ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 1995, p. 1772–1778 Vol. 39, No. 8
0066-4804/95/$04.00+0


# Use of Primers Selective for Vancomycin Resistance Genes To Determine *van* Genotype in Enterococci and To Study Gene Organization in VanA Isolates

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Received 23 November 1994/Returned for modification 11 January 1995/Accepted 23 May 1995

**Vancomycin resistance in enterococci is an emerging therapeutic problem. Resistance is not always detected by standard microbiological methods. Oligonucleotide primers for PCR were designed to target amplification of defined regions of genes of the *vanA* cluster, as well as *vanB* and *vanC1*. These primers correctly identified 30 vancomycin-resistant isolates tested (17 VanA, 7 VanB, and 6 *Enterococcus gallinarum*). No amplification was observed with *Enterococcus casseliflavus* or vancomycin-susceptible strains. Using PCR and Southern blotting, we found that all 17 VanA isolates had *orf-1*, *orf-2*, *vanR*, *vanS*, *vanH*, *vanA*, and *vanY* genes in the same sequence and that the intergenic distances in the *vanR-vanA* segments were the same. The described methods should be applicable to the rapid detection of the different vancomycin resistance genotypes in enterococci.**

The glycopeptides vancomycin and teicoplanin constitute an important class of antibiotics for the treatment of severe infections caused by gram-positive bacteria, particularly staphylococci and enterococci. Pediococci, leuconostocs, and some species of lactobacilli are intrinsically resistant to these antibiotics (18, 30). *Erysipelothrix rhusiopathiae* is also generally considered to be intrinsically vancomycin resistant, although teicoplanin MICs for this organism often fall within the susceptible range (14, 18). Vancomycin resistance in enterococci has been increasing over the last several years. Three phenotypic classes (VanA, VanB, and VanC) are defined by the level of resistance to vancomycin and susceptibility or resistance to teicoplanin (15, 33, 35). The genetic and biochemical basis of resistance is best understood for VanA, in which genes carried by Tn*1546* encode transposition functions (*orf-1*, *orf-2*), regulators of the expression of resistance (*vanR*, *vanS*), and enzymes which enable the bacteria to replace D-alanyl-D-alanine with D-alanyl-D-lactate in cell wall peptidoglycan precursor (1, 2, 4). *vanH*, *vanA*, and *vanX* encode enzymes essential for resistance, while *vanY* and *vanZ* behave as accessory proteins, at least in vitro (4). Since the elucidation by Arthur et al. (4) of the structure and function of Tn*1546* in plasmid pIP816 of *Enterococcus faecium* BM4147, *vanA* sequences have been detected by Southern blotting in many other phenotypically VanA isolates (6, 9, 13, 19, 20, 33, 34, 36, 39). The VanA phenotype is defined as high-level resistance to vancomycin and resistance to teicoplanin.

VanB and VanC strains are susceptible to teicoplanin and have, respectively, variable and low-level resistance to vancomycin. Although the genetic and biochemical basis for these phenotypes has yet to be completely elucidated, *vanB* and *vanC* have been sequenced and have been shown to encode ligases which, like the *vanA* ligase of VanA strains, determine the synthesis of altered peptidoglycan precursors (12, 25). Low-level vancomycin resistance is not reliably detected by either commercial susceptibility test systems or the classical disk diffusion method (31, 37).

Because of the limited therapeutic options available for treating vancomycin-resistant enterococci, rapid detection of vancomycin resistance and presumptive determination of teicoplanin susceptibility may be of benefit to some patients, as well as to efforts to control outbreaks in hospitals. Both Southern blotting with probes for the *van* genes (9, 13, 16, 24, 34, 39) and PCR (6) are being applied to the detection and determination of Van phenotypes. Southern blotting requires a series of operations including DNA purification, endonuclease digestion, electrophoresis, blotting, hybridization with labeled probes, and development of the blots. PCR, involving fewer steps, is more rapid and, because of its sensitivity, can more reliably be applied directly to clinical samples as well as to purified DNA.

In designing PCR primers, it is important to establish their specificities and to define the optimal conditions for amplification. Our main goal was to design primers and to define conditions that would reliably correlate the genotypes of vancomycin-resistant enterococci with their phenotypes. In the case of VanA isolates, we were interested in evaluating the potential utility of primers targeting various genes of the *van* cluster, as well as *orf-1* and *orf-2*. Additionally, we used these tools to compare the organization of the *vanA* gene cluster in 17 VanA isolates.

