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# VanD-Type Vancomycin-Resistant *Enterococcus faecium* 10/96A

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**VanD type *Enterococcus faecium* 10/96A is constitutively resistant to vancomycin and to low levels of teicoplanin by nearly exclusive synthesis of peptidoglycan precursors terminating in D-alanyl–D-lactate (L. M. Dalla Costa, P. E. Reynolds, H. A. Souza, D. C. Souza, M. F. Palepou, and N. Woodford, Antimicrob. Agents Chemother. 44:3444-3446, 2000). A $G_{184}S$ mutation adjacent to the serine involved in the binding of D-Ala1 in the D-alanine:D-alanine ligase (Ddl) led to production of an impaired Ddl and accounts for the lack of D-alanyl–D-alanine-containing peptidoglycan precursors. The sequence of the *vanD* gene cluster revealed eight open reading frames. The organization of this operon, assigned to a chromosomal location, was similar to those in other VanD type strains. The distal part encoded the $VanH_D$ dehydrogenase, the VanD ligase, and the $VanX_D$ dipeptidase, which were homologous to the corresponding proteins in VanD-type strains. Upstream from the structural genes for these proteins was the $vanY_D$ gene; a frameshift mutation in this gene resulted in premature termination of the encoded protein and accounted for the lack of penicillin-susceptible D,D-carboxypeptidase activity. Analysis of the translated sequence downstream from the stop codon, but in a different reading frame because of the frameshift mutation, indicated homology with penicillin binding proteins (PBPs) with a high degree of identity with $VanY_D$ from VanD-type strains. The 5′ end of the gene cluster contained the $vanR_D$-$vanS_D$ genes for a putative two-component regulatory system. Insertion of IS*Efa4* in the $vanS_D$ gene led to constitutive expression of vancomycin resistance. This new insertion belonged to the IS*605* family and was composed of two open reading frames encoding putative transposases of two unrelated insertion sequence elements, IS*200* and IS*1341*.**

In susceptible bacteria, the glycopeptide antibiotics vancomycin and teicoplanin form a complex with the D-alanyl–D-alanine (D-Ala–D-Ala) terminus of peptidoglycan precursors at the cell surface, leading to inhibition of transglycosylation and transpeptidation reactions in the cell wall layer (47). Three types, VanA, VanB, and VanD, of acquired resistance to glycopeptides by production of peptidoglycan precursors ending in the depsipeptide D-Ala–D-lactate (D-Ala–D-Lac) instead of the dipeptide D-Ala–D-Ala have been characterized in enterococci (13, 44, 54). Substitution of the C-terminal D-Ala residue by D-Lac eliminates a hydrogen bond critical for binding antibiotics and leads to a ca. 1,000-fold reduction in the affinity of vancomycin for peptidoglycan precursors (10, 19).

The general organization of the *vanD* operon is similar to those present in VanA- and VanB-type strains (12, 21, 24). Three proteins are required for glycopeptide resistance: a dehydrogenase (VanH, $VanH_B$, or $VanH_D$) to reduce pyruvate to D-Lac, a ligase (VanA, VanB, or VanD) to synthesize the depsipeptide D-Ala–D-Lac, and a D,D-dipeptidase (VanX, $VanX_B$, or $VanX_D$) to hydrolyze the D-Ala–D-Ala dipeptide synthesized by the host D-Ala:D-Ala Ddl ligase and thereby limit synthesis of precursors containing the target for glycopeptides (3, 13, 20, 49). In VanA- and VanB-type strains, a penicillin-insensitive and $Zn^{2+}$-dependent D,D-carboxypeptidase (VanY and $VanY_B$) contributes to vancomycin resistance by hydrolyzing the C-terminal D-Ala residue of late peptidoglycan precursors, when elimination of D-Ala–D-Ala by VanX is incomplete (3, 8, 9). Certain PBPs which function as D,D-carboxypeptidases preferentially cleave depsipeptide substrates (46), whereas the $Zn^{2+}$-dependent VanY D,D-carboxypeptidase exhibits a higher catalytic efficiency for hydrolysis of substrates ending in D-Ala–D-Ala (3). The $VanY_D$ D,D-carboxypeptidase is distinct from VanY and $VanY_B$ since it displays substantial identity with some penicillin-binding proteins (17, 21). These catalytic-serine D,D-carboxypeptidases are susceptible to benzylpenicillin (48). VanZ, which confers teicoplanin resistance by an unknown mechanism, and VanW, with an unknown function, encoded by the *vanA* and *vanB* clusters, respectively, do not have counterparts in the *vanD* cluster (6, 24).

