

Reduction in Glycopeptide Resistance in Vancomycin-Resistant Enterococci as a Result of *vanA* Cluster Rearrangements

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Received 24 September 2003/Returned for modification 5 November 2003/Accepted 29 December 2003

The molecular characterization of five clinical isolates of *vanA*-containing vancomycin-resistant enterococci with altered resistance to glycopeptides was examined. One strain represented an *IS1216V* insertion accompanied by partial deletion of the reading frame of *vanX* following a transposition event. The other four strains represented *IS1216V* within the *vanX-vanY* intergenic region associated with deletion of *vanY* or *vanZ*.

VanA glycopeptide resistance is characterized by acquired inducible resistance to both vancomycin and teicoplanin, whereas the VanB phenotype is characterized by variable levels of resistance to vancomycin but with susceptibility to teicoplanin (2, 7). Recently, vancomycin-resistant enterococci (VRE) with the *vanA* genotype that are susceptible to teicoplanin, and hence have the VanB phenotype and the *vanA* genotype, have been detected in Japan and Taiwan (8, 9). Hashimoto et al. (8) suggested that three point mutations in the putative sensor domain of *vanS* could be responsible for the impaired resistance to teicoplanin among VRE isolates possessing the *vanA* gene cluster. Another explanation for the VanB phenotype-*vanA* genotype VRE is impairment of accessory proteins VanY and VanZ (3, 12). In particular, the VanY D,D-carboxypeptidase has been known to contribute to vancomycin resistance by cleaving the C-terminal D-Ala residue of this precursor. The *vanZ* gene has also been shown to confer low-level resistance to teicoplanin, and deletion of *vanZ* could result in loss of teicoplanin resistance (3, 12).

To understand the spread of the *van* gene among enterococci in Korea, we collected 30 isolates of *vanA*-containing *Enterococcus faecium* from eight different university hospitals. Of these, four isolates (13%) were resistant to vancomycin but susceptible to teicoplanin. In contrast, one isolate was susceptible to both vancomycin and teicoplanin, like vancomycin-susceptible enterococci. The aim of present study was therefore to find out the molecular mechanisms responsible for the impaired glycopeptide resistance of VanB phenotype-*vanA* genotype VRE in Korea.

Five clinical isolates of *vanA*-containing VRE, AJ01, CA20, JC03, H23, and AS03, were obtained from five university hospitals in Korea during 2002. Previously well-characterized VRE strain *E. faecium* BM4147 was used as the control (6). The MICs of vancomycin and teicoplanin for all of the isolates are presented in Fig. 1.

Pulsed-field gel electrophoresis (PFGE) was performed with *SmaI* (Gibco BRL, Gaithersburg, Md.) as described by Murray et al. (10). Plasmid analysis was performed as previously de-

scribed (13). The *SmaI*-restricted DNA obtained by PFGE and plasmid DNA were Southern blotted and hybridized with a *vanA*-specific probe. All isolates were genetically unrelated as determined by PFGE typing (data not shown). Hybridization with a *vanA* probe demonstrated that two isolates had plasmid-borne *vanA* elements, whereas the other three isolates carried *vanA* on the chromosome (Fig. 1). These data suggest that the location of the *vanA* gene cluster may not affect the level of resistance to glycopeptides.

For structural analysis of Tn1546, PCR amplification of overlapping internal regions of Tn1546 was performed. The purified PCR products were directly sequenced with an ABI Prism 3100 DNA sequencer (Applied Biosystems, Foster City, Calif.). All isolates were analyzed for detection of the point mutations in *vanR* and *vanS* and the genetic rearrangement at the left and right ends of Tn1546. Genomic DNAs from all isolates were digested with *EcoRI* (Gibco BRL) and hybridized with internal Tn1546 PCR fragments (Fig. 1).

Activities of D,D-dipeptidase and D,D-carboxypeptidase were determined in crude extracts from all of the isolates as described by Arthur et al. (4). Vancomycin-resistant *E. faecium* BM4147 and vancomycin-susceptible *E. faecalis* JH2-2 were used as the controls (5, 6). To estimate D,D-dipeptidase activity, the amount of D-Ala released from D-Ala-D-Ala was determined by using D-amino acid oxidase coupled to peroxidase as the indicator reaction. For estimation of D,D-carboxypeptidase activity, D-Ala-D-Ala was replaced with the pentapeptide L-Ala-D-γ-Glu-L-Lys-D-Ala-D-Ala.

