# Characterization of the genetic environment of the *bla*$_{VEB-4}$ gene, associated with a transposable region in a *Proteus mirabilis* clinical isolate.

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**Running title**: *bla*$_{VEB-4}$ in *Proteus mirabilis* from Spain

Espinal, P.

**Abstract**

*Proteus mirabilis* is the second most common cause of urinary tract infections and is also an important cause of nosocomial infections. TEM-type and CTX-M-type extended-spectrum β-lactamases (ESBLs) are the most widely distributed in this bacterial species, but minor ESBLs such as the VEB-type have also been identified. The aim of this study was to analyse the genetic environment of the $bla_{VEB-4}$ gene found in a *P. mirabilis* clinical isolate recovered in Spain. *P. mirabilis* N2231 showed resistance to penicillins, cephalosporins, and aminoglycosides, remaining susceptible to imipenem, cefoxitin, β-lactamases inhibitors and quinolones. Southern blot analysis revealed that $bla_{VEB-4}$ was located in the chromosome. Analysis of the $bla_{VEB-4}$ genetic context revealed a 15 Kb segment 98 % identical to the multidrug resistance region (MDR) of a *Salmonella* genomic island 1 (SGI1), which included a class 1 integron belonging to the In104 family, previously described in $bla_{VEB-6}$-producing *P. mirabilis* VB1248. $bla_{VEB-4}$ was surrounded by repeat elements (Re), transposon *Tn1721* and located on a class 1 integron containing *aacA4- aadB-dfrA1-orfC* genes. The $bla_{VEB-4}$ gene was inserted in a complex structure of a class 1 integron, which is part of an MDR region of an SGI1 possibly involved in the mobilization of the gene and homologous recombination.

**Keywords:** Class A β-lactamases, transposon *Tn1721*, VEB-enzymes

## Introduction

*Proteus mirabilis* is the second most common cause of urinary tract infections, such as symptomatic cystitis and pyelonephritis, and is present in cases of asymptomatic bacteriuria, particularly in the elderly and patients with type 2 diabetes. *P. mirabilis* is also an important cause of nosocomial infections, infection in the respiratory tract, eye, ear, nose, skin, throat, burns, and wounds, and has been implicated in neonatal meningoencephalitis, empyema and osteomyelitis [1].

Resistance to extended-spectrum cephalosporins in *P. mirabilis* is mainly due to the production of extended-spectrum β-lactamases (ESBLs) that belong to Ambler class A. TEM-type and CTX-M-type ESBLs are the most widely distributed among this species, but minor ESBLs, such as the VEB type, have also been identified [2].

VEB-type β-lactamases, which represent one of the smaller subgroups of class A β-lactamases [3,4], classified as group 2be enzymes [5] are a rare type of enzyme responsible for conferring high-level resistance to ceftazidime, cefotaxime, and aztreonam, although not carbapenems. There are 16 variants of VEB enzymes reported to date, derived from VEB-1 by one or more amino acid substitutions (http://www.lahey.org/studies/other.asp#table1). The *bla*$_{VEB}$ genes have been isolated from various species of Enterobacteriaceae and non-fermenter Gram-negative bacilli (*Pseudomonas aeruginosa, Acinetobacter baumanii, Achromobacter xylosoxidans*) [4,6-8] from Southeast Asia, Europe, the Middle East, Africa, and North and South America [9]. In *P. mirabilis* three variants have been reported: VEB-1 [2], VEB-4 [10] and VEB-6 [11]. They are usually inserted within the variable regions of class 1 integrons and horizontally transmitted at the intra- and interspecific levels [12]. Genetic analysis of *bla*$_{VEB-1}$ has revealed both chromosome and plasmid locations [13]. Interestingly, *bla*$_{VEB-1}$ has also been

reported on a class 1 integron located in an 86 Kb resistance island [14]. The *bla*$_{VEB-1}$ gene was first reported in an *Escherichia coli* isolate from a Vietnamese patient, where it was plasmid- and integron-located [15], and subsequently in two *P. aeruginosa* isolates from Thailand, where it was chromosomally and integron-located [11].

We report here the genetic environment of the *bla*$_{VEB-4}$ gene in a *P. mirabilis* clinical isolate N2231 recovered from a patient treated in a Spanish hospital.

## Materials and Methods

### Bacterial strain and antibiotic susceptibility testing

The *P. mirabilis* N2231 strain resistant to extended-spectrum β-lactams was isolated during a survey from February 2000 to December 2005 at Hospital de la Santa Creu i Sant Pau in Barcelona. Among 1423 *P. mirabilis* clinical isolates studied to determine the prevalence of ESBLs, we had found one VEB-4-producing isolate, the N2231, recovered from a urine culture. Reported here is the first description of this new variant (accession number EF136375) [10].