## MATERIALS AND METHODS

**Bacterial strains.** We used 33 vancomycin-resistant isolates of *Enterococcus* spp. isolated between 1986 and 1992 and obtained from various sources (Table 1), including the following well-characterized reference strains: *E. faecium* BM4147 (VanA) (1, 4, 10, 19), *E. faecium* D366 (VanB) (24, 38), and *Enterococcus gallinarum* BM4174 (VanC) (11). *E. faecium* BM4147-1 (a vancomycin-susceptible derivative of BM4147) (19) and another glycopeptide-susceptible strain, *Enterococcus faecalis* BM4110 (7), were used as negative controls. Species identification was confirmed by using the API 32 Strep system (Bio-Mérieux).

MICs were determined by broth microdilution in cation-adjusted Mueller-Hinton broth (Difco) at 35°C (22). Inocula were approximately $5 \times 10^5$ CFU/ml prepared from overnight cultures on brain heart infusion agar (Difco). The antimicrobial agents tested were vancomycin, teicoplanin, penicillin G, ampicillin, gentamicin, and streptomycin. High-level resistance to penicillins, gentamicin, and streptomycin was determined as ≥64, >500, and ≥2,000 μg/ml, respectively (17). Phenotypic glycopeptide resistance was classified as VanA if the teicoplanin MIC was ≥16 μg/ml; VanC if isolates were identified as *E. gallinarum*, *Enterococcus casseliflavus*, or *Enterococcus flavescens*; and VanB for isolates

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TABLE 1. Properties of bacterial strains studied

| Isolate | Van[a] | | MIC (μg/ml) | | Other resistances[b] | Source[c] (reference) |
|---|---|---|---|---|---|---|
| | Phenotype | Genotype | Vancomycin | Teicoplanin | | |
| *E. faecium* | | | | | | |
| NCTC 12203 | A | A | 1,024 | 64 | Am PenG | I (9) |
| NCTC 12204 | A | A | 1,024 | 256 | Am PenG | I (9) |
| BM4147-1 | S | − | 2 | 0.5 | PenG | II (19) |
| BM4147 | A | A | 1,024 | 512 | PenG | II (19) |
| PIS77 | A | A | 1,024 | 512 | Str | III (23) |
| D399 | A | A | 1,024 | 512 | Str | IV (27) |
| L1666 | A | A | 1,024 | 2,048 | Gm Str | V |
| L1683 | A | A | 512 | 128 | Str | VI |
| L1686 | A | A | 512 | 256 | PenG | VI |
| L2215 | A | A | 1,024 | 512 | PenG Str | V |
| L1833 | B | B | 128 | 1 | Am Gm PenG Str | VII |
| L1836 | B | B | 1,024 | 1 | Am Gm PenG | VII |
| L1839 | B | B | 512 | 1 | Am Gm PenG Str | VII |
| D366 | B | B | 64 | 0.5 | PenG Str | IV (38) |
| *E. durans* | | | | | | |
| L1680 | A | A | 256 | 64 | | VI |
| L1681 | A | A | 512 | 128 | | VI |
| *E. faecalis* | | | | | | |
| BM4110 | S | − | 2 | 0.125 | Str | II (7) |
| BM4166 | A | A | 512 | 512 | Str | II (20) |
| NCTC 12201 | A | A | 512 | 256 | Gm Str | I (9) |
| NCTC 12202 | A | A | 512 | 256 | Gm | I (9) |
| A256 | A | A | 512 | 256 | | IV (28) |
| L1841 | A | A | 256 | 256 | | VIII |
| L2212 | A | A | 256 | 128 | Gm | IX |
| V586 | B | B | 64 | 0.25 | Gm | VII (26) |
| L1837 | B | B | 32 | 0.5 | Str | VII |
| V587 | B | B | 64 | 0.5 | Gm | VII (26) |
| *E. gallinarum* | | | | | | |
| L1689 | C | C1 | 4 | 1 | Str | VI |
| L1831 | C | C1 | 4 | 1 | | VII |
| L1832 | C | C1 | 8 | 0.5 | | VII |
| L1834 | C | C1 | 8 | 1 | | VII |
| BM4174 | C | C1 | 8 | 0.5 | Str | II (11) |
| NCDO 2311 | C | C1 | 8 | 1 | | X |
| *E. casseliflavus* | | | | | | |
| L1829 | C | − | 4 | 0.5 | | VII |
| L1834 | C | − | 0.5 | 0.25 | Str | VII |
| L1835 | C | − | 2 | 0.5 | | VII |

[a] Vancomycin resistance phenotype: S, susceptible; A, VanA; B, VanB; C, VanC. Vancomycin resistance genotype: A, *vanA* PCR product and hybridization to *vanA* gene cluster probes; B, *vanB* PCR product and hybridization to *vanB* probe; C1, *vanC1* PCR product and hybridization to *vanC1* probe; −, no PCR product or hybridization.

[b] High-level resistance; see Material and Methods. Abbreviations: Am, ampicillin; Gm, gentamicin; PenG, penicillin G; Str, streptomycin.