Synthesis of the resistance proteins is regulated at the transcriptional level by two-component regulatory systems (VanR-VanS and $VanR_B$-$VanS_B$) (11, 24). VanS is a putative membrane-associated sensor that controls the level of phosphorylation of VanR (55). Phosphorylation of the VanR and $VanR_B$ response regulators enhances the affinity of the proteins for the regulatory regions of the $P_R$, $P_{RB}$ and $P_H$, $P_{YB}$ promoters, and allows transcription of the regulatory (*vanRS* and $vanR_BS_B$) and resistance (*vanHAX* and $vanH_BBX_B$) genes, respectively (4, 5, 24, 28, 30). The VanR-VanS system activates the $P_H$ promoter for cotranscription of the *vanH*, *vanA*, and *vanX* genes in response to the presence of vancomycin or teicoplanin in the culture medium (8, 11). In contrast, the $VanR_B$-$VanS_B$ system mediates activation of the $P_{YB}$ promoter only in the presence of vancomycin, and lack of induction by teicoplanin accounts for susceptibility of VanB-type strains to

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TABLE 1. Strains and plasmids used in this study

| Strain or plasmid | Relevant properties | Reference or source |
|---|---|---|
| Strains | | |
| *E. coli* Top10 | F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*) *7697 galU galK rpsL endA1 nupG* | Invitrogen |
| *E. faecalis* JH2-2 | Fus$^r$ Rif$^r$ | 32 |
| *E. faecium* | | |
| 10/96A | Vm$^r$ Te$^r$ (VanD type) | 23 |
| BM4416 (N97-330) | Vm$^r$ Te$^r$ (VanD type) | 17, 43 |
| BM4339 | Vm$^r$ Te$^r$ (VanD type) | 45 |
| BM4409 | BM4339/pAT662 ($P_2$*ddlcat*) containing *ddl* gene of BM4147 | 21 |
| BM4512 | BM4339/pAT640 ($P_2$*ddl*$_{G184S}$*cat*) containing *ddl* gene of 10/96A | This study |
| Plasmids | | |
| pCR-Blunt | Km$^r$, Zeocin$^r$, *oriR* from *colE1*, *lacZ*α, *ccdB* | Invitrogen |
| pAT79 | *oriR* from pAMβ1, *oriR* from pUC, *oriT* from RK2, Sp$^r$ *lacZ*α $P_2$*cat* | 11 |
| pAT637 | 632-bp PCR fragment (*vanD′ vanX*$_D$) of 10/96A cloned into pCR-Blunt | This study |
| pAT638 | 1,060-bp PCR fragment (*vanS*$_D$*′ vanY*$_D$*′*) of 10/96A cloned into pCR-Blunt | This study |
| pAT639 | 3,725-bp PCR fragment (*vanR*$_D$ *S*$_D$*′* ORFA ORFB *vanS*$_D$*′ vanY*$_D$*′*) of 10/96A cloned into pCR-Blunt | This study |
| pAT640 | 1,135-bp *Sac*I-*Xba*I PCR fragment (*ddl*$_{G184S}$) of 10/96A cloned into pAT79 | This study |
| pAT635 | 672-bp *Sac*I-*Xba*I PCR fragment (*vanX*$_D$) of BM4339 cloned into pAT79 | This study |
| pAT633 | 1,153-bp *Sac*I-*Xba*I PCR fragment (*vanY*$_D$) of BM4339 cloned into pAT79 | This study |
| pAT636 | 672-bp *Sac*I-*Xba*I PCR fragment (*vanX*$_D$) of 10/96A cloned into pAT79 | This study |
| pAT634 | 1,153-bp *Sac*I-*Xba*I PCR fragment (*vanY*$_D$) of 10/96A cloned into pAT79 | This study |

this antibiotic (8, 24). Low-level resistance to vancomycin in VanB strains results from a limited capacity to synthesize D-Ala–D-Lac and to hydrolyze D-Ala–D-Ala, leading to coproduction of D-Ala and D-Lac-ending peptidoglycan precursors (8).