Antimicrobial susceptibility testing was performed by the broth microdilution method, using GNX2F-layout plates; (Sensititre, Thermo Fisher Scientific Inc) and the results were interpreted by Clinical and Laboratory Standards Institute guidelines (CLSI) [16].

### Conjugation experiments

Transferability of the resistance phenotype was studied by conjugation assays using a broth mating method at 37ºC without shaking. The *P. mirabilis* N2231 VEB-4-producing strain was used as a donor and the modified *E. coli* Hb101 (UA6190) strain (rifampicin- and aminoglycoside-resistant, lactose-negative and green fluorescent protein (GFP)-producing) as a recipient strain. Transconjugants were selected based on fluorescence

production and grown on LB agar plates supplemented with 10 mg/L ceftazidime and 100 mg/L rifampicin.

**Genetic localization of the *bla*$_{\text{VEB-4}}$ gene**

The genetic location of *bla*$_{\text{VEB-4}}$ was analyzed by PFGE and Southern Blot. First, the genomic DNA was digested with the S1 nuclease to define plasmid localization. Second, the genomic DNA was digested with the I-*CeuI* restriction enzyme (New England Biolabs) to observe a chromosomal localization. After Southern transfer to a Hybond-N+ membrane (GE Healthcare) the fragments were hybridized with PCR-generated probes derived from purified DNA products obtained from the PCR of the *bla*$_{\text{VEB}}$ [10] and 16S rDNA genes (primers: 27F: 5´-AGAGTTTGATCMTGGCTCAG-3´; 907R: 5´-CCGTCAATTCMTTTRAGTTT-3´) marked with the PCR DIG probe synthesis kit (Roche, Spain). Detection was performed with antidigoxigenin antibody conjugated to alkaline phosphatase and CDPStar chemiluminescence substrate (Roche) according to the manufacturer's instructions.

**Genetic context of the *bla*$_{\text{VEB-4}}$ gene**

The genetic context of the *bla*$_{\text{VEB-4}}$ gene in *P. mirabilis* N2231 was analyzed by inverse PCR and sequencing as previously described [17]. Briefly, DNA was extracted by the GenElute-Bacterial Genomic DNA Kit (Sigma-Aldrich, Spain) from an overnight culture in LB (Luria Bertani) broth at 37ºC. The *bla*$_{\text{VEB}}$ gene was detected by PCR with primers described by Aragón *et al.* [10]. The genomic DNA from strain N2231 was digested with the *Hind*III (Promega), and the fragments obtained were autoligated using T4 DNA ligase (Promega), following the manufacturers' instructions. The fragment of DNA containing the *bla*$_{\text{VEB-4}}$ gene was used as a template for an inverse PCR with primers designed in this study from the *bla*$_{\text{VEB-4}}$ gene sequence (VEB.inv-FW: 5'-

GCAGAGTCCAAAGAAACTTCG-3' and VEB.inv-R: 5'-GTCAGCTTGAGCATTTGAATAC-3'. Further PCR mapping was performed following the genetic environment of *P. mirabilis* VB1248 (HQ888851). Nucleotide and deduced amino acid sequences were analysed and compared by means of the BLAST programs from the National Center for Biotechnology Information web site (http://blast.ncbi.nlm.nih.gov/Blast.cgi)

The schematic representation of the sequences, including their comparison and visualization, was generated by Easyfig [18].

**Nucleotide sequence accession number**

The genetic environment of the *P. mirabilis* N2231 strain has been deposited in the GenBank database under accession number KX859086.

**Results**

**Susceptibility testing and transfer experiments**

*P. mirabilis* N2231 showed resistance to ampicillin, ceftazidime and cefotaxime (MICs >16, >32 and >16 mg/L, respectively) as well as some aminoglycosides [gentamicin, tobramycin and amikacin (MICs >8, >32 and >32 mg/L, respectively)], tetracycline, ciprofloxacin and trimethoprim/sulphamethoxazole (MICs >8, >2 and >4/76 mg/L, respectively), remaining susceptible to carbapenems (imipenem, and ertapenem), cefoxitin and β-lactamase-inhibitors (Amoxicillin-clavulanate and Piperacillin-tazobactam). No transconjugants were obtained in transfer experiments with this strain.

Espinal, P.

## Genetic location of the *bla*$_{VEB-4}$ gene

Genomic DNA digestion with S1 nuclease and subsequent PFGE analysis and hybridization with the *bla*$_{VEB}$ probe were negative, pointing to a chromosomal localization for this gene. The *bla*$_{VEB-4}$ chromosomal location was screened by Southern hybridization using the I-*Ceu*I-PFGE profile with *bla*$_{VEB}$ and 16S rDNA probes. *bla*$_{VEB}$ and 16S rDNA PCR amplicons hybridized in the same ca. 291 Kb I-*CeuI* fragment, suggesting that *bla*$_{VEB-4}$ was located on the chromosome of *P. mirabilis* N2231 (Fig 1.).

