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Heterogeneity of the *vanA* Gene Cluster in Clinical Isolates of Enterococci from the Northeastern United States

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In several strains of *Enterococcus faecium* isolated in Europe, the cluster of genes encoding high-level resistance to vancomycin (VanA phenotype) resides on a 10.85-kb transposon, Tn*1546*, or closely related elements. To determine whether Tn*1546* was conserved in recent enterococcal isolates from the northeastern United States, seven strains were compared by restriction mapping and DNA hybridization with probes from within the *van* cluster. Two of the seven strains contained intact Tn*1546*-like sequences; however, in five of the strains, the organization of the *van* cluster differed from that of Tn*1546*. Three of the five strains with variations harbored a novel DNA segment within the *van* gene cluster. This 1,496-bp segment was similar to IS*1165* of *Leuconostoc mesenteroides* and IS*1181* of *Staphylococcus aureus* and was flanked by 24- and 23-bp imperfect inverted repeats and 8-bp direct repeats. On the basis of these findings, we propose that this element comprises a novel insertion-like sequence, IS*1251*. Multiple copies of IS*1251* were also present at other sites in both resistant and susceptible clinical isolates. Our findings suggest that the *van* cluster in recent isolates from the northeastern United States differs from that present in the early European VanA phenotype strains.

Glycopeptide antibiotics such as vancomycin act by binding to the D-alanyl-D-alanine terminus of peptidoglycan precursors, preventing cell wall synthesis (26). It had been widely assumed that this terminus was ubiquitous among eubacterial species producing peptidoglycan and that vancomycin resistance was therefore unlikely to emerge in the absence of a barrier to vancomycin binding, such as the outer membrane of gram-negative bacteria. Nonetheless, vancomycin-resistant isolates of *Enterococcus faecium* were identified in France in 1986 (22). Resistant enterococcal strains have subsequently been isolated worldwide, and in 1993 the estimated incidence of glycopeptide resistance among isolates of *Enterococcus* spp. from intensive care units in the United States was 14% (24).

Vancomycin resistance in members of several gram-positive genera is due to the production of peptidoglycan precursors terminating in the depsipeptide D-alanyl-D-lactate, to which vancomycin does not bind (1, 17, 18, 23). In highly resistant (VanA phenotype) *E. faecium* and *Enterococcus faecalis* strains, production of this altered precursor is encoded by a plasmid-borne cluster of genes. Insertional mutagenesis experiments with two vancomycin resistance plasmids, pIP816 and pHKK100, have shown that four genes in the cluster are required for resistance: *vanR*, *vanH*, *vanA*, and *vanX* (3, 4, 15). *vanR* and *vanS* act as a sensor-regulatory pair analogous to such previously characterized systems as *ompR-envZ* and are required for the induction of resistance (3, 29). VanH, a D-specific α-keto acid reductase (8), and VanA, a D-alanine-D-alanine ligase of altered specificity (7), act in concert to form the depsipeptide D-alanyl-D-lactate, which is added in place of D-alanyl-D-alanine to the UDP-muramyl-tripeptide precursor (1, 8, 17, 23). *vanX* encodes a dipeptidase that diminishes the available pool of D-alanyl-D-alanine (5). Two additional genes present in the *van* cluster, the "accessory" genes *vanY* and *vanZ*, do not appear to be required for high-level resistance. *vanY* encodes a membrane-associated D,D-carboxypeptidase that has been shown in vitro to hydrolyze both the normal pentapeptide precursor and the depsipeptide-terminating precursor (14, 30). The function of *vanZ* is unknown.

In several strains isolated in France between 1986 and 1989, Arthur and coworkers (4) showed that the *van* gene cluster resides on a Tn*3*-like 10,851-bp transposon, Tn*1546*. Two open reading frames (ORFs) upstream from *vanR*, ORF1 and ORF2, show homology to transposases and resolvases, respectively, of the Tn*3* family. Tn*1546* is a nonconjugative transposon that transposes at a low frequency ($<10^{-8}$ per donor) in vitro, as determined by plasmid conduction experiments. However, the identification of Tn*1546* or closely related elements on several different plasmids suggests that transposition is one mechanism by which vancomycin resistance disseminates (4).

Although the *vanA* gene has been identified by DNA hybridization in a number of highly resistant enterococcal strains from the United States (6, 9), little information is available about the organization of the *van* genes in such isolates. The aim of the study described here was to determine whether the *van* gene cluster of recent enterococcal isolates from the United States was similar to that of European strains. Our findings suggest that the *van* cluster in some high-level resistant isolates in the United States differs from that present on Tn*1546*.

