at the 3´ end of the *YhiN* gene (Figure 1, B). The latter two characteristics are typical of Tn6230-like transposons, which have been described in *Salmonella enterica* (38). We observed that the Tn6230-like transposon containing the five tandem copies of the bla_{VIM-63} gene was inserted within a genomic island (supplementary figure S3). This genomic island was 49.48 kb in size and located near the origin of replication (positions 240128 to 289615). It encodes several other transport- and resistance-related genes apart from the Tn6230-like transposon. Based on sequence homology (BLAST identity percentage > 95%) searches against the positive reference dataset (i.e. confirmed genomic islands) in the IslandPick database (20), we identified multiple origins for the genes contained in it. A first region of ~20 kb in size was observed, which included the Tn6230-like transposon. Some parts within this region showed homology to a GI characterized in *Acinetobacter baumannii.* str. *AYE* (accession number NC_010410.1). More specifically, the homology included three regions of ~5 kb, ~2 kb and 600 bp, respectively, encoded antibiotic resistance genes against tetracycline (*tetA* and *tetR*) and aminoglycosides (APH(3')-IV and ANT(2')), respectively. Besides, another homologous region of ~4 kb long contained genes involved in transport of mercury was also found. A second identified region of ~16 kb included genes involved in transport systems for different metals (copper, silver, lead, cadmium, zinc, and mercury). Sequence homology results indicated that this region is related to a genomic island characterized in *Shewanella putrefaciens* str. CN-43. From these observations, we concluded that the predicted genomic island of *C. freundii* isolates containing a hybrid combination of multiple resistance and transport-related genes is the result of mobile element insertions of different origins, giving rise to a complex and dynamic genomic island specialized for metals and antibiotic resistance. The bla_{VIM-63} gene encoded by *P. aeruginosa* isolate 20190031 was also inserted in a genomic island of ~26 kb (supplementary figure S3). However, this genomic island is not the same as the one previously described in *C. freundii,* since the one in *P. aeruginosa* lacks heavy metal transporters. This, together with the observed differences in the close genetic surroundings, would rule out

transmission of the *bla*$_{VIM-63}$ gene or its platform between the two species and indicate independent acquisition.

C. freundii isolate typing

Both isolates belonged to ST22. To characterize the genetic relationship between the two isolates, two types of SNP analysis were carried out. First, the genetic distance between the two hybrid genome assemblies of *C. freundii* was calculated by mapping the reads of the second isolate (20200723) to the genome assembly of the first (20200334), resulting in 28 SNPs of difference. We also compared the isolates using the CF8_ST22 strain as the reference genome, performing core and whole-genome SNP alignments by mapping the raw reads of our isolates to the assembled reference sequence. We found 39 SNPs of difference between our isolates, both in the core and whole-genome alignments (supplementary figure S1). The time lapse between the 3 isolates, the lack of overlap between the 3 patient admissions, the different location of the rooms and the number of different SNPs initially ruled out a potential epidemiological link. No further cases have been detected since then.

Phenotypic characterization of clinical isolates and transformants.

Clinical VIM-63-producing *P. aeruginosa* 20190031 was less resistant to ceftazidime and cefepime than clinical VIM-2-producing *P. aeruginosa* 20181090 (Table 1). These differences could be associated with a partial deletion of *mexZ* (regulator of the MexXY pump) and two substitutions in *ampD* (G148A and D183Y) in the case of the VIM-2-producing isolate (Supplementary table S2) according to the mutation analysis in Cabot's study (39). The clinical isolates of VIM-63-producing *C. freundii* showed higher MIC values for cefepime and piperacillin/tazobactam than *P. aeruginosa* (Table 1), which may be explained by the presence of OXA-48 and OXA-1 genes in the two *C. freundii* isolates which are included in IncL and an IncHI2 plasmids, respectively. The presence of the five tandem copies of *bla*$_{VIM-63}$ in *C. freundii* does not seem to increase carbapenem MIC values. No differences in MIC values between