[c] Sources of isolates: I, A. Uttley, Public Health Laboratory, Dulwich Hospital, London, United Kingdom; II, P. Courvalin, Institut Pasteur, Paris, France; III, G. C. Schito, Institute of Microbiology, University of Genoa, Genoa, Italy; IV, D. Shlaes, Case Western Reserve University School of Medicine, Cleveland, Ohio; V, B. Pasticci, Institute of Infectious Disease and Microbiology, University of Perugia, Perugia, Italy; VI, P. Van der Auwera, Institut J. Bordet, University of Brussels, Brussels, Belgium; VII, D. F. Sahm, Medical Center, University of Chicago, Chicago, Ill.; VIII, M. Venditti, Institute of Hematology, University "La Sapienza" Rome, Rome, Italy; IX, G. Ravizzola, Institute of Microbiology, University of Brescia, Brescia, Italy; X, Bio-Mérieux, Rome, Italy.

of other species for which the vancomycin MIC was ≥16 μg/ml and the teicoplanin MIC was ≤8 μg/ml.

**DNA preparation.** Bacteria were cultured in 30 ml of brain heart infusion broth (Difco) and were harvested during logarithmic growth (optical density at 590 nm of 0.8). DNA was prepared by using a modification of the initial steps of the method of Crosa and Falkow (8) to recover both chromosomes and large plasmids. The bacterial pellet was suspended in 1.5 ml of 25% sucrose–0.05 M Tris–0.001 M EDTA (pH 8); 0.2 ml of a freshly prepared 40-mg/ml solution of egg white lysozyme (Boehringer Mannheim) in 0.25 M Tris (pH 8) was added. After incubation for 30 min at 37°C, the bacteria were lysed by adding 0.1 ml of 0.25 M EDTA (pH 8) and 0.1 ml of 20% sodium dodecyl sulfate (SDS). NaCl was added to 1 M; this was followed by incubation on ice for 30 min, centrifugation at 30,000 × *g* and 4°C for 30 min, and incubation of the decanted clear lysate with 5 μl of a 5-mg/ml solution of bovine pancreatic RNase (Boehringer Mannheim) for 30 min at 37°C.

**PCR primers.** Oligonucleotides were designed by using OLIGO version 4.0 software (National Bioscience, Hamel, Maine) on the basis of the published nucleotide sequences of *orf-1* and *orf-2* (4), *vanR* and *vanS* (2), *vanH* (5), *vanA* (10), *vanY* (3), *vanB* (12), and *vanC1* (11). *orf-1* and *orf-2* are too large to be amplified in single fragments; therefore, this region was divided into five over-