MATERIALS AND METHODS

Bacterial strains. *E. faecium* 228 was isolated in Barcelona, Spain, in 1987 and harbors the previously described plasmid pHKK100 (16). *E. faecalis* TJ153 and *E. faecium* E100 were isolated in 1991 and were obtained from Henry Fraimow, Thomas Jefferson Hospital, Philadelphia, Pa.; *E. faecium* GUC was isolated in 1991 and was obtained from Timothy E. Kiehn, Memorial Hospital, New York, N.Y.; *E. faecium* Nova was isolated in 1992 and was obtained from Brian Walters, Beth Israel Medical Center, New York, N.Y.; *E. faecium* Bat was isolated in 1993 and was obtained from Judy Berger, St. Barnabas Hospital, Bronx, N.Y.; *E. faecium* Fair was isolated in 1993 and was obtained from Amy Kresel, Bronx Municipal Hospital, Bronx, N.Y.; and *E. faecium* King was isolated in 1992 and was obtained from Marilou Corpuz, Our Lady of Mercy Hospital, Bronx, N.Y. Strains were grown in brain heart infusion (BHI; Difco Laboratories). MICs were determined by broth macrodilution in BHI. Teicoplanin was a gift from Marion Merrell Dow, Inc., Cincinnati, Ohio.

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Filter matings. For transfer of resistance to plasmid-free strains, filter matings were performed overnight as described by Clewell et al. (10), with a donor: recipient ratio of approximately 1:10. Recipient strains were *E. faecalis* JH2-2 (resistant to rifampin and fusidic acid [20]) and OG1X (resistant to streptomycin [19]).

DNA isolation. Plasmid DNA was isolated by a modification of the method of Ehrenfeld and Clewell (12). Briefly, cells from 5-ml overnight cultures were pelleted, washed in 50 mM Tris HCl (pH 8.0)–10 mM EDTA–2% glucose, and resuspended in 100 μl of this buffer with 50 mg of lysozyme per ml. After incubation for 30 min at 37°C, 200 μl of 1% sodium dodecyl sulfate (SDS)–0.2 N NaOH was added, and the samples were mixed gently. A total of 150 μl of potassium acetate (3 M, pH 5) was added, and the samples were chilled at −20°C for at least 30 min. After centrifugation, the supernatant was extracted with phenol-chloroform and then with chloroform and was precipitated with two volumes of ethanol for at least 30 min. After centrifugation, the pellets were washed three times in 70% ethanol, dried, and resuspended in 20 μl of water. For each restriction digestion reaction, 8 μl was used, and the entire digest was loaded on an agarose gel for analysis.

Isolation of genomic DNA was performed as described by Wilson (27), except that washed cells were incubated with 10 mg of lysozyme per ml for 30 min prior to the addition of SDS. The DNA extracted from a 5-ml culture was suspended in a final volume of 160 μl, and 8 μl of this was used for restriction digestion and analysis by agarose gel electrophoresis.

Preparation of DNA probes. Probes for *van* cluster genes were prepared from pHKK100 DNA. The *vanHAXY* probe (the 3.2-kb *Sal*I-*Eco*RI fragment) was purified from restriction-digested DNA cloned in *Escherichia coli* and was purified by agarose gel electrophoresis. The *vanY* probe (0.56-kb *Xba*I-*Hin*dIII fragment) was similarly purified. The *vanR* probe (a 0.50-kb fragment internal to *vanR*) was synthesized by PCR amplification of pHKK100 DNA with primers based on the previously obtained sequence (15). The primers, which were synthesized by GenoSys (Woodlands, Tex.), were 5′-ACAAGTCTGAGATT GACC-3′ and 5′-GGATTATCAATGGTGTCG-3′. The *vanH* probe (0.48 kb) was similarly constructed by using the primers 5′-GGCAGATGCATTC CATGC-3′ and 5′-CTCTATACTTCGGCTGCG-3′, which were based on the sequence obtained from pHKK100 (see below). Primers for the ORF1 probe (0.74 kb), 5′-TAGATCCGTCTCATGATG-3′ and 5′-GATACATGGAAT CAATCG-3′, were based on the published sequence of Tn*1546* (4).

PCR was performed with a DNA Thermal Cycler 480 (Perkin-Elmer Cetus, Norwalk, Conn.). Reagents were obtained from Perkin-Elmer Cetus, and the protocol used was that recommended by the manufacturer. Amplified fragments were purified by gel electrophoresis. Probes were labelled with digoxigenin-UTP by using random-primed labelling (Genius; Boehringer Mannheim, Indianapolis, Ind.).

Southern hybridization. DNA was transferred to nylon membranes (Hybond-N; Amersham, Arlington Heights, Ill.) by vacuum blotting, and membranes were baked at 80°C for 1 h. Hybridization and colorimetric detection were as described by the manufacturer (Boehringer Mannheim). Hybridization was performed overnight at 42°C in buffer containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 2% blocking reagent, 0.1% *N*-lauroylsarcosine, 0.2% SDS, and 50% formamide.

Restriction mapping. Restriction maps were generated by Southern hybridization of restriction-digested DNA by using five probes from within the *van* cluster: ORF1, *vanR*, *vanH*, *vanHAXY*, and *vanY*. At least five restriction enzymes (*Eco*RI, *Hin*dIII, *Ava*I, *Bam*HI, *Nde*I) were used alone and in combination for the mapping of each strain; additional enzymes were used as needed to clarify the maps. Restriction digestions were performed as recommended by the manufacturer (Gibco-BRL, Gaithersburg, Md.).

