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Genetic Linkage of the *vanB2* Gene Cluster to Tn1549-like Transposon in a *Clostridium* sp. Strain Isolated from the Normal Flora of Human Bowel

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ABSTRACT

Background: Vancomycin-resistant enterococci (VRE) are increasingly being reported, causing great concern in the hospital environment. We recently developed a rapid real-time PCR assay for the detection of VRE directly from fecal specimens. During a hospital surveillance program to detect VRE carriers, 11 of 197 fecal specimens were *vanB* positive with the PCR assay while showing no culturable VRE. One of the 11 specimens was shown to contain an anaerobic vancomycin-resistant *Clostridium* species containing the *vanB* gene which was further characterized.

Methods: Thirty PCR primers complementary to known *vanB* operons (*vanRB* to *vanXB* genes) were designed and used to amplify and/or sequence the complete *vanB* gene cluster of the *Clostridium* sp. strain. Amplification primers specific to Tn1547 and Tn1549 transposons were then used to characterize the genetic elements associated with the *vanB* gene cluster.

Results: Nucleotide sequence analysis showed that the organization of the *Clostridium* sp. *vanB* gene cluster was identical to those of *E. faecalis* V583 containing *vanB1* and *E. faecalis* E93/268 containing *vanB2*. The complete DNA sequence of the *Clostridium* sp. *vanB* operon exhibited 95.2% identity with that of *vanB1* and 98.9% identity with that of *vanB2*. The deduced amino acid sequences of VanRB, VanSB, VanYB, VanW, VanHB, VanB and VanXB from the *Clostridium* sp. strain displayed, respectively, 95.9%, 95.9%, 92.9%, 92.7%, 94.4%, 95.9% and 95.8% identity with those of the corresponding proteins from the *vanB1* operon and 100%, 99.7%, 100%, 96%, 98.7%, 99.1% and 95% identity with those from the *vanB2* operon. No PCR product was obtained with the primers specific to Tn1547 while specific amplification products were produced with the Tn1549-specific primers.

Conclusions: These results showed that resistance to vancomycin in this *Clostridium* sp. strain is of the *vanB2* genotype and is carried by a Tn1549-like transposon.

INTRODUCTION

Resistance to glycopeptide antibiotics such as vancomycin in enterococci is due to incorporation of D-alanyl-D-lactate (VanA-, VanB- and VanD-type) or of D-alanyl-D-serine (VanC- and VanE-type) into peptidoglycan precursors that have reduced affinity for vancomycin. VanA and VanB phenotypes are the most commonly encountered forms.

The *vanB* gene cluster confers resistance to vancomycin but not teicoplanin. Three subtypes, based on sequence variability in the *vanB* ligase gene, have been designated *vanB1*, *vanB2*, and *vanB3*, respectively, and have been found within different mobile genetic elements. The *vanB1* gene cluster has been shown to be part of a 64-kb composite transposon, Tn1547, delineated by insertion sequence elements IS16 and IS256-like in *E. faecalis* BM4281 (Quintiliani and Courvalin, 1996. Gene 172:1-8.). An approximately 27-kb putative conjugative transposon,

Tn5382, containing the *vanB2* gene cluster has been described in *Enterococcus faecium* C68. Integration of Tn5382 in the chromosome of *E. faecium* has occurred in the region downstream of the *pbp5* gene, which encodes high-level ampicillin resistance (Carias *et al.*, 1998. J. Bacteriol. 180:4426-4434). A 34-kb transposon, Tn1549, containing the *vanB2* gene cluster and borne by conjugative plasmids pIP834 and pIP835 has also been described in *Enterococcus faecalis* 93/268 and 654, respectively. Sequence comparison of both ends revealed that Tn1549 was highly similar to Tn5382 (Garnier *et al.*, 2000. Microbiology 146:1481-1489). The *vanB* genes have also been detected in non-enterococcal bacteria of the commensal bowel flora of humans and animals such as *Streptococcus*, *Eggerthella*, and *Clostridium* species and this suggested that these bacterial species may play a role in the dissemination of vancomycin resistance (Poyart *et al.*, 1997. Antimicrob. Agents Chemother. 41:24-29; Melvius *et al.*, 1998. J. Antimicrob. Chemother. 42:275-279; Stinear *et al.*, 2001. Lancet 357:855-856).

During a hospital surveillance program to detect VRE carriers using a DNA-based assay specific to *vanA* and *vanB* genes described by our group, 11 of 197 fecal specimens were *vanB*-positive with the PCR assay while showing no culturable VRE. One of the 11 specimens was shown to contain an anaerobic vancomycin-resistant *Clostridium* species containing the *vanB* gene which was further characterized.

