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DNA Sequence Variation within *vanA*, *vanB*, *vanC-1*, and *vanC-2/3* Genes of Clinical *Enterococcus* Isolates

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We studied the DNA sequence variation of *van* genes of 34 isolates of *Enterococcus* spp. The isolates containing the *vanB* gene exhibited between 0 and 41 base pair changes per 801 bp studied when the *vanB* sequences were compared to that of the reference strain *Enterococcus faecalis* V583. The isolates carrying the *vanC-2* gene exhibited between 0 and 23 base pair changes per 346 bp studied when the *vanC-2* sequences were compared to that of the reference strain *E. casseliflavus* ATCC 25788. Little variation was noted in the *vanA* and *vanC-1* genes.

We have previously described a multiplex PCR-restriction fragment length polymorphism (RFLP) assay which detects and discriminates *vanA*, *vanB*, *vanC-1*, and *vanC-2/3* genes in *Enterococcus* spp. (5). In our original study we noted that in 4 of 63 isolates of vancomycin-resistant enterococci (VRE), a PCR product was produced but that it was produced with an amplicon which had RFLPs which differed from those found with the reference *vanA*, *vanB*, *vanC-1*, and *vanC-2* strains. We detected sequence variability to account for the unique *Msp*I restriction enzyme patterns observed. Since our assay detected sequence variation only in resistance genes that had variations located at their restriction enzyme sites, we hypothesized that there would be further sequence variation present in the *van* genes of enterococci. The objective of the present study was to determine the sequence variation of the *vanA*, *vanB*, *vanC-1*, and *vanC-2/3* genes in VRE.

Thirty-four clinical isolates of *Enterococcus* spp. were studied (5). The 34 isolates included 10 isolates which we previously identified as carrying the *vanA* gene (*E. faecium* [$n = 9$] and *E. gallinarum* [$n = 1$] [vancomycin MIC, ≥256 μg/ml; teicoplanin MIC, >16 μg/ml]), 8 isolates previously identified as carrying the *vanB* gene (*E. faecium* [$n = 5$] and *E. faecalis* [$n = 3$] [vancomycin MIC, 128 to >256 μg/ml; teicoplanin MIC, ≤8 μg/ml]), 9 isolates previously identified as carrying the *vanC-1* gene (*E. gallinarum*), and 7 isolates previously identified as carrying the *vanC-2* gene (*E. casseliflavus* [$n = 6$] and *E. flavescens* [$n = 1$] [vancomycin MIC, 4 to 8 μg/ml; teicoplanin MIC, ≤8 μg/ml]) (5). The following reference strains were used: *E. faecium* B7641 (*vanA*), *E. faecalis* V583 (*vanB*), *E. gallinarum* GS (*vanC-1*), and *E. casseliflavus* ATCC 25788 (*vanC-2*) (all of which were kindly provided by Daniel F. Sahm) (4–6).

Identification and antimicrobial susceptibility testing, PCR, and amplicon sequencing of isolates of *Enterococcus* spp. were performed as previously described (5). For pulsed-field gel electrophoresis (PFGE), a 7-ml sample of log-phase bacterial cells in brain heart infusion broth was centrifuged, the supernatant was discarded, and the cell pellet was added to 500 μl of 0.5 M EDTA–0.1 M EGTA–1 M Tris (pH 8) in distilled water. One-half milliliter of 1.6% SeaPlaque GTG agarose in the same solution was added. An insert was prepared, removed, and placed in a solution of 6 mM Tris-hydrochloride (pH 7.6), 1 M NaCl, 0.1 M EDTA, 0.5% Brij 58 (Sigma, St. Louis, Mo.), 0.2% deoxycholate (Sigma), 0.5% *N*-lauroylsarcosine (Sigma), 20 μg of RNase per ml, and 1 mg of lysozyme (Sigma) per ml in distilled water. Following overnight incubation, the lysis buffer was discarded and 1 ml of 0.5 M EDTA–0.1 M EGTA–1 M Tris (pH 8)–1% sodium dodecyl sulfate and 1 mg of proteinase K (Boehringer Mannheim, Indianapolis, Ind.) per ml in distilled water were added. This mixture was incubated at 50°C in a water bath, and then the insert was washed four times with Tris-EDTA wash buffer. For restriction endonuclease digestion, the inserts were removed from the buffer and equilibrated for 10 min in a solution containing 450 μl of distilled water and 50 μl of restriction buffer. *Sma*I (3 μl) was added, and the mixture was incubated at 37°C overnight. The inserts were then placed in 0.5× TAE. Thin slices of the inserts were loaded into the gel, and PFGE was performed. Previously published guidelines for interpreting chromosomal DNA restriction patterns produced by PFGE were used for the interpretation of PFGE findings (7).