DNA cloning and sequencing. Two adjacent *Eco*RI fragments (5.6 and 4.1 kb) of pHKK100 harboring *van* genes were identified by mapping vancomycin-susceptible Tn*917* insertional mutants of *E. faecalis* 221 (JH2-2[pHKK100]) as described previously (15). Each fragment was purified from agarose gels, cloned into pACYC184, and introduced by transformation into *E. coli* XL1-Blue for DNA sequencing.

The 4.5-kb *Eco*RI fragment of *E. faecium* GUC containing *vanH*, *vanA*, *vanX*, a portion of *vanY*, and the 3′ region of IS*1251* (described below) was cloned into LambdaZapII (Stratagene, La Jolla, Calif.). *Eco*RI-digested genomic DNA from *E. faecium* GUC was ligated to lambda arms, packaged, and used to infect *E. coli* XL1-Blue MRF′ as directed by the manufacturer. Plaques were screened by hybridization with the *vanHAXY* probe. Positive clones were rescued by helper phage and pBluescript excised in vivo from LambdaZapII as suggested by the manufacturer.

The 5′ region of IS*1251* was cloned by PCR amplification of a 1.4-kb fragment partially overlapping the 4.5-kb *Eco*RI fragment of *E. faecium* GUC. The PCR primers used were 5′-CTCAGCTTTCCCACATGG-3′, a sequence 520 bp upstream from the end of *vanS* (based on the pHKK100 sequence), and 5′-TGT CAGCGGACCTTTGTCG-3′, 212 bp downstream from the *Eco*RI terminus of the cloned 4.5-kb fragment (based on the sequence obtained from this fragment). The 1.4-kb amplification fragment was cloned into pBluescript for sequencing.

Plasmid DNA from *E. coli* was sequenced by the dideoxy chain termination method of Sanger et al. (25) with incorporation of [^{35}S]dATP. The Sequenase 2.0 kit was used according to the manufacturer's protocol (United States Biochemical Corp., Cleveland, Ohio). Starting primers were the T3 20-mer and the T7 22-mer oligonucleotides designed for use with pBluescript (Stratagene). Sequencing was continued with internal sequence-specific primers synthesized on a Biosearch 8700 DNA Synthesizer (New Brunswick Scientific Co., New Brunswick, N.J.). One strand was sequenced. DNA and deduced amino acid sequences were analyzed by using BESTFIT and PILEUP (University of Wisconsin Genetics Computer Group package, version 7.0-UNIX). The BLAST algorithm (2) was used to identify GenBank sequences with similarities.

Nucleotide sequence accession number. The nucleotide sequence of IS*1251* has been assigned GenBank accession number L34675.

RESULTS AND DISCUSSION

Genetic heterogeneity of clinical isolates. All seven clinical isolates were highly resistant to vancomycin (MIC, >256 μg/ml) and were resistant to teicoplanin (MIC, >16 μg/ml).

DNA sequencing of the 4.1-kb *Eco*RI fragment of pHKK100 yielded a sequence for this fragment (which includes *vanH*, *vanA*, and *vanX* and portions of *vanS* and *vanY*) that was

TABLE 1. Sizes of restriction fragments that hybridized with the ORF1, *vanR*, and *vanHAXY* probes

DNA source	Size (kb) of restriction fragments hybridizing with[a]:				
	ORF1, *Nde*I-*Ava*I	*vanR*		*vanHAXY*	
		*Ava*I[a]	*Eco*RI[a]	*Hin*dIII	*Eco*RI
Tn*1546*, predicted[b]	4.1	>4.3, 1.1	>5.5	7.2, 1.2	4.1
E. faecium 228	4.1	22, 1.1	5.6	7.2, 1.2	4.1
E. faecium E100	4.1	6, 1.1	7.0	7.2, 1.2	4.1
E. faecalis EJ1 (E100 × JH2-2)[c]	4.1	6, 1.1	7.0	7.2, 1.2	4.1
E. faecium Nova	4.1	4.5, 1.1	5.8	7.2, 1.2	4.1
E. faecalis N35 (Nova × JH2-2)[c]	4.1	4.5, 1.1	5.8	7.2, 1.2	4.1
E. faecalis TJ153	**4.6**[d]	**2.8**, 1.1	**3.8**	7.2, **3.6**, 1.2	**4.7**, 4.1
E. faecalis T31 (TJ153 × JH2-2)[c]	**4.6**	**2.8**, 1.1	**3.8**	7.2, 1.2	4.1
E. faecium King	4.1	**5.6**, 4.4, 1.1	**16**, 5.7	**6.5**, **5.8**, 1.2	**4.0**
E. faecium GUC	4.1	4.4, 1.1	5.7	**4.0**, 1.2	**4.5**
E. faecalis GUCJ7 (GUC × JH2-2)[c]	4.1	4.4, 1.1	5.7	**4.0**, 1.2	**4.5**
E. faecium Bat	4.1	4.4, 1.1	5.7	**4.0**, 1.2	**4.5**
E. faecium Fair	4.1	4.4, 1.1	5.7	**4.0**, 1.2	**4.5**