Four VanD-type strains of *Enterococcus faecium* have been reported so far, and clinical isolates BM4339 and BM4416 (also designated N97-330) have been extensively studied (17, 22, 39, 43, 45). These two VanD-type strains are characterized by constitutively expressed resistance to moderate levels of vancomycin (MIC, 16 to 256 μg/ml) and teicoplanin (MIC, 2 to 64 μg/ml) despite the presence of the *vanR*$_D$ and *vanS*$_D$ genes expressed from the $P_{RD}$ promoter (22, 43, 45).

Strain 10/96A is resistant to vancomycin (MIC, 256 μg/ml) and to low levels of teicoplanin (MIC, 4 μg/ml) (23). A PCR product, obtained using the D-Ala:D-X ligase universal degenerate primers V1 and V2, was sequenced and revealed 83 to 85% identity with structural genes for VanD ligases (17, 21, 39). The *vanD* cluster of strain 10/96A was sequenced partially and found to contain two open reading frames (ORFs) encoding a dehydrogenase, VanH$_D$, and a D-Ala:D-Lac ligase, VanD (23). The operon, which is not transferable, confers resistance by constitutive synthesis of peptidoglycan precursors ending in D-Ala–D-Lac, which represent the main components of cell wall cytoplasmic precursors. In contrast to the VanY$_D$ activities in strains BM4339 and BM4416, the VanY$_D$ D,D-carboxypeptidase activity in membrane fractions of strain 10/96A was not inhibited by penicillin G (23, 43, 45).

We report the organization of the *vanD* gene cluster in *E. faecium* 10/96A and the regulation of expression of the resistance genes. We also show that a single mutation in the chromosomal *ddl* gene accounts for the lack of precursors terminating in D-Ala–D-Ala in this strain and that an insertion sequence in the *vanS*$_D$ gene is likely to be responsible for constitutive expression of resistance.

## MATERIALS AND METHODS

**Strains, plasmids, and growth conditions.** The origin and characteristics of the bacterial strains and plasmids used in this study are listed in Table 1. *E. faecium* 10/96A was isolated in 1996 from the blood of a 9-year-old girl with aplastic anemia (23). *Escherichia coli* Top10 (Invitrogen, Groningen, The Netherlands) was used as a host for recombinant plasmids. *Enterococcus faecalis* JH2-2 is a derivative of strain JH2 that is resistant to fusidic acid and rifampin (31). Kanamycin (50 μg/ml) was used as a selective agent for cloning PCR products into the pCR-Blunt vector (Invitrogen). Spectinomycin (60 μg/ml) was added to the medium to prevent the loss of plasmids derived from pAT79 (11). Strains were grown in brain heart infusion (BHI) broth or agar (Difco Laboratories, Detroit, Mich.) at 37°C. The MICs of glycopeptides were determined by the method of Steers et al., with $10^5$ CFU per spot on BHI agar after 24 h of incubation at 37°C (52).

**Recombinant DNA techniques.** Plasmid DNA isolation, digestion with restriction endonucleases (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, England and Gibco BRL-life Technologies Inc.), amplification of DNA by PCR with *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.), ligation of DNA fragments with T4 DNA ligase (Amersham Pharmacia Biotech), and transformation of *E. coli* Top10 with recombinant plasmid DNA were performed by standard methods (14). Total DNA from enterococci was prepared according to the method of Le Bouguénec et al. (33).

**Plasmid construction.** The plasmids were constructed as follows (Fig. 1).

**(i) Plasmid pAT637.** To amplify the *vanX*$_D$ gene of 10/96A, the specific primer D4$_{COOH}$ (5′ ACTTTCACTTAGGAGGTAAC), complementary to a previously sequenced portion of the 3′ end of the *vanD* gene from this strain, was used in combination with specific primer XD1 (5′ CTAGGCAATGCAAAAATC), designed from comparative analysis of sequences downstream from the *vanX*$_D$ genes of *E. faecium* BM4339 and BM4416 (17, 21). The PCR product, with the expected size of 632 bp, obtained from 10/96A total DNA as a template, was cloned into pCR-Blunt, leading to plasmid pAT637.

**(ii) Plasmid pAT638.** A strategy similar to that used for construction of pAT637 was followed to clone the *vanY*$_D$ gene of strain 10/96A. A sequence deduced from the alignment of the 3′ end of the *vanS*$_D$ genes of BM4339 and BM4416 was used to design primer SD1 (5′ GTTTTGAGGTTACATTGC). YD4 (5′ GGTAATAGGGACTGTTCGGAT) contained 21 bases complementary to the sequence of the 3′ end of the *vanY*$_D$ gene from strain 10/96A. These primers, used with total DNA from 10/96A as a template, yielded a product with the expected size of 1,060 bp that was cloned into pCR-Blunt generating plasmid pAT638.