We sequenced 825-bp DNA sequences for the *vanA* isolates, 801-bp DNA sequences for the *vanB* isolates, 360-bp DNA sequences for the *vanC-1* isolates, and 346-bp DNA sequences for the *vanC-2* isolates.

Nine of the 10 *vanA*-containing isolates had identical *vanA* amplicon sequences, and these sequences differed from *vanA* of reference strain B7641 by one base pair and one amino acid change. One isolate had a *vanA* amplicon sequence identical to that of the reference strain. PFGE on the 10 *vanA* isolates revealed that two isolates were closely related (one fragment in each isolate differed from the other) and that the remainder were different (7).

Marked sequence variation was found among the *vanB*-containing isolates (Fig. 1). One *E. faecalis* and one *E. faecium* isolate had sequences identical to the *vanB* sequence of the reference strain V583, which was used to determine the published sequence of the *vanB* gene (1, 2). The other six isolates, however, exhibited 41 base pair changes (5%) and 13 amino

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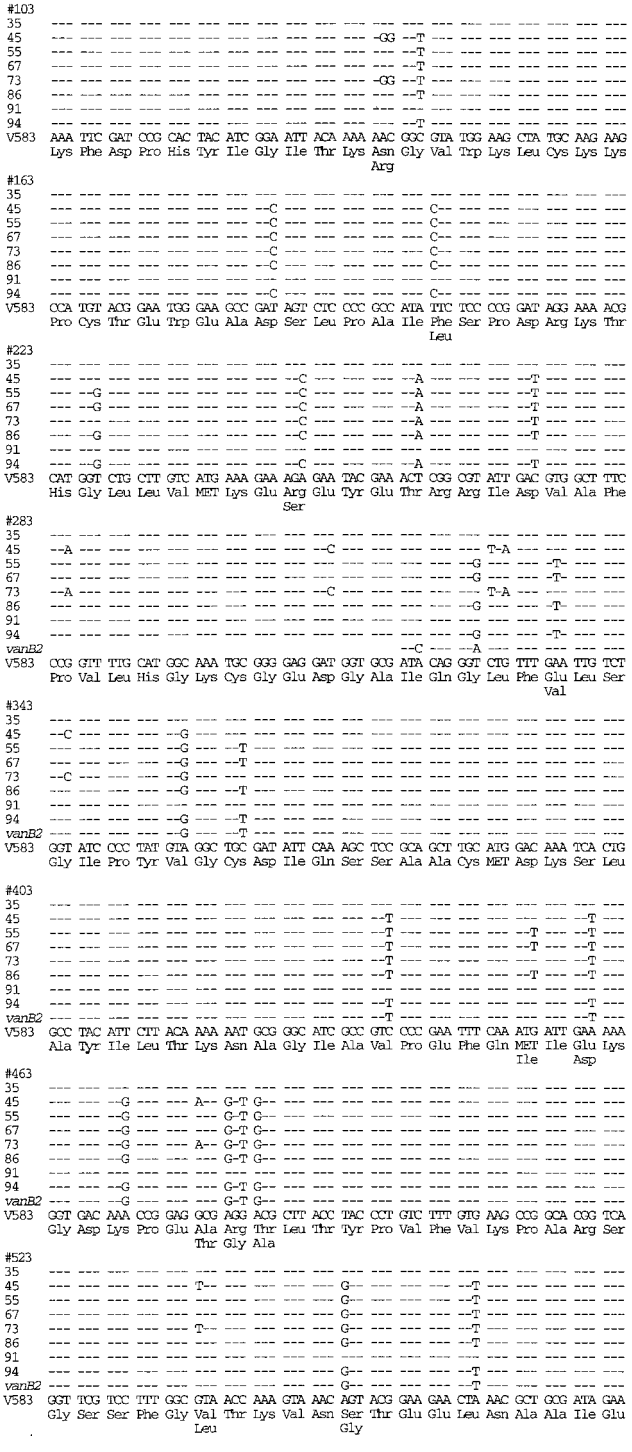