[a] Restriction fragments that hybridized contained one site external to the *van* cluster.
[b] Fragments predicted from the sequence of Tn*1546* (4).
[c] Vancomycin-resistant transconjugants obtained by filter mating with *E. faecalis* JH2-2.
[d] Results that differ from the predicted map of Tn*1546* and the observed map of the pHKK100 *van* cluster, either in the predicted size or number of fragments, are shown in boldface.

identical to that of Tn*1546* (4). Sequencing of a portion (2,524 nucleotides, including *vanR* and *vanS*) of the 5.6-kb fragment also showed a sequence identical to that of Tn*1546*. The regions corresponding to ORF1, ORF2, and *vanZ* were not sequenced, but restriction mapping showed sites identical to that of Tn*1546*. Thus, the *van* cluster present on pHKK100 appears to be very similar, if not identical, to that of Tn*1546*.

Table 1 compares the results of DNA hybridizations with the ORF1, *vanR*, and *vanHAXY* probes against selected restriction digests of DNA from the U.S. clinical isolates and *E. faecium* 228, the European wild-type strain that harbors the 58-kb vancomycin resistance plasmid pHKK100. The variety of fragment sizes hybridizing with the *van* probes suggested considerable heterogeneity in the organization of the *van* gene cluster among the U.S. isolates. In strains *E. faecalis* TJ153 and *E. faecium* King, the results were consistent with the presence of two *van* clusters with different organizations. In *E. faecium* GUC, Bat, and Fair, fragment sizes suggested an alteration in the *vanH-vanA-vanX* region. Three representative strains were selected for more detailed mapping: *E. faecium* E100, *E. faecalis* TJ153, and *E. faecium* GUC. Restriction maps of the *van* cluster region of these isolates compared with that of *E. faecium* 228 are shown in Fig. 1.

In *E. faecium* E100, the sizes of the restriction fragments within the *van* cluster hybridizing to each of the probes were identical to those of pHKK100. Restriction fragments with sites external to the *van* cluster (e.g., the larger *Ava*I fragment hybridizing with the *vanR* probe) differed in size from those of pHKK100. The size of the vancomycin resistance plasmid of E100 was estimated to be 26 kb on the basis of the sum of restriction fragment sizes. These observations suggest that the Tn*1546*-like element present in E100 resided on a plasmid different from pHKK100. Vancomycin-resistant transconjugants obtained by filter mating (E100 × *E. faecalis* JH2-2) showed restriction and hybridization patterns identical to those of the donor. Similar results were obtained for *E. faecium* Nova (data not shown). Thus, in some *E. faecium* strains recently isolated in the United States, the *van* genes appear to reside within a Tn*1546*-like element on a conjugative plasmid.

Plasmid DNAs from two isolates showed more than the expected number of fragments hybridizing with the *van* probes, suggesting that more than one configuration of the *van* cluster was present. One such strain, *E. faecalis* TJ153, was selected for further mapping. In matings of TJ153 with both JH2-2 and OG1S, transconjugants selected for vancomycin resistance contained only one version of the *van* cluster, consistent with the presence of more than one plasmid carrying *van* genes in the donor strain. Plasmid DNA from one transconjugant, *E. faecalis* T31, was used for restriction mapping of this longer cluster (TJ153-1 in Fig. 1). The map of the shorter cluster