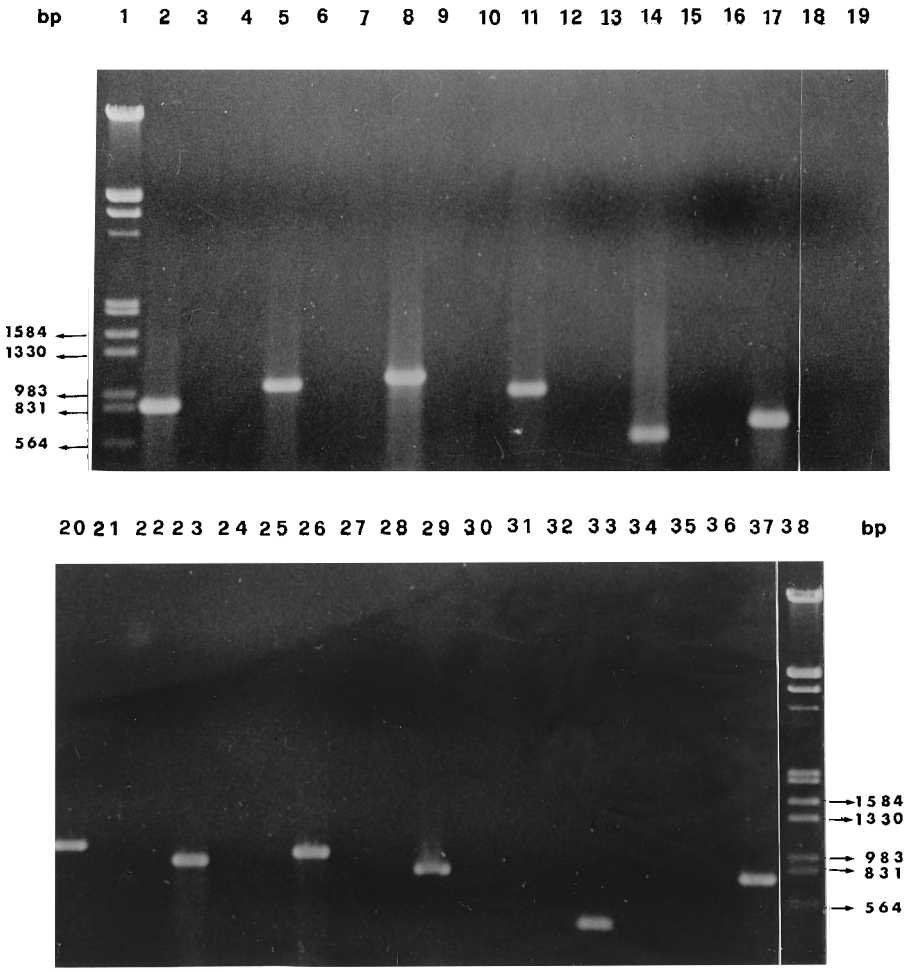


FIG. 1. Specificities of 12 primer pairs for reference strains *E. faecium* BM4147 (VanA), *E. faecium* D366 (VanB), and *E. gallinarum* BM4174 (VanC1). (Primers) Lanes 2 to 4, ORF1A-ORF1A1; lanes 5 to 7, ORF1B-ORF1B1; lanes 8 to 10, ORF1C-ORF1C1; lanes 11 to 13, ORF1D-ORF1D1; lanes 14 to 16, ORF2E-ORF2E1; lanes 17 to 19, VANR-VANR1; lanes 20 to 22, VANS-VANS1; lanes 23 to 25, VANH-VANH1; lanes 26 to 28, VANA-VANA1; lanes 29 to 31, VANY-VANY1; lanes 32 to 34, VANB-VANB1; lanes 35 to 37, VANC-VANC1. (Source of DNA) Lanes 2, 5, 8, 11, 14, 17, 20, 23, 26, 29, 32, and 35, strain BM4147; lanes 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, and 36, strain D366; lanes 4, 7, 10, 13, 16, 19, 22, 25, 28, 31, 34, and 37, strain BM4174. Lanes 1 and 38, *Hin*dIII-*Eco*RI-cleaved bacteriophage λ DNA.

lapping sequences, each of which was amplified with a different pair of primers (see Table 3). The primers were synthesized on an OLIGO 1000 DNA synthesizer (Beckman Instruments, Palo Alto, Calif.), vacuum dried, and stored at −20°C in double-distilled water. The sequences and properties of the primers are listed in Tables 2 and 3. The specificities of the primers for PCR amplification were tested by using the reference strains described above.

**DNA amplification and electrophoresis.** PCR amplifications were carried out on a Gene Amp System 9600 instrument (Perkin-Elmer Cetus, Norwalk, Conn.) in 0.2-ml New Micro Amp reaction tubes by using the Gene Amp DNA amplification kit containing *Thermus acquaticus* DNA polymerase (Ampli Taq; Perkin-Elmer). Amplifications were performed in 10 mM Tris-HCl (pH 8.3)–50 mM KCl–2 mM $MgCl_2$–0.15 mM (each) deoxynucleoside triphosphate–1.2 U of Ampli Taq. The final reaction volume of 0.05 ml contained 50 pmol of each primer and 500 ng of enterococcal DNA. The PCR program consisted of an initial denaturation step at 94°C for 3 min; this was followed by 40 cycles of DNA denaturation at 94°C for 30 s, primer annealing at the appropriate temperature for each set of primers (see Tables 2 and 3) for 2 min, and DNA extension at 72°C for 2 min. After the last cycle, the reaction was terminated by incubation at 72°C for 6 min, and the products were stored at 4°C. The PCR products (5 μl) were analyzed by electrophoresis in TAE buffer (0.04 M Tris-acetate, 0.002 M EDTA [pH 8.5]) on 1% (wt/vol) agarose gels, and the gels were stained with ethidium bromide. *Eco*RI-*Hin*dIII fragments of bacteriophage λ DNA (Boehringer-Mannheim) were used as molecular mass standards. The PCR products were identified by at least one of the following methods: estimation of the size by gel electrophoresis (21), analysis with a suitable restriction enzyme (Boehringer Mannheim), and hybridization with specific probes. Restriction endonuclease digestions were performed as specified by the supplier of the enzyme.

**Probe generation.** Probes for the *vanA* gene cluster, *orf-1*, *orf-2*, *vanB*, and *vanC1*, were generated by PCR amplification of DNA from the reference strains by using the appropriate primers (see Tables 2 and 3). The PCR products were resolved by electrophoresis at 70 V for 3 h on 1% agarose gels and were stained with ethidium bromide. The desired portions of the gels were cut out and the DNA was extracted with a Geneclean II kit (Bio 101, Inc., La Jolla, Calif.). The probes were labeled with [α-$^{32}$P]dATP by using a random priming labeling kit (Boehringer Mannheim).