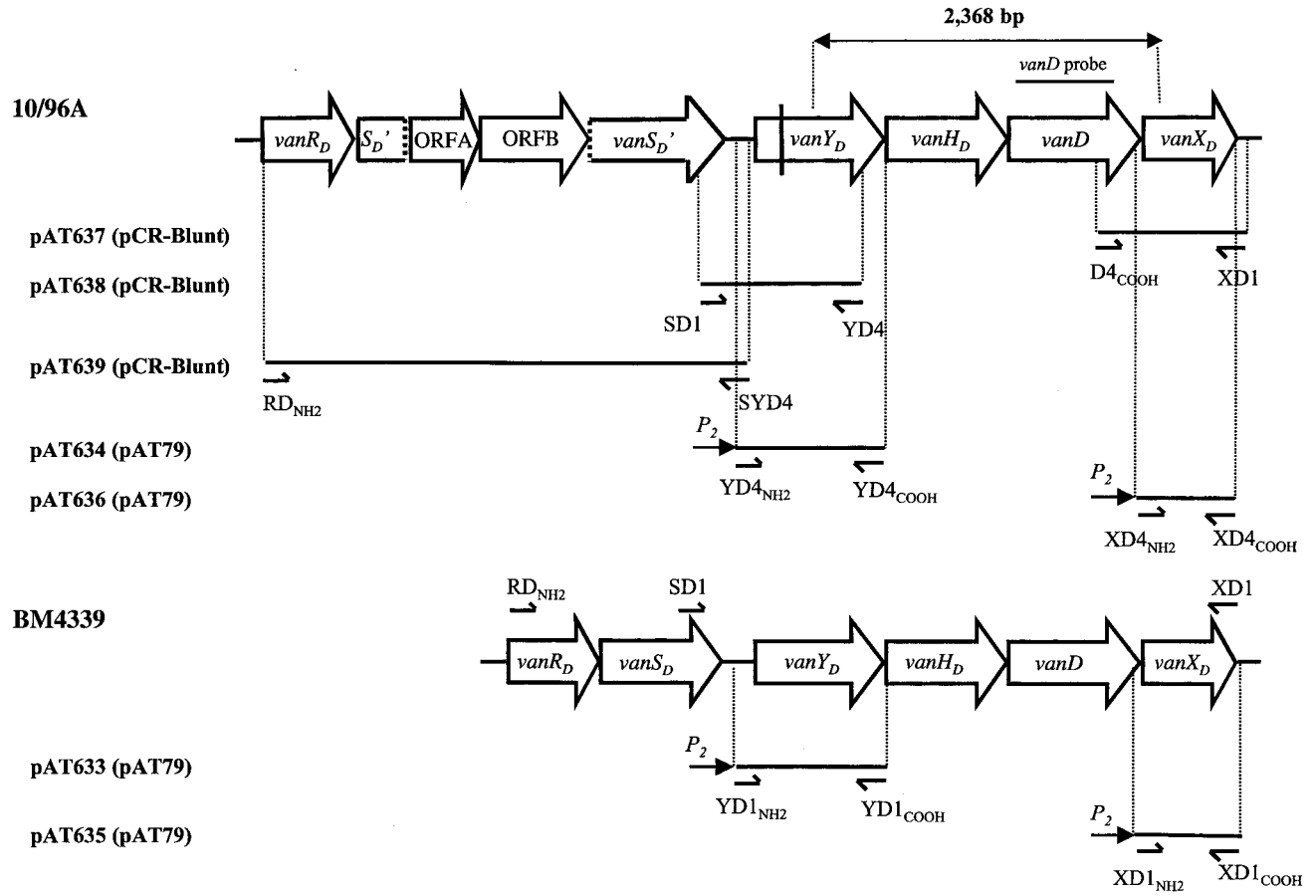


FIG. 1. Schematic representation of *vanD* gene clusters and of recombinant plasmids. Open arrows represent coding sequences and indicate the direction of transcription. The double-headed arrow indicates the 2,368-bp PCR product previously sequenced (23). The PCR fragment internal to the *vanD* gene of strain 10/96A used as a probe in hybridization experiments is indicated above the corresponding region. Vertical dashed lines indicate separation of the $vanS_D$ gene of 10/96A into two parts $S_D'$ and $vanS_D'$. The vertical bar in the $vanY_D$ gene of 10/96A indicates the position of the frameshift mutation leading to a predicted truncated protein. The inserts in recombinant plasmids are represented by solid lines, and the vectors are indicated in parentheses. Arrowheads represent binding sites and orientation of oligodeoxynucleotides used for amplification.