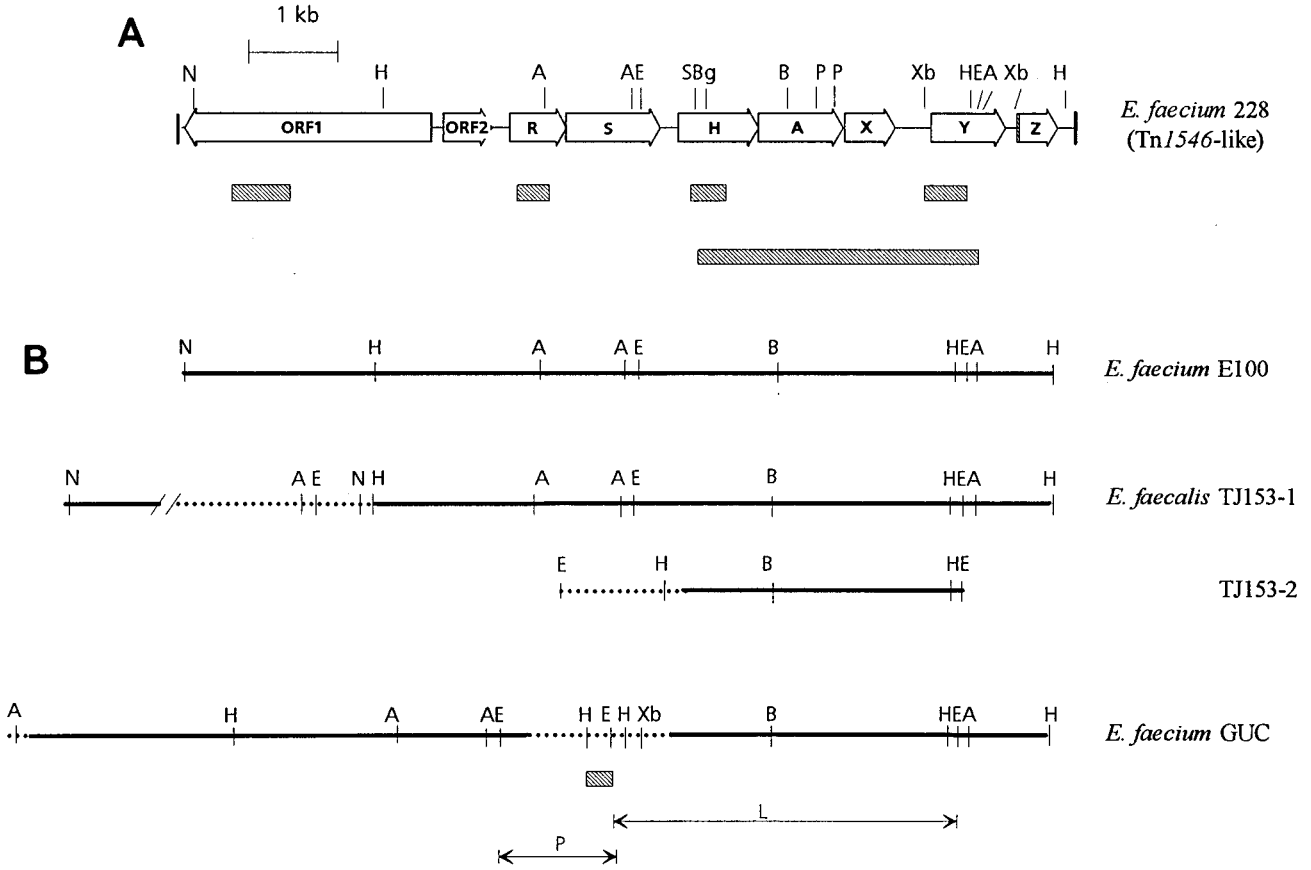

FIG. 1. (A) Restriction map of the *van* cluster of *E. faecium* 228, which harbors a Tn*1546*-like element on pHKK100. Locations of the probes used are indicated by the hatched rectangles. N, *Nde*I; H, *Hin*dIII; A, *Ava*I; E, *Eco*RI; S, *Sal*I; Bg, *Bgl*I; B, *Bam*HI; P, *Pst*I; Xb, *Xba*I. (B) Restriction maps of the *van* regions of three U.S. clinical isolates. Solid lines indicate regions similar to those in pHKK100 by Southern hybridization of restriction fragments; dotted lines indicate regions that differ. TJ153-1 and TJ153-2 indicate versions of the *van* cluster carried on two different plasmids in *E. faecalis* TJ153. The extent of the insertion or rearrangement in the ORF1 region of TJ153-1 is unknown, as indicated by the line break. The location of the IS*1251* probe is indicated by the hatched rectangle under *E. faecium* GUC. Arrows show the locations of fragments for DNA sequencing obtained by cloning into LambdaZapII (fragment L) and by PCR amplification (fragment P).

