

vanO, a New Glycopeptide Resistance Operon in Environmental *Rhodococcus equi* Isolates

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We describe here the sequence and gene organization of a new glycopeptide resistance operon (*vanO*) in *Rhodococcus equi* from soil. The *vanO* operon has low homology to enterococcal *van* operons and harbors a *vanHOX* cluster transcribed in the direction opposite that of the *vanS-vanR* regulatory system and composed of three open reading frames with unknown function. This finding has clinical interest, since glycopeptides are used to treat *R. equi* infections and resistance has been reported in clinical isolates.

Glycopeptides such as vancomycin and teicoplanin are last-resort drugs for treatment of nosocomial infections caused by methicillin-resistant *Staphylococcus aureus* and enterococci (1). Resistance is due to synthesis of low-affinity peptidoglycan (PG) precursors that terminate with either D-Ala–D-Lac or D-Ala–D-Ser (2). In enterococci, four D-Ala–D-Lac operons (*vanA*, *vanB*, *vanD*, and *vanM*) and five D-Ala–D-Ser operons (*vanC*, *vanE*, *vanG*, *vanL*, and *vanN*) have been described to date (3–7). It has been hypothesized that glycopeptide resistance operons originated from glycopeptide producers and other Gram-positive bacteria residing in soil (8).

The aim of this study was to elucidate the genetic organization of glycopeptide resistance operon in *Rhodococcus equi* RE-S7B isolated from garden soil in Denmark in 2004 (9). *Rhodococcus equi* is a Gram-positive soil saprophytic coccobacillus associated with severe bronchopneumonia in foals (10–12) and rare but fatal infections in immunocompromised patients (13, 14). Vancomycin is a last-resort drug for treatment of *R. equi* infections in humans (13), and resistance to vancomycin has been reported in clinical isolates from Taiwan (15), but the genetic mechanism of resistance was not investigated.

Whole-genome sequencing of RE-S7B was performed using Illumina paired-end (PE) technology (500-bp library) (BGI, Hong Kong). *De novo* genome assembly of the reads was performed using Geneious v7.3, and putative glycopeptide resistance genes were annotated using CLC Genomics Workbench v6.8.2, NCBI BLAST, and the NCBI conserved domain search. The Illumina 500-bp PE library generated 700 Mb of data and 7,777,778 paired reads, and *de novo* genome assembly produced 427 contigs of $\geq 1,000$ bp with a mean length of 18,575 bp. RE-S7B was confirmed to be *R. equi* based upon 98.6% 16S rRNA gene sequence identity to reference strain *R. equi* DSM20307. By use of the partial ligase gene sequence obtained by Guardabassi et al. (9), a putative glycopeptide resistance operon was localized on a 28,806-bp contig harboring 30 open reading frames (ORFs).

The operon in RE-S7B, designated *vanO*, had unique gene organization in comparison with the *vanA* operon in enterococci and displayed structural similarities to putative *vanHAX* operon-like clusters detected in actinomycete *Amycolatopsis* sp. strain ATCC 39116 (16) and *Frankia* sp. strain EAN1pec (GenBank accession no. YP_001511364) (Fig. 1). The *vanO* operon harbored a *vanHOX* resistance gene cluster transcribed in the direction opposite that of the *vanS-vanR* two-component regulatory system.

The percentages of nucleotide sequence identity to the *vanHAX* gene cluster in enterococci ranged between 60 and 63%, whereas higher homology (76 to 84%) to the corresponding *vanHAX* clusters in the actinomycetes was observed (Fig. 1). Upstream of *vanHOX*, *orf3* was annotated, and this was determined to be a truncated *murF* homologue sharing 83% predicted amino acid identity to the 3' end of the *murF*-like gene described in the teicoplanin producer *Actinoplanes teichomyceticus* (17). Downstream of *vanHOX*, *orf1* and *orf2* were identified, and these are not similar to any genes described in the *van* operons known to date. Orf1 is 84% identical to a putative MurG-like protein described in *Frankia* sp. EAN1pec (accession no. YP_001511362), while Orf2 does not have a protein homologue in publicly available databases. Homology modeling using structure guided sequence profiles (18) showed that Orf2 is a peptidase C39-like family protein (data not shown).

We identified upstream of the *vanS-vanR* two-component regulatory system an IS3-like transposase gene with 83% nucleotide identity to putative IS3-like elements (*orfA* and *orfB*) in pyridine-degrading *Rhodococcus* sp. strain PY11 (accession numbers ZP_09309307.1 and ZP_09309306.1). IS3-like elements are known to catalyze the “figure-eight” form of transposition across bacterial species (19–21). Acquisition of glycopeptide resistance in clinically important bacteria is generally due to horizontal transfer of plasmids carrying Tn3 transposons, which contain transposases that are structurally and functionally distinct from the IS3 type (22–24). Thus, it appears that the *vanO* operon in RE-S7B is distantly related to and has evolved separately from *van* operons in enterococci. This was confirmed by the GC content (61 to 71%) of the *vanO* operon genes, which is higher than the GC contents of enterococcal genes in *vanA* operons but similar to those of genes in actinomycetes. This is not surprising, since *R. equi* is phyloge-