**(iii) Plasmid pAT639.** To complete the sequence of the *vanD* operon of strain 10/96A, the portion upstream from $vanY_D$ was amplified using primers $RD_{NH2}$ (5′ ATGAATGAAAAAATCTTAGTGG) and SYD4 (5′ TTACGATTTTCCT ACGG) and total DNA as a template. Alignment of the $vanR_D$ genes from BM4339 and BM4416 was used to design the $RD_{NH2}$ primer, complementary to a sequence conserved at the 5′ end of these genes. When combined with primer SYD4, specific for the intergenic region upstream from the $vanY_D$ gene of 10/96A, $RD_{NH2}$ led to amplification of a 3,725-bp fragment. This PCR fragment, with an unexpectedly large size, was cloned into pCR-Blunt, generating plasmid pAT639, and was sequenced.

**(iv) Plasmids pAT635 and pAT633.** For construction of pAT635($P_2vanX_Dcat$) and pAT633($P_2vanY_Dcat$), the $vanX_D$ and $vanY_D$ genes of BM4339 were amplified using primer pairs $XD1_{NH2}$-$XD1_{COOH}$ and $YD1_{NH2}$-$YD1_{COOH}$, respectively, and BM4339 total DNA as a template. Oligodeoxynucleotides $XD1_{NH2}$ (5′ CAGTGAGCTCCGGTTTTACGCTTTCTG) and $YD1_{NH2}$ (5′ CAGTGAGCTCGCGAAAACATAAATCGC) harbored a *Sac*I restriction site (underlined) and 17 bases complementary to the sequence upstream from $vanX_D$ or $vanY_D$ of BM4339, respectively. Oligodeoxynucleotides $XD1_{COOH}$ (5′ AGTGTCTAGA*C TA*GGCAATGCAAAAAT) and $YD1_{COOH}$ (5′ AGTGTCTAGA*TTAC*TGGG CTTTGATTT) contained an *Xba*I restriction site (underlined), the stop codon (italicized), and 14 bases complementary to the 3′ end sequence of $vanX_D$ or $vanY_D$, respectively. The *Sac*I and *Xba*I restriction sites allowed directional cloning of $vanX_D$ or $vanY_D$ upstream from the *cat* reporter gene of the shuttle vector pAT79 carrying the $P_2$ promoter to generate pAT635($P_2vanX_Dcat$) and pAT633($P_2vanY_Dcat$). The 672-bp insert of plasmid pAT635($P_2vanX_Dcat$) corresponded to nucleotides 5057 to 5728 of the *vanD* operon of BM4339 and included the ribosome binding site (RBS), the initiation codon, the $vanX_D$ coding sequence, and the stop codon of the gene. The 1,153-bp insert of pAT633($P_2vanY_Dcat$) corresponded to nucleotides 1950 to 3102 of the *vanD* operon of BM4339 and consisted of the $vanY_D$ coding sequence with its RBS and initiation and stop codons.