## Genetic context of the *bla*$_{VEB-4}$ gene

Sequence analysis derived from the inverse PCR and PCR mapping in *P. mirabilis* N2231 revealed that the obtained 15 Kb segment corresponded to the multidrug resistance region (MDR) of a *Salmonella* genomic island 1 (SGI1), and included a previously described complex class 1 integron [19]. The *bla*$_{VEB-4}$ described herein was located in a similar integron as part of a truncated gene cassette in opposite orientation (Fig 2). Interestingly, this 15 Kb segment were 98 % identical to those described by Siebor *et al.* [19] in a *bla*$_{VEB-6}$-producing *P. mirabilis* VB1248 (HQ888851) and 100 % identical to the deposited sequence described by Zong *et al.*, [2] who also found *bla*$_{VEB-6}$ in the *P. mirabilis* JIE273 chromosome. The *bla*$_{VEB-4}$ gene was bracketed by two 135-bp repeated elements named Re1 and Re2, found in opposite orientation in comparison with the *P. mirabilis* VB1248. A 28-bp segment found between Re1 and the *bla*$_{VEB-4}$ was absent in the same region in the *P. mirabilis* VB1248. The structure found downstream of Re1 until the insertion sequence IS*6100* (transposase) was identical to *P. mirabilis* VB1248 (Fig 2).

In opposite orientation to *bla*$_{VEB-4,}$ a 432 bp segment corresponding to a hypothetical protein was found, followed by the Tn*1721*Δ-like truncated tetracycline resistance transposon, including the *tet*(A) gene (encoding a class A tetracycline efflux protein), [20]

158 which was bracketed by the repeated elements Re2 and Re3.. Upstream to Re3 was
159 detected a third copy of a 3´conserved segment (3´-CS1) and the conservative structure
160 of a class 1 integron containing four gene cassettes: *aacA4* and *aadB* genes (conferring
161 resistance to aminoglycosides), *dfrA1 (*conferring resistance to trimethoprim) and *orfC*
162 with unknown function. At the terminal left-hand end, a fragment of a 5´-conserved
163 segment (5´-CS) containing *intI1* (integrase) was found.

164 **Discussion**

165 The aim of this study was to characterize the genetic surroundings of the $bla_{\text{VEB-4}}$ gene
166 found in the *P. mirabilis* N2231 clinical isolate [10]. The well known VEB β-lactamase has
167 a wide dissemination and an increasing number of novel variants recovered from humans
168 or food indicate a rapid evolution and spread [19,21]. *P. mirabilis* isolates naturally lack *bla*
169 genes on their chromosome, and are therefore generally susceptible to all β-lactam
170 antibiotic agents. However, a progressive increase of multiresistant strains has been
171 occurring in this species [22], which has demonstrated a great ability to acquire resistance
172 genes, such as inhibitor-resistant TEM β-lactamases (IRTs), ESBLs and ampC β-
173 lactamases [23].

174 Sequence analysis of the genetic context of the $bla_{\text{VEB-4}}$ described herein revealed a 15
175 Kb segment corresponding to an MDR region of a *Salmonella* genomic island 1 (SGI1),
176 and including a previously described class 1 integron belonging to the In104 family [19].
177 SGI1-like variants have been detected among bacteria other than *Salmonella,* including
178 *P. mirabilis* [24-26]. This MDR region included frequently described resistance genes, such
179 as *aacA4, aadB, sul1, dfrA1* and *tet(A)* in addition to the $bla_{\text{VEB-4}}$ and *qnrA* genes. Our
180 results were very similar (98 % identity) to those reported by Siebor *et al*., [19] who found
181 $bla_{\text{VEB-6}}$ in the MDR region of a SGI1-V from a *P. mirabilis* clinical isolate, and 100 %

identical to the reported sequence described by Zong *et al.* [2]. The latter found *bla*$_{\text{VEB-6}}$ in the *P. mirabilis* JIE273 chromosome in a truncated gene cassette, similarly flanked by three repeated elements Re1, Re2 and Re3, including the Tn*1721*Δ-like transposon and duplications of the 3´-conserved segments of a class 1 integron.