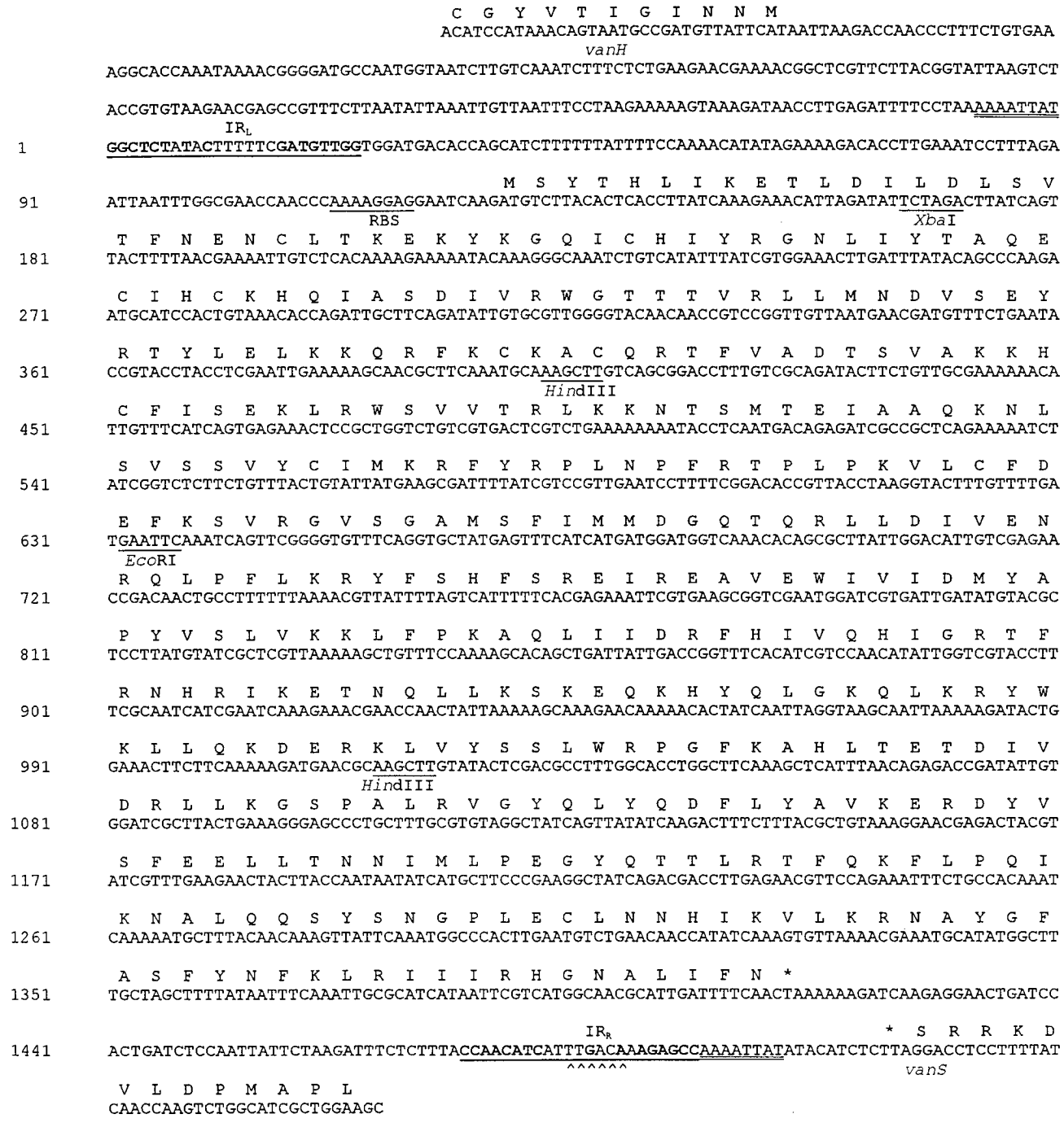

FIG. 2. DNA and deduced amino acid sequences of IS*1251*. Imperfect inverted repeats of 24 bp (IR_L) and 23 bp (IR_R) are underlined, with the conserved nucleotides shown in boldface. An outward-facing −35 promoter sequence within IR_R is indicated by arrowheads. The 8-bp direct repeats are double underlined. The deduced amino acid sequence of the ORF is shown above the nucleotide sequence. Stop codons are indicated by asterisks. The putative ribosome-binding site (RBS) and selected restriction sites are underlined.

(TJ153-2) was deduced by subtraction of fragments present in the transconjugant from those present in the donor strain.

Southern analysis of plasmid DNA from strain T31 digested with *Ava*I, *Nde*I, or the two in combination showed that in each case fragments hybridizing with the ORF1 probe differed in size from those hybridizing with the *vanR* probe. Since Tn*1546* contains no *Nde*I or *Ava*I sites in the region between these two probes, the results suggested an arrangement of the ORF1 or ORF2 region different from that of Tn*1546*. The *vanHAXY* and *vanR* probes both hybridized with a *Hin*dIII fragment of 7.2 kb, the size predicted from the Tn*1546* sequence, suggesting that the *Hin*dIII site of ORF1 was conserved. The *vanR* probe hybridized with *Ava*I fragments of 2.8 and 1.1 kb, *Nde*I-*Ava*I fragments of 2.1 and 1.1 kb, and an *Eco*RI fragment of 3.8

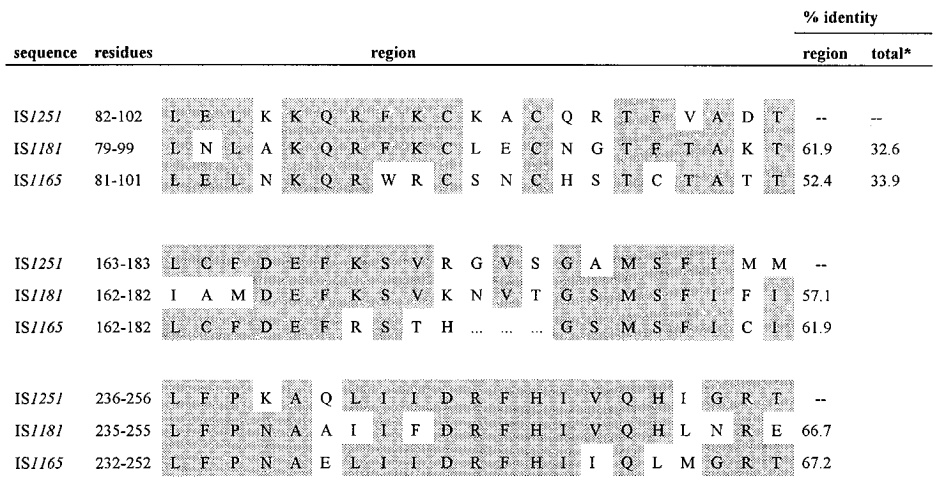

FIG. 3. Sequence similarity among deduced amino acid sequences of putative transposases from IS*1251* of *E. faecium*, IS*1181* of *S. aureus* (11), and IS*1165* of *L. mesenteroides* (21). The PILEUP routine of the University of Wisconsin Genetics Computer Group package was used to align the sequences. Total* refers to amino acid identity over the entire ORF. Alignments are shown for three regions of each ORF. Identical amino acids are indicated by shaded boxes. Dashes represent gaps introduced to optimize similarity.