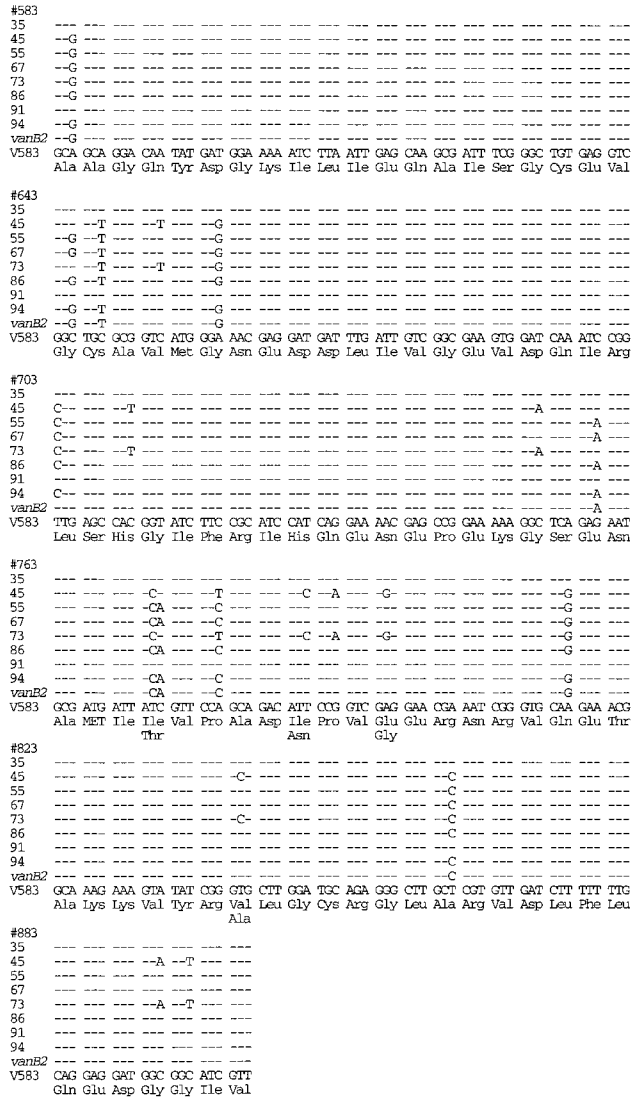

FIG. 1. Comparison of DNA sequences of *vanB* genes from VRE isolates to the *vanB* sequence from the reference strain V583 and to *vanB2* (3).

acid changes (two *E. faecalis* isolates, i.e., isolates 45 and 73), 31 base pair changes (4%) and 9 amino acid changes (three *E. faecium* isolates), and 30 base pair changes (4%) and 8 amino acid changes (one *E. faecium* isolate) compared to the *vanB* sequence of the reference strain V583. We found 22 base pair changes in each of these six isolates that were identical among these isolates but that differed from the *vanB* sequence of the reference strain V583. PFGE on the eight *vanB* isolates revealed that isolates 45 and 73 were indistinguishable, isolates 67 and 86 were closely related (one fragment in each isolate differed from the other), and the remaining four isolates were different (7).

The 584-bp sequence common to *vanB2* (3) was compared to those of our *vanB* study isolates with marked sequence differences from the reference *vanB* strain V583 (Fig. 1). We found 16 base pair changes in each of our six *vanB* study isolates with marked sequence differences from the reference *vanB* strain that were identical among these isolates and that were also found in the *vanB2* sequence. Isolates 45 and 73 had 16 base pair changes in common and 21 base pair changes (3.6%) that were different (resulting in five amino acid differences) from the published sequence of *vanB2*. The sequences of the remaining four isolates (isolates 55, 67, 86, and 94) shared 21 base pair changes with *vanB2* and differed from *vanB2* by only 3 to 4 (0.5 to 0.7%) base pair changes.

Our *vanB* isolates 45 and 73, which were identical by *van* gene sequencing and PFGE, showed marked sequence diversion from both the *vanB* gene and the *vanB2* gene. We have designated the genes found in isolates 45 and 73 *vanB3*. Gold and associates proposed the *vanB2* genotype based on a 3.6%