MATERIALS AND METHODS

Isolation and identification of the non-enterococcal *vanB*-positive bacterial strain

A fecal sample obtained during a VRE surveillance program was shown to be *vanB* positive by PCR analysis but negative for VRE by culture method. To isolate the organism harboring *vanB*, an aliquot of the fecal specimen was incubated anaerobically in enriched Brain Heart Infusion Broth (eBHI). An aliquot of the primary enrichment broth was sequentially passaged several times through eBHI broth. Subsequently, an aliquot of the broth was subcultured in Enriched Cooked Meat broth containing 60 µg/ml aztreonam and 60 µg/ml vancomycin. The broth was then subcultured onto Wilkins-Chalgren agar containing 60 µg/ml vancomycin. Identification of the isolated anaerobic bacteria (CCRI-9842) containing *vanB* was done by sequencing and analyzing its 16S rRNA genes.

RESULTS

Identification and characterization of the isolated *vanB*-positive anaerobic bacteria

Comparison of the 16S rRNA gene sequence from the *vanB*-positive anaerobic strain (CCRI-9842) with databases revealed a potentially novel species that was most similar (96.8% identity) to an unidentified *Clostridium* sp. strain DR6A (Genbank accession number Y10028) (Figure 1). The

isolated *Clostridium* strain CCRI-9842 was resistant to vancomycin (MIC, >256 µg/ml) and sensitive to teicoplanin (MIC, 1.5 µg/ml) which corresponds to the VanB phenotype. The strain was also sensitive to ampicillin (MIC, 0.38 µg/ml).

Characterization of the *vanB* gene cluster

Nucleotide sequencing of the *vanB* gene cluster showed that the order of the *vanB* gene cluster in the *vanB*-positive *Clostridium* strain CCRI-9842 was similar to those of *vanB1* and *vanB2* (Figure 2).

The complete DNA sequence of the *Clostridium vanB* operon (6337 kb) exhibited 95.2% identity with that of *vanB1* and 98.9% identity with that of *vanB2* (Table 1).

Characterization of the genetic element containing the *vanB* gene cluster

No PCR products were obtained when one of the primers used for amplification was complementary to the region upstream from *vanR_B* or downstream from *vanX_B* of the *vanB1* gene cluster. Also, no PCR products were generated with primers specific to the insertion sequences 1S16 and 1S256-like and to the *pbp5* gene from *E. faecium* C68 containing Tn5382 (data not shown). These results suggest that the *vanB* gene cluster of *Clostridium* strain CCRI-9842 is not linked to Tn1547 and to 1S16 or 1S256-like insertion sequences and is not associated with *pbp5*.

PCR mapping with primers complementary to the left extremity of Tn1549 and to the region upstream from *vanR_B* (Figure 2) showed that genetic organization of this region in the *Clostridium* sp. strain was similar to that of Tn1549.

PCR amplification with primers specific to *xis* and *int* genes located to the right extremity of both Tn5382 and Tn1549, and encoding an excisase and an integrase, respectively, showed the presence of these two genes in *Clostridium* strain CCRI-9842 (Figure 2).