**Southern blotting and hybridization.** DNA digests or PCR products were resolved by electrophoresis on 0.8% agarose and were transferred to Hybond-N membranes (Amersham, Buckinghamshire, United Kingdom) as described by Smith and Summers (29). Prehybridization was overnight at 65°C in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt's solution–0.5% SDS–0.1 mg of salmon sperm DNA per ml. Hybridization was in the same buffer overnight at 65°C. The membranes were washed twice for 10 min at room temperature with 2× SSC–0.1% SDS and once for 40 min at 65°C with 0.2× SSC–0.1% SDS. Membranes containing PCR products were washed once more at 65°C with 0.1× SSC–0.1% SDS. The wet membranes were exposed to hyperfilm-β max (Amersham) for various periods of time at −80°C.

## RESULTS

**Susceptibility patterns of isolates.** The MICs of vancomycin and teicoplanin and the susceptibilities of the test strains to the other antibiotics tested are given in Table 1. For the 17 VanA

TABLE 2. Properties and nucleotide sequences of PCR primers for *vanA* cluster genes (*vanRSHAY*), *vanB*, and *vanC*

| Primer pair | Target gene (function) | Nucleotide sequences | Position[a] | Reference | $T_a$ (°C)[b] | Size of PCR product (bp) |
|---|---|---|---|---|---|---|
| VANR | *vanR* (regulation) | 5′-AGCGATAAAATACTTATTGTGGA | 4–26 | 2 | 62 | 645 |
| VANR1 | *vanR* (regulation) | 5′-CGGATTATCAATGGTGTCGTT | 648–627 | | 62 | |
| VANS | *vanS* (regulation) | 5′-AACGACTATTCCAAACTAGAAC | 28–49 | 2 | 60 | 1,094 |
| VANS1 | *vanS* (regulation) | 5′-GCTGGAAGCTCTACCCTAAA | 1121–1102 | | 60 | |
| VANH | *vanH* (dehydrogenase) | 5′-ATCGGCATTACTGTTTATGGAT | 10–31 | 5 | 60 | 943 |
| VANH1 | *vanH* (dehydrogenase) | 5′-TCCTTTCAAAATCCAAACAGTTT | 952–930 | | 60 | |
| VANA | *vanA* (ligase) | 5′-ATGAATAGAATAAAAGTTGCAATAC | 1–25 | 10 | 62 | 1,029 |
| VANA1 | *vanA* (ligase) | 5′-CCCCTTTAACGCTAATACGAT | 1029–1009 | | 62 | |
| VANY | *vanY* (carboxypeptidase) | 5′-ACTTAGGTTATGACTACGTTAAT | 44–66 | 3 | 55 | 866 |
| VANY1 | *vanY* (carboxypeptidase) | 5′-CCTCCTTGAATTAGTATGTGTT | 909–888 | | 55 | |
| VANB | *vanB* (ligase) | 5′-CCCGAATTTCAAATGATTGAAAA | 440–462 | 12 | 59 | 457 |
| VANB1 | *vanB* (ligase) | 5′-CGCCATCCTCCTGCAAAA | 896–879 | | 59 | |
| VANC | *vanC1* (ligase) | 5′-GCTGAAATATGAAGTAATGACCA | 93–115 | 11 | 58 | 811 |
| VANC1 | *vanC1* (ligase) | 5′-CGGCATGGTGTTGATTTCGTT | 904–924 | | 58 | |

[a] The positions given are from the first base of the coding sequence (see the references cited).
[b] $T_a$, annealing temperature.

isolates, the MICs of vancomycin and teicoplanin were ≥256 and ≥64 μg/ml, respectively. Vancomycin MICs ranged from 32 to 1,024 μg/ml for the seven VanB isolates, from 4 to 8 μg/ml for six VanC1 isolates, and from 0.5 to 4 μg/ml for three *E. casseliflavus* isolates; teicoplanin MICs were ≤1 μg/ml for all of these strains. High-level resistance to aminoglycosides was common among the VanA and VanB isolates, and three of the VanC strains were highly resistant to streptomycin. High-level resistance to penicillins was observed only among VanA and VanB *E. faecium* isolates.

**Specificities of the primers.** The specificity of each primer pair was tested by PCR by using 500 ng of DNA from the VanA, VanB, and VanC1 reference strains as templates in different reactions. DNA from BM4147 (VanA) was amplified by primers for *vanR*, *vanS*, *vanH*, *vanA*, *vanY*, *orf-1*, and *orf-2* (yielding in each case a single product of the expected size), but not by the VANB-VANB1 or VANC-VANC1 primer pairs (Fig. 1; Tables 2 and 3). DNA from D366 (VanB) was amplified only with the VANB primer pair, and DNA from BM4174 (VanC-1) was amplified only with the VANC primer pair; both products were of the expected sizes (Fig. 1; Table 2).