284 bla_{VIM-2} and bla_{VIM-63} transformants were observed (Table 1), except in the case of ampicillin,
285 whose MIC values were 3 times higher for bla_{VIM-2} than for bla_{VIM-63}.
286 The purity of the VIM-2 and VIM-63 proteins was greater than 90%, as estimated by SDS-PAGE
287 (data not shown). Carbapenemase activity was tested, except for piperacillin-tazobactam. Both
288 enzymes were able to hydrolyze all β-lactams tested except for aztreonam. The kinetic
289 parameter analysis indicated that VIM-63 showed less affinity for cefotaxime and meropenem
290 than VIM-2, and higher *Km* values for ceftazidime and cefepime (Table 1). VIM-63 also had
291 superior catalytic activity, with higher *kcat* values for cefotaxime, ceftazidime, imipenem and
292 ertapenem (Table 1). On the other hand, inhibition assays using imipenem as substrate
293 showed that the IC50 value of EDTA was slightly higher in VIM-2 than VIM-63 (3.62 µM *vs* 2.01
294 µM, respectively).
295 With respect to sequence similarity, there is only one amino acid difference between VIM-2
296 and VIM-63: Ile226Leu. This residue located in the α4 helix is in close proximity to the L3 loop,
297 which seems to play a role in substrate binding and is involved in the catalytic activity of the
298 VIM family of enzymes (40) (Figure 2). The results described in this study could indicate that
299 Iso226Leu plays a role in kinetic activity, since the *Kcat* and *Km* values were modified in VIM-
300 63. However, these changes did not contribute to the different MIC values of carbapenem or
301 third- and fourth-generation cephalosporins for the two enzymes.
302 In summary, this is the first study describing 5 tandem copies of a chromosomal
303 carbapenemase, stable across subcultures, as well as the first characterization of VIM-63. The
304 new variant was detected in multicopy in two genetically related *C. freundii* isolates and in
305 single copy in a clinical *P. aeruginosa* isolate belonging to a low frequency clone at the same
306 center. This *bla* gene was found in two different genomic islands in each species, indicating at
307 least two different acquisition events at the same center.
308 **Data availability**

The nucleotide sequence of the novel *bla*$_{VIM63}$ reported in this study was deposited in the GenBank nucleotide database under accession no. NG_064785.1. The protein sequence was deposited in GenPept under accession number WP_136512108.1. Citrobacter genomes were deposited in BioProject PRJNA768321 in the NCBI database. Raw sequencing reads were deposited in the Sequence Read Archive under accession numbers SAMN22027648 for isolate 20190031, SAMN22027648 for isolate 20200334, SAMN22027650 for isolate 20200723 and SAMN22027651 for PacBio-sequenced isolate 20200334.

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Transparency declarations.

None to declare.

Figure and table legends

Figure 1. Schematic map of the two genetic environments of *bla*$_{VIM-63}$. (A) Homology between the genome of *Pseudomonas aeruginosa* 20190031 and *Citrobacter freundii 20200334*, obtained using *EasyFig* v2.2.5. The blaVIM-63 gene is shown in red; intI1 in green; the resistance genes in light blue; genes associated with resistance to metals in dark blue; the integration sequences in pink; the antitoxin-toxin system in yellow. (B) Comparison of the promoter region. Pc, P2, and *P*intI1 promoters are indicated by horizontal brackets, the −35 and −10 promoter elements are boxed. The integrase and the *bla*$_{VIM-63}$ start codons are bold and colored in green and red, respectively.

Figure 2. Three-dimensional structure of VIM proteins obtained using the Swiss-Model platform (24,25)

Table 1. MICs of β-lactams for VIM-2-producing *Pseudomonas aeruginosa* (20181090), VIM-63-producing *Pseudomonas aeruginosa* (20190031), and VIM-63-producing *Citrobacter freundii* (20200334) and their corresponding *Escherichia coli* transformants expressing both *bla* genes and kinetic parameters of VIM-2 and VIM-63 proteins.

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