kb, indicating *Ava*I, *Nde*I, and *Eco*RI sites just beyond the *Hin*dIII site within ORF1. These results suggest an insertion or rearrangement spanning approximately 1 kb or more containing the *Nde*I, *Eco*RI, and *Ava*I sites. The precise extent of the rearrangement could not be determined with the probes used. The *van* cluster on another plasmid in *E. faecalis* TJ153 was markedly truncated, lacking ORF1, ORF2, and *vanR* (TJ153-2 in Fig. 1). Conjugal transfer of the plasmid containing the shorter *van* cluster was not detected. It is unclear whether the shorter cluster confers resistance.

The third strain studied in detail was *E. faecium* GUC. Results of hybridization studies showed the presence of approximately 1.5 kb of additional DNA between *vanS* and *vanH*. This pattern was also present in the single transconjugant obtained by filter mating with *E. faecium* GUC with *E. faecalis* JH2-2 (frequency of approximately 10^{-8} per donor). *E. faecium* GUC also differed from the other isolates tested in that only faint hybridization was seen with the *van* probes in Southern blot experiments with plasmid DNA preparations. Although strain GUC harbored several plasmids, the hybridization signals did not correlate with any visible plasmid DNA bands on ethidium bromide-stained agarose gels (data not shown). This suggested that hybridization may have been due to contamination of plasmid preparations with chromosomal DNA. Accordingly, genomic DNA was isolated from both the wild-type strain and the JH2-2 transconjugant. By using the *vanHAXY* probe, the hybridization signal obtained was markedly increased with genomic DNA compared with that when plasmid-enriched DNAs from both the donor and the transconjugant were used (data not shown), suggesting that the *van* genes may be located on the chromosome or on a plasmid that is difficult to isolate.

Characterization of IS*1251*. To further characterize the *van* cluster of *E. faecium* GUC, the region extending from *vanS* to *vanY* was cloned in two steps. First, the 4.5-kb *Eco*RI fragment hybridizing with the *vanHAXY* probe (fragment L, Fig. 1B) was cloned into LambdaZapII, and the phagemid was excised and sequenced. Sequence analysis revealed *vanH*, *vanA*, and *vanX* genes identical in sequence to those of Tn*1546* (4). However, 206 bp upstream from the *vanH* start codon, the sequence diverged from that of Tn*1546*. The sequence of the remainder of the *vanS-vanH* intergenic region was obtained by PCR amplification of GUC genomic DNA by using one primer from the sequence near the terminus of *vanS* and another from the 5′ region of the cloned 4.5-kb *Eco*RI fragment. A 1.4-kb PCR product (fragment P, Fig. 1B) was obtained and cloned into pBluescript, and the DNA sequence was obtained.

Sequence analysis revealed a 1,496-bp insertion present between *vanS* and *vanH* (Fig. 2) beginning 17 nucleotides beyond the stop codon of *vanS*. An 8-bp direct repeat corresponding to nucleotides 5813 to 5820 of Tn*1546* (4) was present at each end of the new sequence, suggestive of target sequence duplication at the site of the insertion sequence insertion (13). The largest ORF was identified beginning 333 bp upstream from the start of *vanH* on the complementary strand, transcribed in the opposite direction, corresponding to a deduced protein of 428 amino acids. A search of GenBank databases for similar amino acid sequences with the BLAST program (2) yielded the highest scores for the putative transposases of two insertion sequences from gram-positive bacteria: IS*1181* (11) from *Staphylococcus aureus* and IS*1165* (21) from *Leuconostoc mesenteroides*. Alignment of the ORF identified with these sequences with BESTFIT revealed 32.6% identity and 53.1% similarity with the transposase of IS*1181* and 33.9% identity and 54.2% similarity with the transposase of IS*1165* (Fig. 3). This homology, coupled with the 8-bp target sequence duplication and the identification of identical or closely related nucleotide sequences in a number of clinical isolates (see below), suggested that the ORF identified was part of an insertion-like sequence, subsequently designated IS*1251*. The ends of IS*1251* are imperfect inverted repeats of 24 (IR_L) and 23 (IR_R) bp, with 16 bp conserved. These inverted repeats also show homology with those of IS*1181* and IS*1165* (Fig. 4).

As in IS*1165* (21), a perfect prokaryotic promoter −35 consensus region (TTGACA) is present within the right inverted repeat of IS*1251*. Outward-directed −35 regions have been identified in a number of insertion sequences and may form hybrid promoters upon insertion (13). In the 3′ region of the *vanS* gene, a potential consensus −10 region (TACATC) is separated by 16 bp from the −35 sequence of IS*1251* (Fig. 2). However, the functional significance of this is unclear, because *vanS* is transcribed in the opposite direction.