When the PCR products were labeled with $^{32}$P and used to probe DNA digests from the appropriate strain, they all hybridized with restriction fragments of the correct size. Presumptive *vanR*, *vanS*, *vanH*, *vanA*, and *vanY* products identified a 6.2-kb *Bgl*II fragment (4) of BM4147 DNA; the *orf-2* product recognized the same fragment plus a larger fragment which also hybridized to the *orf-1* product (data not shown). The product obtained with the VANB-VANB1 primer pair identified the expected 7.5-kb band (24) in a *Hin*dIII-*Kpn*I digest of D366 DNA, and the product of VANC-VANC1 priming identified the 690-bp internal fragment of *vanC1* (11) in an *Eco*RI-*Hin*cII digest of DNA from *E. gallinarum* BM4174.

**Determination of vancomycin resistance genotypes by PCR.** All of the primer pairs targeting the *vanA* gene cluster, *orf-1*, and *orf-2* yielded products of the appropriate sizes (Tables 2

TABLE 3. Primers for the amplification of transposase (*orf-1*) and resolvase (*orf-2*) genes of Tn*1546*

| Primer pair | Target gene (function) | Nucleotide sequences | Position[a] | $T_a$ (°C)[b] | Size of PCR products (bp) |
|---|---|---|---|---|---|
| ORF1A | *orf-1* (transposase) | 5′-AGGGCGACATATGGTGTAACA | 170–190 | 58 | 844 |
| ORF1A1 | *orf-1* (transposase) | 5′-GGGCGACGGTACAACATCTT | 1014–994 | 58 | |
| ORF1B | *orf-1* (transposase) | 5′-TGGTGGCTCCTTTTCCCAGTTC | 907–928 | 60 | 1,007 |
| ORF1B1 | *orf-1* (transposase) | 5′-CGTCCTGCCGACTATGATTATTT | 1913–1891 | 60 | |
| ORF1C | *orf-1* (transposase) | 5′-ACCGTTTTTGCAGTAAGTCTAAAT | 1871–1894 | 60 | 1,066 |
| ORF1C1 | *orf-1* (transposase) | 5′-AAACGGGATTTAGAAATAGTTAAT | 2936–2913 | 60 | |
| ORF1D | *orf-2* (resolvase) | 5′-CCATTTCTGTATTTTCAATTTATTA | 3053–3077 | 58 | 925 |
| ORF2D1 | *orf-2* (resolvase) | 5′-CATAGTTATCACCCTTTCACATA | 3978–3956 | 58 | |
| ORF2E | *orf-2* (resolvase) | 5′-TTGCGGAAAATCGGTTATATTC | 3187–3208 | 56 | 540 |
| ORF2E1 | *orf-2* (resolvase) | 5′-AGCCCTAGATACATTAGTAATT | 3726–3705 | 56 | |

[a] The positions given are from the first base of the published sequence (4).
[b] $T_a$, annealing temperature.