Siebor *et al.* [26] have hypothesized that *P. mirabilis* is involved in the dissemination of SGI1, since they found that the same modifications of the SGI1 backbones occur in *Salmonella*. A schematic comparison of the MDR regions among *P. mirabilis* strains, N2231 from this study, SGI1-PmSCO [26] and VB1248 [19] (Fig. 2), might suggest the presence of an SGI1 element in our strain, considering the similarity of the structures, particularly with *P. mirabilis* VB1248 (98 % identity). Interestingly, the *bla*$_{\text{VEB-6}}$ variant is essentially identical to *bla*$_{\text{VEB-4}}$, varying in only one nucleotide (A52G), which predicts only a conservative amino acid substitution (Ile18Val) in the leader peptide.

The *bla*$_{\text{VEB-1}}$ gene is often part of a gene cassette located in class 1 integrons [27]. Worthy of mention, the immediate genetic environment (3´-CS-Re3-Tn1721Δ-Re2-*bla*$_{VEB}$-Re1-3´-CS) of the *bla*$_{\text{VEB-4}}$ in *P. mirabilis* N2231 included the 135-bp repeat elements (Re1, Re2 and Re3) bracketing the *bla*$_{\text{VEB-4}}$ gene and duplications of the 3´-CS of class 1 integrons. Notably, this structure is similar to that observed for *bla*$_{VEB\text{-1a}}$ in *P. aeruginosa* [6], *bla*$_{VEB\text{-1b}}$ in *P. stuartii* B1 [28], and *bla*$_{\text{VEB-6}}$ in *P. mirabilis* JIE273 [2] and *P. mirabilis* VB1248 [19] (Fig. 2), suggesting that *bla*$_{\text{VEB}}$ is associated with highly conserved genetic structures. In addition, the presence of *bla*$_{\text{VEB}}$ in different bacterial species illustrates how resistance genes may spread via conjugative plasmids and/or integrons [11], as well as transposable elements. This is of concern in the context of dissemination of resistance genes via horizontal gene transfer.

The high similarity between these genetic contexts, particularly in three *P. mirabilis* strains with a chromosomally located *bla*$_{VEB}$ gene, JIE273 [2], VB1248 [19] and N2231 of our study, indicate a common ancestry, possibly mediated by SGI1 elements, taking into account that *P. mirabilis* is an important host of SGI1 [26]. The association of *bla*$_{VEB}$ variants with MDR regions of SGI1, cassette arrays, repeat elements bracketing the *bla*$_{VEB}$ genes, duplications of the 3´CS of a class 1 integron and components of transposons further support this hypothesis. The presence of these components could also play a role in the movement of the *bla*$_{VEB}$ gene and suggest possible recombinations and rearrangements of a limited set of different elements, as described by Zong *et al.* [2].

In addition, differences in genes at both ends of the *bla*$_{VEB}$ structures could be explained by these recombination events, which can mediate large evolutionary jumps in bacterial genomes by rapidly spreading variants associated with increased virulence, fitness and antibiotic resistance. Interestingly, a recent study reported transmission of *bla*$_{VEB-6}$ through the food chain to humans: Seiffert *et al.* [21] identified *bla*$_{VEB-6}$ in a *P. mirabilis* isolate from poultry meat in Switzerland. The *bla*$_{VEB-6}$ from that study had a nucleotide sequence identical to that found in the human VEB-6-positive *P. mirabilis* VB1248 reported in France.

This is the first report describing the genetic environment of the *bla*$_{VEB-4}$ gene, detected in a *P. mirabilis* N2231 clinical isolate from Spain. The chromosomally located *bla*$_{VEB-4}$ gene was inserted in a complex structure of a class 1 integron, which is part of an MDR region of an SGI1, possibly involved in the mobilization of the gene and homologous recombination.

This structure carried several genes whose products confer resistance to aminoglycosides, tetracycline, sulphonamides and all tested β-lactams, except carbapenems. Therefore,

multiresistant strains harboring several resistant genes and complex structures require special attention.

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## Disclosure Statement

No competing financial interests exist.

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Espinal, P.

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**FIG. 1.** Southern hybridization of I-*Ceu*I-PFGE. a. PFGE-I*CeuI,* b. Hybridization with 16S rDNA probe, c. Hybridization with *bla*$_{VEB}$ probe. M. Lambda Ladder PFE marker.

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348 **FIG. 2.** Schematic view of the genetic context of the *bla*$_{\text{VEB-4}}$ gene in *P. mirabilis* N2231
349 and comparative structures with the MDR region within the SGI1 in *P. mirabilis* SGI1-
350 PmSCO (GenBank accession number JX121639), *P. mirabilis* VB1248 (SGI1-V)
351 (GenBank accession number HQ888851) {Siebor, 2011 #16} and *P. stuartii* B1 {Aubert,
352 2005 #26}. Conserved segments (5´CS and 3´CS). The repeat elements (Re1, Re2 and
353 Re3). Regions of homology are shaded in gray scale.[19,28]

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