Prevalence of IS*1251*. To determine whether IS*1251* or related elements were present in other clinical isolates of entero-

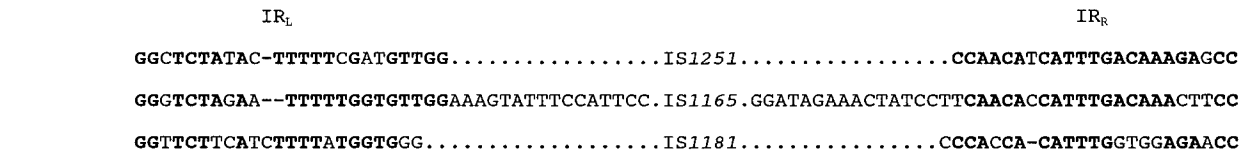

FIG. 4. Sequence similarity among terminal inverted repeats of IS*1251*, IS*1181*, and IS*1165*. Nucleotides identical in at least two sequences are shown in boldface. Dashes represent gaps introduced to optimize similarity.

coccus, an internal 385-bp probe was constructed by digesting the 1.4-kb PCR product (fragment P, Fig. 1B) with *Eco*RI and *Hin*dIII. DNA hybridizations were performed with *Eco*RI-digested and *Hin*dIII-digested genomic DNAs from the 8 clinical isolates listed in Table 1 and from 13 additional clinical isolates obtained from five hospitals in the New York City area (10 of the VanA phenotype, 1 of the VanB phenotype, and 2 vancomycin susceptible). Among the eight original clinical isolates, strains *E. faecium* GUC, Bat, Fair, and Nova showed hybridization with the IS*1251* probe. The sizes of the *Hin*dIII (4.0 kb), *Eco*RI (4.5 kb), and *Xba*I (3.4 kb) restriction fragments hybridizing with the *vanHAXY* probe were identical in strains GUC, Bat, and Fair. These fragment sizes are consistent with a *vanS-vanH* intergenic location for the insertion sequence, with new sites introduced by the insertion of IS*1251*. The possibility that more than one copy of the insertion sequence was present in this location in strains Bat and Fair could not be excluded. In strain Nova, multiple genomic copies of IS*1251* were present, but vancomycin-resistant transconjugants (Nova × JH2-2) showed no hybridization, suggesting that IS*1251* was not present on the plasmid bearing the *van* cluster. Among the 11 additional resistant isolates tested, seven (all of the VanA phenotype) showed hybridization with the IS*1251* probe. Restriction digestion patterns in all seven isolates were consistent with a *vanS-vanH* intergenic location for the insertion sequence, but in four of the strains, additional copies of the insertion sequence were present at sites outside of the *van* cluster. One susceptible isolate also showed hybridization with the IS*1251* probe. Thus, in addition to its location within Tn*1546* in some strains, IS*1251* is commonly present in the genomes of *E. faecium* clinical isolates.

In summary, our results suggest that considerable heterogeneity is present within the *van* cluster of resistant enterococcal isolates from the northeastern United States. In one strain (TJ153), a portion of the ORF1 region (the putative transposase of Tn*1546*) was rearranged. The *van* cluster in this isolate was present on a conjugative plasmid. Since conjugation is likely to be an efficient means of dissemination of the *van* gene cluster, ORF1 and ORF2 might be dispensable in such strains. Although analysis of clinical isolates from France has shown that dissemination of resistance occurs by transposition of Tn*1546* onto different replicons (4), recent evidence suggests that in some locales plasmid transfer is also important in the spread of resistance. For example, 17 isolates of *E. faecium* obtained from six hospitals in the northeastern United States and California showed 10 different chromosomal patterns, but in all strains the *vanA* gene resided on either a 34- or a 60-kb plasmid (9). In a similar study 21 of 23 isolates of *E. faecium* from an outbreak in a London hospital, representing eight different ribotypes, harbored a 24-MDa plasmid that hybridized with the *vanA* probe (28). Our finding that Tn*1546*-like elements were present on plasmids differing in size and restriction patterns indicates that transposition of the *van* cluster also remains an important mode of dissemination of resistance.

A novel insertion-like sequence, IS*1251*, was present within the *van* cluster in 3 of the original isolates tested and in 7 of 10 additional VanA phenotype isolates from a number of hospitals in the New York City area, suggesting that this modified form of the *van* cluster has begun to spread throughout the region. Since it is likely that insertion of IS*1251* into the Tn*1546*-like element was a unique event, the distinctive pattern of the *van* cluster in these isolates may provide an opportunity for epidemiologic study of the spread of this resistance transposon. Alternatively, the *vanS-vanH* region of Tn*1546* may represent a "hot spot" for IS*1251* insertion. The role, if any, of IS*1251* in the dissemination or expression of resistance is unclear. It is tempting to speculate that IS*1251* may facilitate integration of the *van* cluster into the chromosome by homologous recombination. Although the forces involved in the evolution of the *van* cluster are unknown, the present findings demonstrate that in some recent U.S. isolates the *van* cluster differs from that present in European isolates of the late 1980s and may continue to evolve.

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