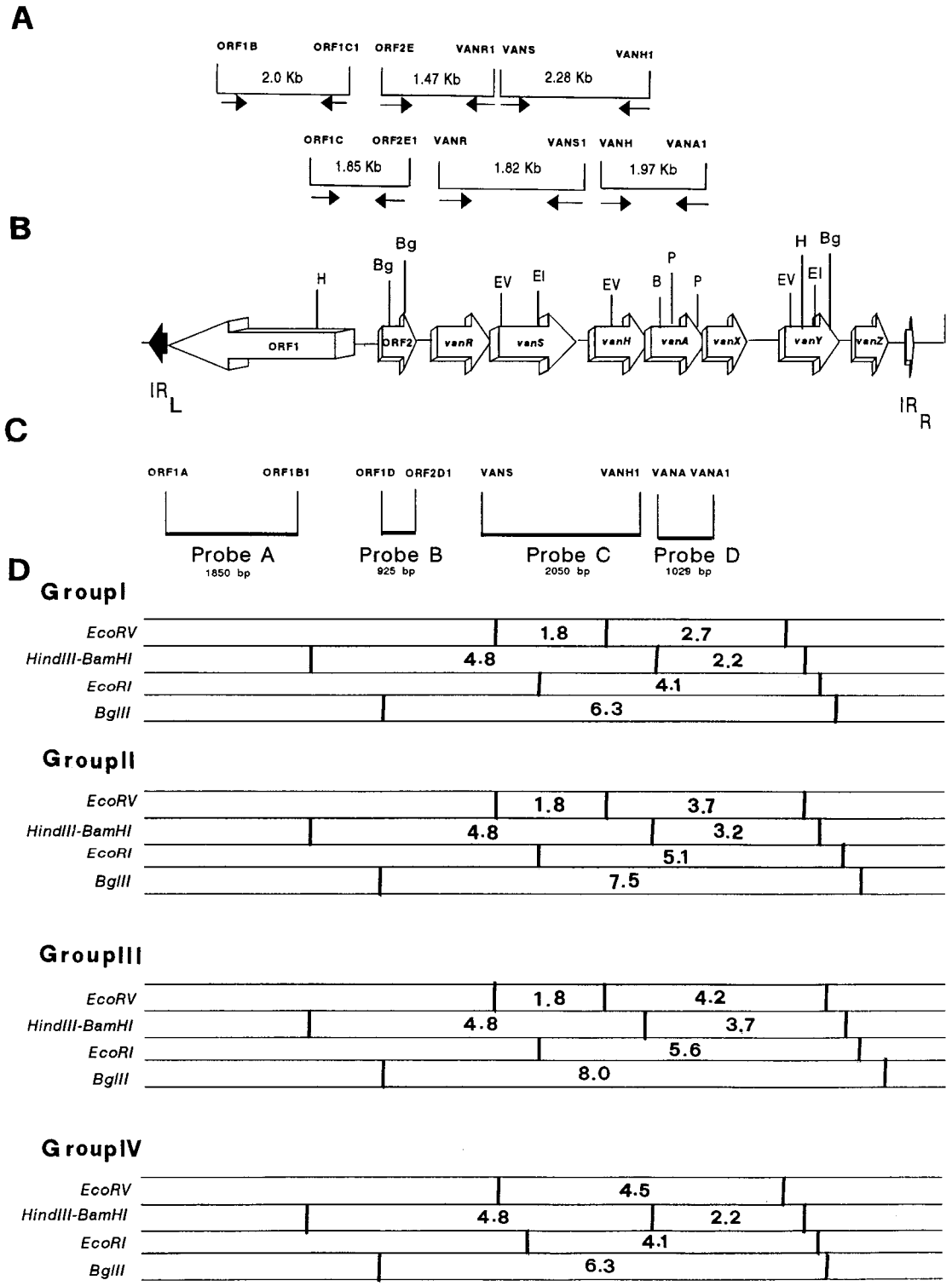


FIG. 2. Characterization of Tn*1546*-related elements. (A) Amplification of overlapping fragments of Tn*1546*. Horizontal bars delimit the PCR products. The arrows show the position of the primers and the direction of DNA synthesis. (B) Map of Tn*1546* and flanking regions in pIP816 (4). Restriction sites: B, *Bam*HI; Bg, *Bgl*II; EI, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; P, *Pst*I. (C) Fragments used as probes obtained by PCR amplification from DNA of BM4147. (D) Restriction fragments detected by Southern blot hybridization in different groups of strains: group I, 10 strains (BM4147, D399, PIS77, L1666, L1686, L1841, L2212, BM4166, NCTC 12201, A256); group II, 3 strains (NCTC 12202, NCTC 12203, NTCC 12204); group III, 1 strain (L2215); group IV, 3 strains (L1680, L1681, L1683). The sizes of the restriction fragments are given in kilobases.

and 3) when DNA from each of 17 phenotypically VanA isolates was used as the template; no products were obtained when DNAs from phenotypically VanB or VanC isolates were used (data not shown). Similarly, the VANB-VANB1 and VANC-VANC1 primer pairs identified the seven phenotypically VanB and the six *E. gallinarum* isolates, respectively, but no others. None of the primers was productive when template DNA was from vancomycin-susceptible or *E. casseliflavus* isolates (data not shown).

The PCR products were tested by Southern blot hybridization with radioactive probes for the *vanA* gene cluster, *orf-1*, *orf-2*, *vanB*, and *vanC* generated from the reference strains as described in Materials and Methods. Under highly stringent conditions, each probe hybridized to PCR products from isolates of the same phenotypic class but to no others (data not shown). In addition, the PCR products from the different isolates were digested with various restriction endonucleases and were analyzed by agarose gel electrophoresis. With one exception, restriction fragment length polymorphism was not detected, indicating conservation of restriction sites, including the *Hin*dIII site in *orf-1*, the two *Bgl*II sites in *orf-2*, the *Hin*cII site in *vanR*, the *Eco*RI and *Eco*RV sites in *vanS*, the *Bam*HI and *Pst*I sites in *vanA*, the *Eco*RV, *Hin*dIII, and *Eco*RI sites in *vanY*, and the *Eco*RI and *Hin*cII sites in *vanC1*. The exception was an *Eco*RV site which was not detected in *vanH* amplified from strains L1680, L1681, and L1683, although it was present in the products obtained from the other VanA isolates (data not shown).

By using the probes described above and highly stringent conditions, Southern blotting also correctly identified VanA, VanB, and VanC isolates.