**(v) Plasmids pAT634 and pAT636.** To construct pAT636($P_2vanX_Dcat$) and pAT634($P_2vanY_Dcat$) from 10/96A, a strategy identical to that used for construction of pAT635($P_2vanX_Dcat$) and pAT633($P_2vanY_Dcat$) from BM4339 was followed. The $vanX_D$ and $vanY_D$ genes of strain 10/96A were amplified using primer pairs $XD4_{NH2}$-$XD4_{COOH}$ and $YD4_{NH2}$-$YD4_{COOH}$, respectively, with 10/96A total DNA as a template. Primers $XD4_{NH2}$ (5′ CAGTGAGCTCAGGGTTTA CGCTTTCTG) and $YD4_{NH2}$ (5′ CAGTGAGCTCGCGAAAAAATAAATC GC) harbored a *Sac*I site (underlined) and 17 bases complementary to the sequence upstream from $vanX_D$ or $vanY_D$ of strain 10/96A, respectively. Primers $XD4_{COOH}$ (5′ AGTGTCTAGA*CTA*GGCAATGCAAAAAT) and $YD4_{COOH}$ (5′ AGTGTCTAGA*TCA*CTGGGCCTTGATTT) contained an *Xba*I site (underlined), the stop codon (italicized), and 14 bases complementary to the 3′ end sequence of $vanX_D$ or $vanY_D$ of strain 10/96A, respectively. The $vanX_D$ and $vanY_D$ PCR products were digested with *Sac*I and *Xba*I and cloned under the control of the $P_2$ promoter of the shuttle vector pAT79, leading to plasmids pAT636($P_2vanX_Dcat$) and pAT634($P_2vanY_Dcat$), respectively (11). The 672- and 1,153-bp inserts of pAT636($P_2vanX_Dcat$) and pAT634($P_2vanY_Dcat$) corresponded, respectively, to nucleotides 6875 to 7546 and nucleotides 3765 to 4917 of strain 10/96A and consisted of the $vanX_D$ or $vanY_D$ coding sequences of 10/96A with their RBS and initiation and stop codons.

TABLE 2. MICs of glycopeptides and cytoplasmic peptidoglycan precursors synthesized by VanD-type strains

| *E. faecium* strain | MIC (μg/ml)[a] | | % of peptidoglycan precursors[b] | | | |
|---|---|---|---|---|---|---|
| | VM | TE | UDP-MurNAc-tripeptide | UDP-MurNAc-tetrapeptide | UDP-MurNAc-pentapeptide | UDP-MurNAc-pentadepsipeptide |
| BM4339 | 64 | 4 | 19 | 21 | 2 | 58 |
| BM4416 | 128 | 64 | 7 | 24 | <1 | 69 |
| 10/96A | 256 | 4 | 0 | 2 | 3 | 95 |

[a] MICs were determined by the method of Steers et al. (52). Abbreviations: VM, vancomycin; TE, teicoplanin.
[b] The bacteria were grown without vancomycin to the mid-exponential phase, and peptidoglycan synthesis was inhibited by addition of ramoplanin to the cultures for 15 min.

**(vi) Plasmid pAT640.** The chromosomal *ddl* gene from *E. faecium* 10/96A with its RBS was amplified by PCR from total DNA with the previously described 4147-1 and 4147-2 oligodeoxynucleotides (26). Primers 4147-1 and 4147-2 contain, respectively, *Sac*I and *Xba*I restriction sites that allow directional cloning of the *ddl* gene under the control of the constitutive $P_2$ promoter and upstream from the *cat* reporter gene of the shuttle vector pAT79 (11). The 1,135-bp insert of the resulting pAT640($P_2ddl_{G184S}cat$) plasmid contained the mutated *ddl* gene with the single $G_{184}S$ mutation and its own RBS.

The nucleotide sequences of the amplified fragments were redetermined.

**Strain constructions.** Plasmids pAT635($P_2vanX_Dcat$) and pAT633($P_2vanY_Dcat$) from BM4339 and plasmids pAT636($P_2vanX_Dcat$) and pAT634($P_2vanY_Dcat$) from 10/96A were introduced into *E. faecalis* JH2-2 by electrotransformation. *E. faecium* BM4512 was obtained by introduction of plasmid pAT640($P_2ddl_{G184S}cat$) into *E. faecium* BM4339 by electrotransformation (Table 1). Transformants selected on spectinomycin, 60 μg/ml for JH2-2 or 120 μg/ml for BM4339, were screened for resistance to chloramphenicol. Plasmid DNA from chloramphenicol-resistant clones was digested with *Eco*RI plus *Hin*dIII and compared to the restriction profiles of pAT635($P_2vanX_Dcat$) and pAT633($P_2vanY_Dcat$) and those of pAT636($P_2vanX_Dcat$), pAT634($P_2vanY_Dcat$), and pAT640($P_2ddl_{G184S}cat$) purified from *E. coli* Top10 to screen for DNA rearrangements.

**Nucleotide sequencing.** Plasmid DNA was extracted with the commercial Wizard Plus Minipreps DNA purification system (Promega, Madison, Wis.), labeled with a dye-labeled ddNTP Terminator cycle sequencing kit (Beckman Coulter UK Ltd., High Wycombe, United Kingdom), and the samples were sequenced and analyzed with a CEQ 2000 automated sequencer (Beckman).

