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Short communication

Whole genome characterization of a naturally occurring vancomycin-dependent *Enterococcus faecium* from a patient with bacteremia

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ABSTRACT

Vancomycin-dependent enterococci are a relatively uncommon phenotype recovered in the clinical laboratory. Recognition and recovery of these isolates are important, to provide accurate identification and susceptibility information to treating physicians. Herein, we describe the recovery of a vancomycin-dependent and revertant *E. faecium* isolates harboring *vanB* operon from a patient with bacteremia. Using whole genome sequencing, we found a unique single nucleotide polymorphism (S186N) in the D-Ala-D-Ala ligase (*ddl*) conferring vancomycin-dependency. Additionally, we found that a majority of *in vitro* revertants mutated outside *ddl*, with some strains harboring mutations in *vanS*, while others likely containing novel mechanisms of reversion.

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1. Introduction

Enterococcus faecium can become resistance to the glycopeptide class of antibiotics through the acquisition of the *van* operon (Depardieu et al., 2007). A variety of *van* operons (*vanA*, *vanB* and *vanD*) have been described which confer varying levels of vancomycin resistance and different resistance profiles (Quintiliani et al., 1993; Arthur et al., 1996; Evers and Courvalin, 1996; Millan et al., 2009). The *van* operons encode for a set of genes responsible for the degradation of the wild-type peptidoglycan penta-peptide subunit (D-Ala-D-Ala) and the synthesis of an alternative peptidoglycan structure, ending in a D-Ala-D-Lac (Evers and Courvalin, 1996; Millan et al., 2009). This alternative peptidoglycan structure allows the bacterium to grow in the presence of vancomycin, as this drug only recognizes and binds D-Ala-D-Ala (Bugg et al., 1991). Expression of this operon is controlled by the *vanRS* two-component system, which recognizes and binds vancomycin, resulting in activation of the VanR response regulator by the sensor kinase, VanS (Depardieu et al., 2007; Depardieu et al., 2005; Depardieu et al., 2003). Once activated, VanR drives expression of both *vanRS* and the *van* operon from their respective promoters.

Rarely, *E. faecium* isolates from patients receiving vancomycin, harboring either *vanA* or *vanB* operons, have been described to be vancomycin dependent, thus requiring the presence of vancomycin to survive (Millan et al., 2009; Van Bambeke et al., 1999; Kerbauy et al., 2011). Vancomycin-dependency results from a spontaneous mutation that occurs in the wildtype D-Ala-D-Ala ligase gene (*ddl*), rendering the gene non-functional (Baptista et al., 1997; Sifaoui and Gutmann, 1997). Consequently, the bacterium requires the presence of vancomycin to stimulate expression of the *van* operon in order to produce a functional cell wall. Interestingly, revertants that no longer require vancomycin have been described and are due to three main mutations: regaining *ddl* activity, constitutively activating *vanS*, or promoting transcriptional read-through from the *vanRS* promoter into the *van* operon by weakening the stem-loop terminator (Millan et al., 2009; Van Bambeke et al., 1999; Baptista et al., 1997).

Using whole genome sequencing (WGS), we have characterized two *E. faecium* isolates recovered from a bacteremic patient: a vancomycin-dependent and a naturally occurring revertant isolate. Additionally, to further uncover the mechanisms of reversion and the impact these mutations may have on antibiotic resistance, we determined the frequency of revertants, molecularly characterized these strains, and determined the minimal concentration of inhibition (MICs) for various antibiotics.

2. Methods

E. faecium clinical isolates were recovered from routine aerobic and anaerobic blood culture bottles (BD Diagnostics BacTec™ FX System),

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that were incubated at 37 °C until positive. Positive blood was plated to blood agar media, in the presence and absence of a 5 μg/ml vancomycin impregnated disk, and incubated overnight at 37 °C in the presence of 0.5% CO_2. The isolate from the aerobic bottle grew in the absence of vancomycin (wildtype revertant), while the isolate from the anaerobic bottle only grew in the presence of vancomycin (vancomycin-dependent).

For WGS, clinical isolates were grown in 200 ml of Trypic Soy Broth (TSB) at 37 °C with aeration in the presence of 5 μg/ml vancomycin. Bacteria were pelleted at mid-log stage of growth. Genomic DNA was extracted using the Qiagen Genomic-tip DNA extraction kit (Qiagen). Genomic library preparation and whole genome sequencing was performed on the PacBio RSII using DNA sequencing reagent 4.0 v2 (Pacific Biosciences). Genome assembly was conducted using HGAP.2 in SMRT Analysis software version 2.3.0 (Pacific Biosciences). WGS of these isolates were deposited at DDBJ/ENA/GenBank with the accession numbers SAMN06850204 and SAMN06850205 for the vancomycin-dependent and aerobic revertant isolates, respectively.