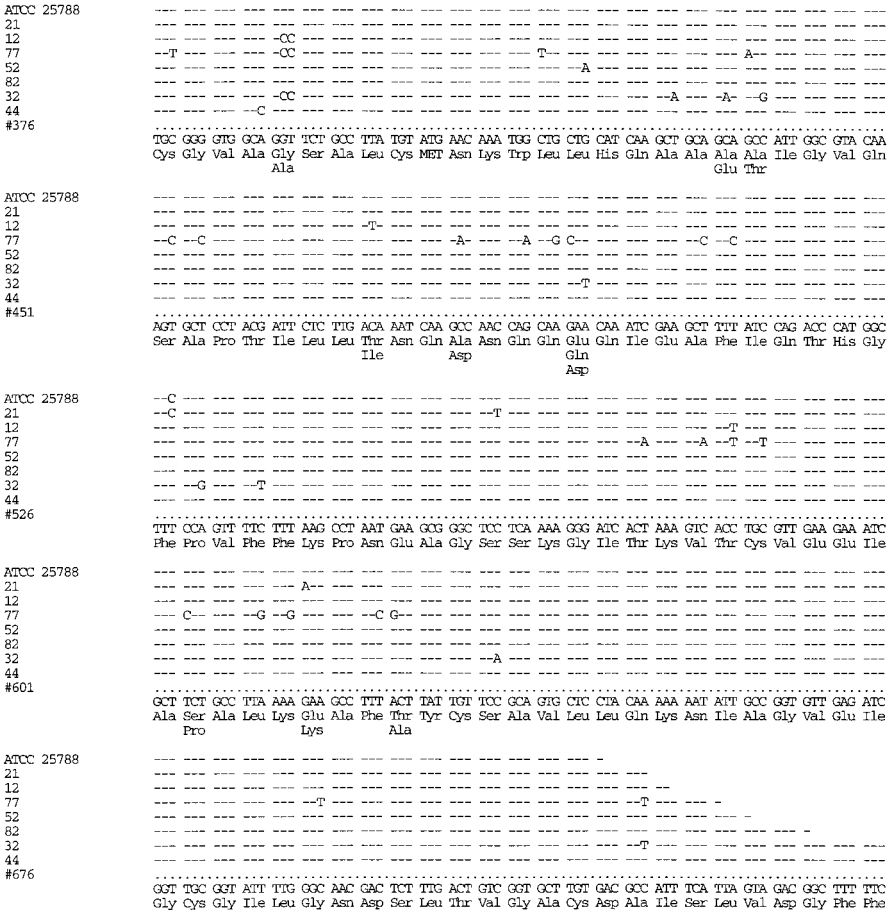

FIG. 2. Comparison of DNA sequences of *vanC-2* genes from VRE isolates, the reference strain ATCC 25788, and the consensus sequence (shown at the bottom) of these isolates.

base pair difference from *vanB* (3). Our isolates 45 and 73 had a 5% base pair difference from the *vanB* sequence from the reference strain V583 and a 3.6% base pair difference from the published sequence of *vanB2*.

Sequence variation was found in the *vanC-1* amplicon in four of nine *vanC-1* isolates. These four isolates exhibited between one and six base pair changes (up to 2% variation) resulting in no amino acid changes when they were compared to the *vanC-1* sequence of the reference strain *E. gallinarum* GS. PFGE on the *vanC-1* isolates revealed that two isolates were closely related (at most, one fragment was different from the corresponding fragment in the other isolate), one isolate had an uninterpretable PFGE pattern, and the remaining six isolates had different RFLPs (7).

Sequence variation was found in the *vanC-2* amplicons of all of the *vanC-2* isolates (Fig. 2). The sequence of the reference *vanC-2* strain ATCC 25788 (GenBank accession no. L29639) was confirmed. Our seven isolates exhibited between 0 and 23 base pair changes (up to 7% variation), resulting in zero to six amino acid changes from the consensus *vanC-2* sequence of these isolates and of the reference strain ATCC 25788 (Fig. 2). PFGE on the *vanC-2* isolates revealed that all had different RFLPs (7).

The finding of sequence variation among the *vanC-2* genes is important. Current breakpoint guidelines of the National Committee for Clinical Laboratory Standards do not accurately detect *vanC*-containing organisms. Therefore, in order to reliably detect *vanC*-containing organisms, molecular techniques must be used. The sequence variation which we have noted, however, indicates that caution is needed when ostensibly stringent molecular approaches for the detection of vancomycin resistance genes are applied to this group of microorganisms. That is, probes and PCR primers which anneal to areas of *van* genes prone to sequence variation may fail to detect these genes.

The sequence variation noted raises the question of whether sequencing of genes associated with vancomycin resistance may be used to determine relatedness of genes of VRE carrying *vanB* and *vanC-2* genes for epidemiologic purposes. The greater the variation in a genetic sequence, the greater the opportunity to see relatedness and differences.

In conclusion, we have found relatively large sequence variation in the *vanB* and *vanC-2* genes in enterococci but not, to any great extent, in the *vanA* and *vanC-1* genes by a PCR sequencing assay. We have designated the gene found in two of our isolates *vanB3*. Knowledge of the sequence variation in *van* genes which we have identified will assist in the accurate design of PCR primers to amplify these genes.

Nucleotide sequence accession numbers. The nucleotide sequences of isolates 45, 55, 94, 12, 21, 52, 77, 82, 44, and 32 have been submitted to GenBank and have been given accession no. U72704, U94528, U94526, U94521, U94522, U94523, U94524, U94525, U72706, and U72705, respectively.

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