**Organization of the VanA gene cluster.** Figure 2B shows the organization of the genes of the *vanA* cluster, *orf-1*, and *orf-2* on Tn*1546* as determined by Arthur et al. (4) in *E. faecium* BM4147. Using primer pairs that covered overlapping portions of the *orf-1–vanA* region (Fig. 2A), we amplified the DNAs of the other 16 VanA isolates in our collection. We obtained products of the expected size that comigrated with the corresponding products obtained from BM4147 when we used the following primer pairs: ORF1B-ORF1C1, ORF1C-ORF2E1, ORF2E-VANR1, VANR-VANS1, VANS-VANH1, and VANH-VANA1 (data not shown). Therefore, these fragments are conserved among the 16 isolates, as is the gene order *orf-1*, *orf-2*, *vanR*, *vanS*, *vanH*, and *vanA*, as demonstrated previously for other VanA isolates (4). We were unable to amplify a segment from *vanA* to *vanY*; this difficulty was previously encountered by Arthur et al. (4).

Using Southern hybridization of probes B, C, and D (Fig. 2C) to DNA digested with four enzymes or combinations of enzymes, we classified the VanA isolates into four groups (Fig. 2D). Nine isolates had the same restriction fragment profile as the reference strain BM4147 (group I). Group II (three isolates) and III (one isolate) isolates differ from one another and from group I isolates in the sizes of the restriction fragments spanning the *vanA-vanY* region (Fig. 2D). These data are consistent with a heterogeneity among VanA isolates in the length of the *vanX-vanY* intergenic region, as previously observed by Arthur et al. (4). Group IV isolates (three isolates) had a profile similar to that of group I isolates except with *Eco*RV. In group IV isolates, the region from *vanS* to *vanY* was on a single 4.5-kb *Eco*RV fragment; this is consistent with the fact that the *vanH* PCR products generated from these isolates were not cut by *Eco*RV.

Experiments with probe A (Fig. 2C) showed considerable restriction fragment length heterogeneity among VanA isolates, indicating a high degree of variability in the positions of the nearest restriction sites in DNA external to the transposon (data not shown). However, the three isolates of group IV, one *E. faecium* and two *Enterococcus durans* isolates, which all came from the Institut Jules Bordet, Brussels, Belgium, showed the same restriction pattern in this region, suggesting that transfer of a single plasmid had occurred among these isolates.

## DISCUSSION

Nucleic acid amplification by PCR has applications in many fields of biology and medicine, including the detection of agents of infection (32). The application of this technique to the rapid detection of resistance should permit more appropriate initial treatment of patients and should aid in controlling the spread of resistant organisms within hospitals.

Using the primers described here, we found complete coincidence between genotype and phenotype for 30 vancomycin-resistant enterococci (17 VanA, 7 VanB, and 6 VanC *E. gallinarum* isolates) from nine different sources. Because of the high degree of homology (74%) between *vanB* and *vanA*, it is important that primers be based on divergent portions of these genes. In initial experiments, we found that some primers based on *vanB* sequences also amplified the DNAs of some VanA isolates (including the reference strain BM4147), yielding fragments of the same size obtained from VanB DNA templates; however, the products obtained from VanA DNA hybridized more strongly, under stringent conditions, to the *vanA* probe and more weakly to the *vanB* probe. This sort of phenomenon may explain some of the discrepant results reported in the literature for *vanA* and *vanB* probes (6, 13, 36). The primers for the other genes of the *vanA* cluster and for *orf-1* and *orf-2* did not cross-react with VanB, VanC, or susceptible isolates. This suggests that as sequences of additional VanB- and VanC-specific genes become available, primers for genes other than the ligases may prove to be useful in PCR identification of vancomycin resistance phenotypes.

The results of our analysis of 16 VanA isolates confirm previous observations of Arthur et al. (4) as to the heterogeneity of the position of insertion of the transposon and therefore support their conclusion that the international spread of VanA is due to dissemination of the transposon rather than of a plasmid or a bacterial clone.

The work described here was aimed at demonstrating the specificity of the PCR method in comparing the genetic organizations of VanA isolates from different sources. We therefore used purified DNA prepared from broth cultures. However, preliminary experiments indicate that the primers and the methodology used will be applicable to crude lysates obtained by boiling suspensions of single colonies.

## ACKNOWLEDGMENTS

We thank P. Courvalin, B. Pasticci, G. Ravizzola, D. F. Sahm, G. C. Schito, D. Shlaes, A. Uttley, P. Van der Auwera, and M. Venditti for kindly providing the isolates used in the study. We also thank E. Bossi for assistance with primer synthesis, R. Williams, K. Islam, and A. Lupinetti for critical reading of the manuscript, and G. Pozzi for helpful discussions.

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