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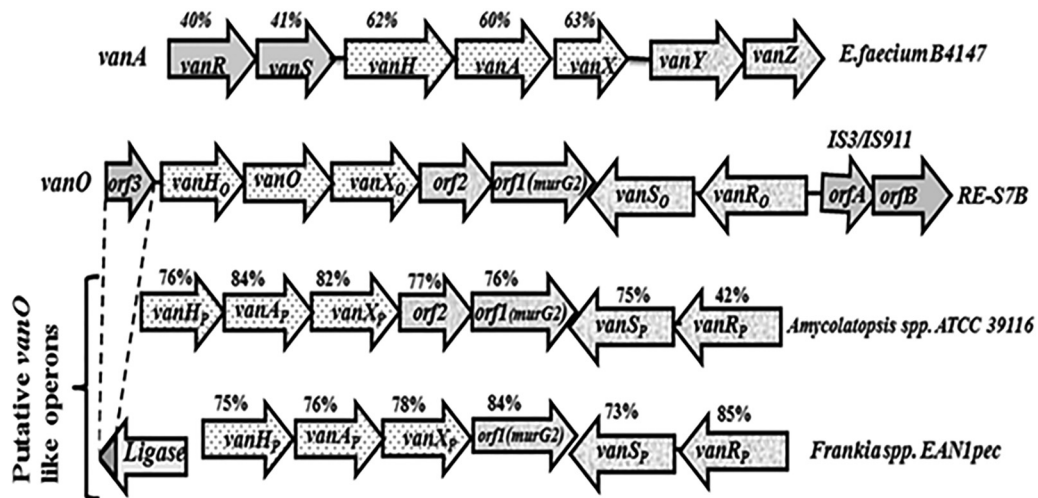


FIG 1 Organization of *vanA* in *Enterococcus faecium* (accession no. M97297), *vanO* in *Rhodococcus equi* RE-S7B (accession no. KF478993), and putative *van*-like clusters in *Amycolatopsis* sp. ATCC 39116 (accession no. WP_020417885) and *Frankia* sp. EAN1pec (accession no. YP_001511364). The arrows show directions of transcription. Percentages above the arrows indicate identity of the corresponding gene to *vanO* operon in RE-S7B. “P” refers to “putative,” to indicate the operons in *Amycolatopsis* and *Frankia* species have never been reported, and their accession numbers refer to the putative ligases (*vanA_P*).

netically more closely related to actinomycetes than to enterococci.

Transfer of *vanO*-mediated glycopeptide resistance was attempted by filter mating experiments (16) using vancomycin-susceptible (Van^s), rifampin-resistant (Rif^r) *Enterococcus faecium* BM4105RF and by transformation of RE-S7B plasmid DNA into Van^s Rif^r *R. equi* SD64 (this study). All attempts to transfer glycopeptide resistance by conjugation and transformation failed. Southern hybridization was performed to investigate whether *vanO* was localized on the chromosome or on a plasmid. Briefly, genomic (Easy-DNA kit; Invitrogen) and plasmid (HiPure Plasmid Midiprep kit; Invitrogen) DNAs were digested with BamHI and hybridized with a digoxigenin (DIG)-labeled *vanX* probe according to the manufacturer’s instructions (Roche Applied Science). Signals were detected from the genomic DNA only, indicating the chromosomal location of the *vanO* operon (see Fig. S1 in the supplemental material).

Expression of glycopeptide resistance in RE-S7B was inducible, and vancomycin MIC values increased proportionally depending on the amount of vancomycin to which cells were exposed prior to MIC determination. RE-S7B cells pregrown in brain heart infusion broth supplemented with 8 μ g/ml of vancomycin grew faster in the presence of vancomycin than nonexposed cells (see Fig. S2 in the supplemental material). The vancomycin MICs measured by Etest (bioMérieux, Denmark) using colonies from agar plates without and with 8 and 20 μ g/ml vancomycin were 0.5, 48, and 98 μ g/ml, respectively (see Fig. S3 in the supplemental material). Altogether, these data indicate that glycopeptide resistance was induced by exposure to vancomycin. Analysis of PG precursors (25) showed that 11% percentage of peptidoglycan precursors ended with D-Ala–D-Lac and the remaining 5% of peptidoglycan precursors ended with D-Ala–D-Ala (data not shown).

In conclusion, this study describes a novel glycopeptide resistance operon with a unique structure, designated *vanO*. This is the 11th *van* operon type detected to date, the first one described in the genus *Rhodococcus*. The finding of a novel glycopeptide resistance determinant in *R. equi* has potential implications in clinical

practice, since glycopeptides are used for therapy of nosocomial infections caused by this Gram-positive species and resistance has been reported in clinical isolates (13). Future work will focus on elucidating the functions of *vanO*-associated *orf1*, *orf2*, and *orf3* genes.

Nucleotide sequence accession number. Newly determined sequence data for the RE-S7B strain are deposited in GenBank under accession no. KF478993.

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We have no transparency declarations to declare.

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