No polymorphism or size variation was found among PCR products from the central regions of Tn1546, i.e., *vanR*, *vanS*, *vanH*, *vanA*, and *vanX*. However, the remaining parts of Tn1546, including the right and left ends, were heterogeneous. The major rearrangements investigated among all of the isolates were the insertions of *IS1542* and *IS1216V*, accompanied by deletions adjacent to the insertion site (Fig. 1). All isolates were characterized by *IS1542* or *IS1216V-IS1542* insertions in the *orf2-vanR* region and *IS1216V* insertions within or downstream of *vanX*. It has therefore been suggested that no association was found between the resistant phenotype and the genetic rearrangement in the left end of Tn1546. In contrast, the genetic rearrangement of the right part of the transposon including *vanX*, *vanY*, and *vanZ* was associated with the resistant phenotype, consistent with previous studies (3, 11, 12).

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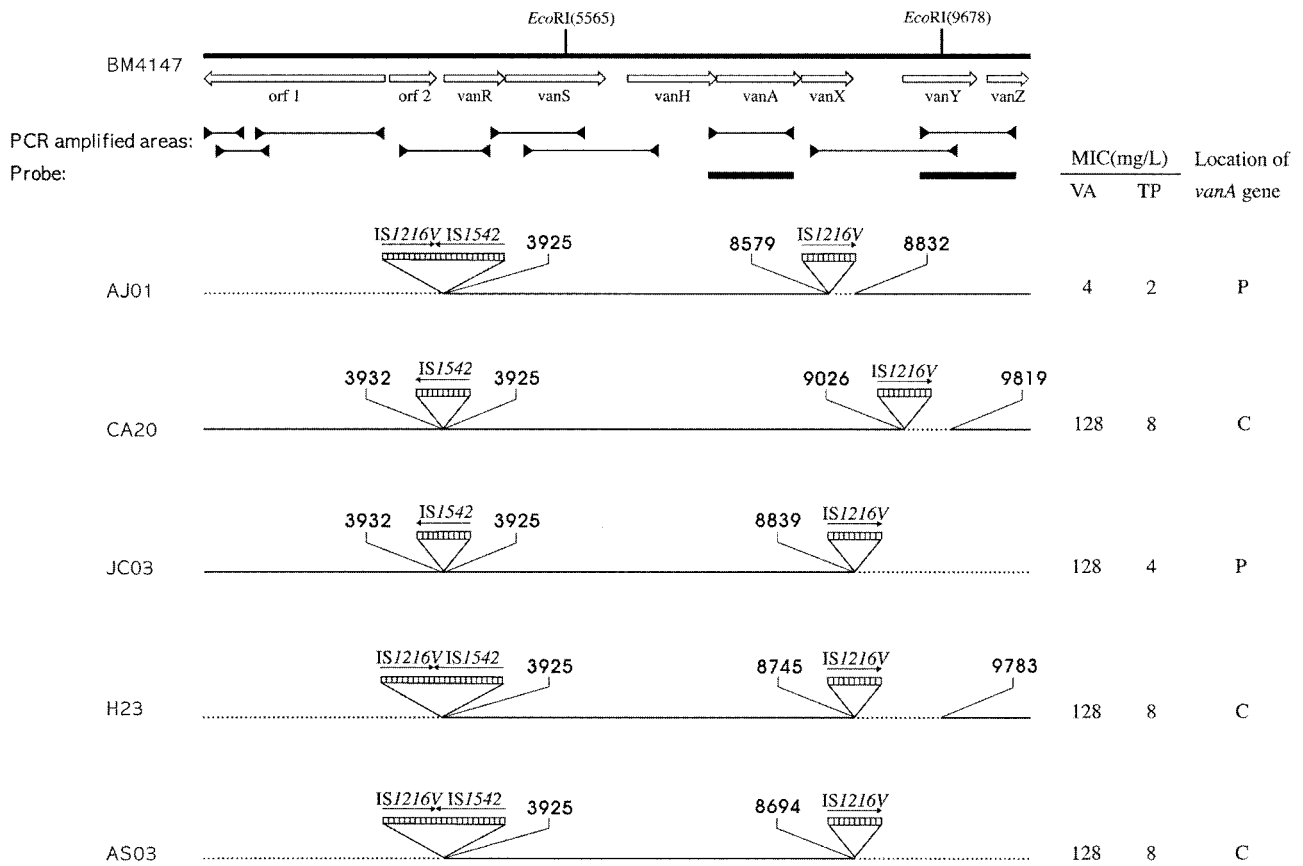


FIG. 1. Genetic maps of Tn1546 with integration of IS1216V and IS1542 in five clinical isolates including the MICs of vancomycin and teicoplanin and the location of the *vanA* gene. The positions of genes and open reading frames (*orf1* and *orf2*) and the direction of transcription are marked by open arrows at the top. The positions of the *EcoRI* sites are shown. Black horizontal bars indicate the positions of the internal Tn1546 fragments used as probes. Dotted boxes represent IS elements. The positions of the first nucleotide upstream and the first nucleotide downstream from the IS insertion sites are depicted. Solid arrows indicate the transcriptional orientation of the inserted IS elements. Deletions are indicated by dotted lines. L, liter; VA, vancomycin; TP, teicoplanin; P, plasmid; C, chromosome.