In vitro revertant mutants were recovered by incubating vancomycin-dependent isolates in TSB in the absence of vancomycin at 37 °C, statically, for up to 5 days. Once the cultures became turbid, they were plated to blood agar in the presence and absence of vancomycin to confirm vancomycin independence and resistance. For isolates that gave the correct phenotype, DNA was extracted by resuspending bacterial colonies in PrepMan sample preparation reagent (Thermo-Fisher). Nucleic acid was released by bead beating the sample for 3 min at 5000 rpm and then heated for 10 min at 100 °C. PCR and Sanger sequencing was performed using the supernatant for *ddl*, *vanS*, or the intragenic region between the *vanRS* and the *vanB* operons (*vanSY* intragenic region). Primers used for PCR and sequencing are as follows (5′-3′): *ddl* (F-CGATTTAGAATACAGGAGGAC and R-TACGCAATCACTCCAGC), *vanS* (F-TATCAAGGGCTGACAATTCGG and R-TGACATTGGATAGCGCCTTTT), *vanSY* (F-CGCTGGAAAACACCTCAGAT and R-ACTGTGAACGAGATAATGAACG). PCR was performed using the illustra™ puReTaq Ready-To-Go PCR kit (GE LifeSciences). Sequencing alignments were made using CLC Main Genomics Workbench (Qiagen). Antibiotic MICs were determined by broth micro-dilution using the Sensititre Panel (Thermo-Scientific) for ampicillin, daptomycin, linezolid, vancomycin, penicillin, gentamicin and streptomycin. Teicoplanin MICs were determined by E-Test (bioMérieux) on blood Mueller-Hinton agar (BD) after 24 h of growth at 37 °C.

3. Results

Genomic comparison of the vancomycin-independent (aerobic) and the vancomycin-dependent (anaerobic) isolates revealed a single point mutation, G527A, in the *ddl* gene of the vancomycin-dependent isolate. This mutation lies within the protein's ATPase domain and results in a S186 N nonsynonymous mutation. Given the significance of this amino acid change, we hypothesize that this mutation rendered the *ddl* non-functional and resulted in vancomycin dependency. Indeed, the S186 amino acid is highly conserved across many Gram-positive cocci bacteria, including enterococci, streptococci and staphylococci, suggesting this residue is functionally important (Fig. 2). Additionally, both isolates harbored identical *vanB* operons, conferring resistance to vancomycin with MIC ≥ 16 μg/ml. Furthermore, the *vanRS* and *vanSY* intragenic region were also identical between the two isolates.

To determine the frequency reversion, 24 independent *in vitro* reversion experiments were conducted in the absence of vancomycin. Of those, 13 (54%) reverted with the expected phenotype (Fig. 1). Reversion occurred quickly (within 18 h). Of the 13 revertants, 3 (23%) restored *ddl* function by reverting the single nucleotide polymorphism (SNP) at 527 back to guanine (Fig. 1). Another 3 (23%) isolates reverted the 527 SNP but also gained an additional point mutation upstream at G522 T, which resulted in a nonsynonymous mutation of glycine to cysteine (Fig. 1). One isolate (7.7%) reverted the 527 SNP but also harbored a 12-basepair deletion, resulting in the removal of glutamic acid, phenylalanine, serine, methionine at positions 307–310.

Interestingly, 6 (46.2%) isolates retained the vancomycin-dependent *ddl* mutation (G527A), suggesting the mechanism of reversion lie outside the *ddl* gene, such as *vanS* and *vanSY* intragenic region. Of these isolates, 2 harbored a single point mutation in the *vanS* gene at C713A and C712T (Fig. 1). Although both isolate's mutations resulted in an amino acid change at position P238, the outcomes were different; one resulted in a P238S mutation, while the other resulted in P238H. These mutations are in close proximity to the conserved histidine kinase active site and likely effect kinase activity of VanS. One isolate was unique in that it acquired a 21-basepair duplication in *vanS* after nucleotide position 240–260 (Fig. 1). Moreover, there was no significant difference in the antibiotic profile between these revertants, with the exception of teicoplanin (Table 1). While low MICs were observed for teicoplanin in 3/4 isolates tested, revertant 3 (*ddl* G527A and *vanS* C713A) was highly resistant with an MIC of >256 μg/ml (Table 1). Interestingly, 3 isolates

Gene	Reversion Mutation	No. Isolates
ddl	wildtype (G527)	3/13 (23%)
	G527 + G522T	3/13 (23%)
	G527 + nt Δ919-931 (aa Δ306-310)	1/13 (7.7%)
	G527A	6/13 (46.2%)

Gene	Reversion Mutation	No. Isolates
vanS	nt C713A, C712T (aa P238S/H)	2/6 (33.3%)
	Duplication (nt 240-260, aa 207-213)	1/6 (16.7%)
	wildtype	3/6 (50%)

Gene	Reversion Mutation	No. Isolates
vanSY	wildtype	3/3 (100%)

Fig. 1. Genomic variances of revertant strains compared to the vancomycin-dependent isolate. Description and frequency of *in vitro* revertant mutations. nt = nucleotide; aa = amino acid.

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