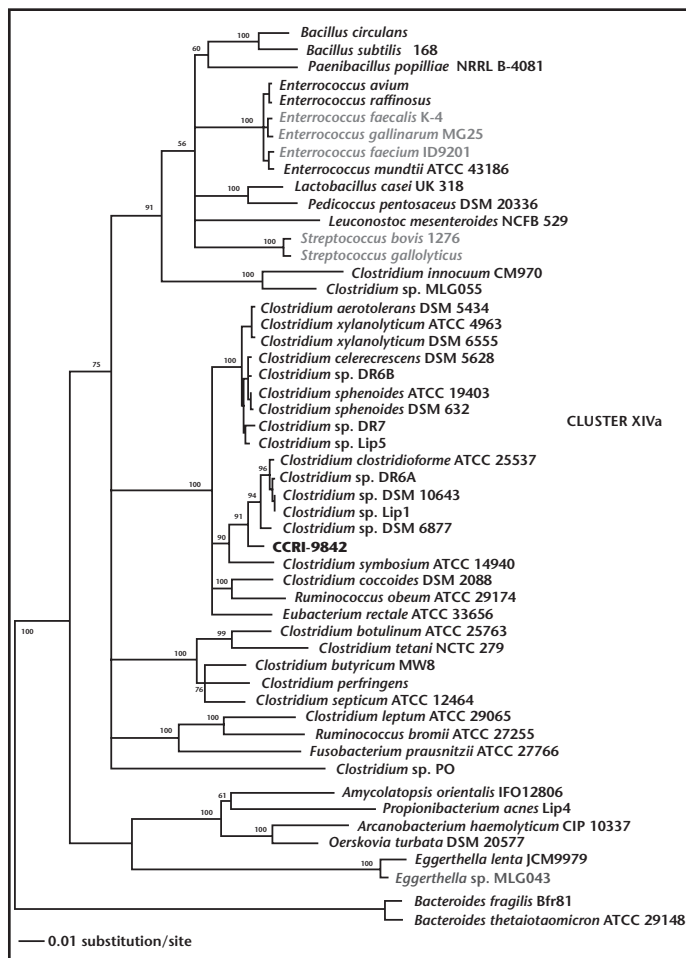


Figure 2: Organization of the Tn1549 transposon and localization of the PCR amplification primers used to map the transposon containing the *vanB* gene cluster of the *Clostridium* strain CCRI-9842. Open arrows represent ORFs. The primers used for amplification are indicated by thin half-arrows. All the primers shown generated PCR products with DNA from the *Clostridium* strain. Numbering of primers is from accession number AF192329. Primers from Garnier *et al.*, 2000. (Figure adapted from Garnier *et al.*, 2000).

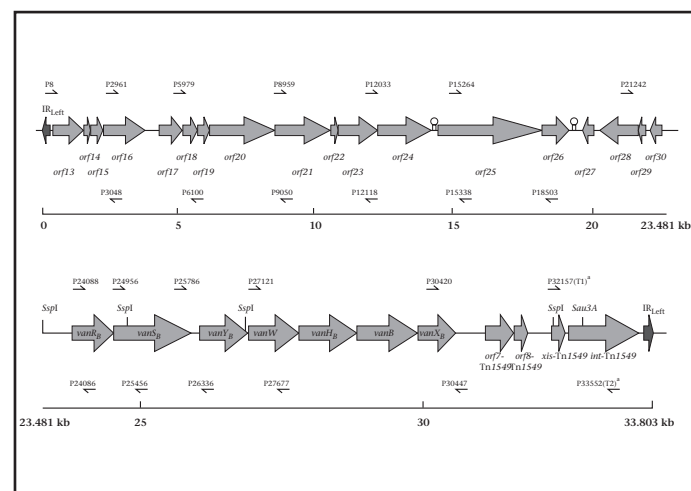


Figure 1: Phylogenetic tree derived from the partial 16S rDNA sequence data showing the relationships of the isolated *vanB*-positive anaerobic bacterial strain CCRI-9842 with members of the *Clostridium* group and various vancomycin-resistant bacterial species. The tree was constructed by the neighbor-joining method using PAUP 4.0b10. Bootstrap values were calculated from 1000 replications. The *Bacteroides* species served as outgroup. Some strains of the bacterial species highlighted in blue have been shown to contain the *vanB* gene.

Gene	Nucleotide/Amino acid identity %	
	<i>vanB1</i> ^a	<i>vanB2</i> ^b
<i>vanR_B</i>	94.4/95.9	99.3/100
<i>vanS_B</i>	96.2/95.9	99.7/99.7
<i>vanY_B</i>	94.1/92.9	100/100
<i>vanW_B</i>	95.5/92.7	97.5/96.0
<i>vanH_B</i>	95.0/94.4	99.4/98.7
<i>vanB</i>	95.7/95.9	99.1/99.1
<i>vanX_B</i>	95.3/95.8	96.5/95.8

^aSequences are from accession number EFU35369
^bSequences are from accession number AF192329

Table 1: Identity of the *vanB* gene cluster of *Clostridium* sp. Strain CCRI-9842 with those of *vanB1* and *vanB2*.

CONCLUSIONS

- We have isolated and identified from the normal flora of human bowel a potentially new *Clostridium* species which is a member of the cluster XIVa of *Clostridium*.
- This *Clostridium* strain (CCRI-9842) exhibits the *VanB*-type glycopeptide resistance phenotype.
- Resistance to vancomycin in this *Clostridium* strain is of the *vanB2* genotype and is carried by a Tn1549-like transposon suggesting a role of this bacterial species as a reservoir of vancomycin resistance.
- These results suggest that Tn1549-like transposons may transfer to a variety of gram-positive bacteria and play a significant role in the dissemination of vancomycin resistance.
- Work is in progress to characterize the plasmidic or chromosomal location of this transposon.

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