vanX encodes D,D-dipeptidase, which breaks the D-Ala-D-Ala dipeptide that is a product of the chromosomal ligase gene *ddl*. The essential role of *vanX* in the expression of glycopeptide resistance has been determined in several reports (1, 11). Strain AJ01 represented IS1216V in the *vanX* region with a 252-bp deletion of the right end of the *vanX* gene (corresponding to Tn1546 nucleotides 8580 to 8831). Insertion at this position would be predicted to cause the loss of 14 amino acid (DEPYPN SYFDFPVK) of the VanX peptide. Moreover, according to enzyme assay, strain AJ01 demonstrated extremely low D,D-dipeptidase activity (Fig. 2), indicating that the expression of *vanX* was significantly altered in AJ01. Therefore, partial deletion of the reading frame of *vanX* following a transposition event produced susceptibility to both vancomycin and teicoplanin.

In contrast, four isolates (JC03, AS03, CA20, and H23) were resistant to vancomycin but susceptible to teicoplanin. Strains JC03 and AS03 were characterized by IS1216V insertions in the *vanX-vanY* intergenic region accompanied by deletion of the region downstream of the *vanX-vanY* intergenic region including *vanY* and *vanZ* (Fig. 1). A probe specific for the *vanY* and *vanZ* genes did not hybridize with these two strains lacking the *vanY* and *vanZ* genes (data not shown). Strains CA20 and

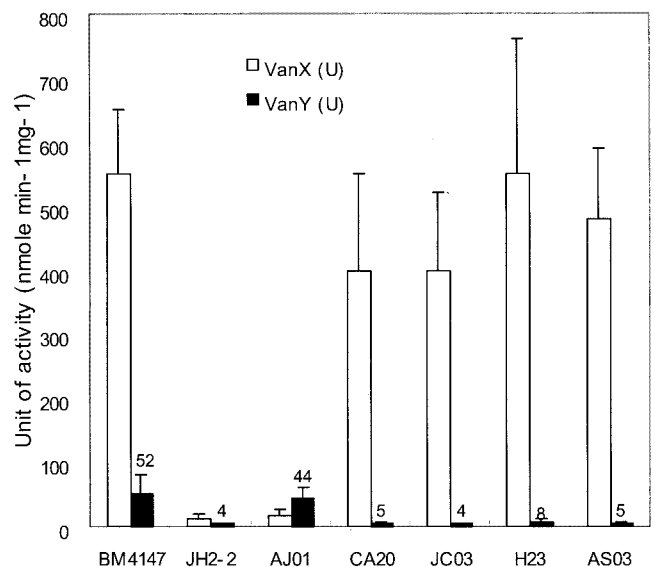


FIG. 2. VanX D,D-dipeptidase and VanY D,D-carboxypeptidase activities in VRE isolates. Induction was performed with 50 μ g of vancomycin ml⁻¹. Results are means \pm standard deviations obtained from a minimum of three independent extracts.

H23 revealed insertions of *IS1216V* in the *vanX-vanY* intergenic region associated with deletions of the *vanX-vanY* intergenic region and most of *vanY* (corresponding to Tn1546 nucleotides 9027 to 9818 and 8746 to 9782, respectively). As shown in Fig. 2, low levels of D,D-carboxypeptidase activity were present in these four strains, indicating that expression of *vanY* was significantly altered. Consistent with this, earlier data also suggested that deletion of *vanY* or *vanZ* could play a role in the loss of teicoplanin resistance (3, 12). It is conceivable that deletion of *vanY* affects the transcription of *vanZ*, resulting in a lower MIC of teicoplanin, since *vanZ* has been shown to be involved in teicoplanin resistance.

No isolates had point mutations in the *vanS* regulatory gene, and this finding suggested that the VanB phenotype-*vanA* genotype of the VRE isolates in the present study was not due to the point mutations of *vanS* observed in previous studies (8, 9). All of the isolates investigated were collected from geographically different areas of Korea and were genetically unrelated. Therefore, genetic rearrangement of *vanY* or *vanZ* or partial or complete deletion of both genes following insertion of *IS1216V* may be a common cause of VanB phenotype-*vanA* genotype VRE in Korea. Recently, the prevalence of this transposon type is increasing and these strains may be mistaken for VanB VRE by phenotypic testing.

This work was supported by grant R05-2002-000-00649-0 from the Basic Research Program of the Korea Science and Engineering Foundation.

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