**Computer analysis of sequence data.** Determination of the degrees of identity and similarity with known proteins was carried out using BLASTN, BLASTX, and BLASTP (2) and FASTA (42) from the Genetics Computer Group suite of programs.

**Contour-clamped homogeneous electric field gel electrophoresis.** Genomic DNA embedded in agarose plugs was digested for 3 h at 37°C with 0.01 U of I-*Ceu*I, an intron-encoded endonuclease specific for rRNA genes (34). Fragments were separated on a 0.8% agarose gel using a CHEF-DRIII system (Bio-Rad Laboratories, Hercules, Calif.) under the following conditions: total migration, 24 h; initial pulse, 60 s; final pulse, 120 s; voltage, 6 V/cm; included angle, 120°; and temperature, 14°C. The DNA fragments were transferred to a nitrocellulose membrane and hybridized successively under stringent conditions at 68°C to an α-$^{32}$P-labeled 16S rRNA (*rrs*) probe obtained by amplification of an internal portion of the *rrs* gene with primers RWO1 and DG74 (27) and to a *vanD* probe obtained by PCR with primers D4-1 and D4-2 and 10/96 total DNA as a template (Fig. 1). The amplification product used to generate the probe was labeled with [α-$^{32}$P]dATP (3,000 Ci/mmol; Amersham Pharmacia Biotech) by Megaprime using a commercially available kit (Amersham).

**Analysis of peptidoglycan precursors.** Extraction and analysis of peptidoglycan precursors was performed as described previously (37, 49). Enterococci were grown in BHI medium without or with vancomycin (4 μg/ml) to the mid-exponential phase ($A_{600}$ = 1). Ramoplanin was added to inhibit peptidoglycan synthesis, and incubation was continued for 15 min to cause accumulation of peptidoglycan precursors. The bacteria were harvested, and the cytoplasmic precursors were extracted with 8% trichloroacetic acid (15 min, at 4°C), desalted, and analyzed by high-performance liquid chromatography. Results were expressed as the percentages of total late peptidoglycan precursors represented by UDP-MurNAc-tripeptide, UDP-MurNAc-tetrapeptide, UDP-MurNAc-pentapeptide, and UDP-MurNAc-pentadepsipeptide that were determined from the integrated peak areas.

**D,D-Dipeptidase (VanX) and D,D-carboxypeptidase (VanY) activities.** The enzymatic activities in the supernatant and in the resuspended pellet fraction were assayed as described previously (8, 48). Strains were grown until the optical density at 600 nm reached 0.7 in the absence or presence of vancomycin at various concentrations (1, 8, and 64 μg/ml) for induction of 10/96A or with spectinomycin (60 μg/ml) to counterselect loss of derivatives of pAT79. Bacteria were then lysed by treatment with lysozyme (2 mg/ml) at 37°C, followed by sonication, and the membrane fraction was pelleted (100,000 × *g*, 45 min). The supernatant (S100) and resuspended pellet (C100) were collected and assayed for D,D-peptidase (VanX or VanY) activities by measuring the D-Ala released from substrate hydrolysis (D-Ala–D-Ala, 6.56 mM, or UDP-MurNAc-L-Ala–D-Glu–L-Lys–D-Ala–D-Ala, 5 mM) through coupled indicator reactions using D-amino acid oxidase and horseradish peroxidase (8, 48). Specific activity was defined as the number of nanomoles of product formed at 37°C per minute per milligram of protein contained in the extracts.

**Preparation of membrane fragments and binding of benzyl[$^{14}$C]penicillin.** The membrane fragments were prepared and labeling was carried out as described elsewhere (48). Briefly, a culture at an optical density at 600 nm of 1.0 was centrifuged, the pellet was washed in 50 mM Tris HCl (pH 7.2) and resuspended, and osmotic lysis was achieved in the presence of lysozyme (400 μg/ml) and muramidase (70 μg/ml) after incubation at 37°C. DNase (25 μg/ml) and $MgCl_2$ (5 mM) were added, and after 3 min at 37°C, the suspension was cooled to 4°C, centrifuged, and washed. The membrane fraction was resuspended in 50 mM Tris HCl (pH 7.2) and incubated at 37°C with benzyl[$^{14}$C]penicillin (1 μg/ml). After addition of unlabeled penicillin G (3 mg/ml) and sample buffer (New England Biolabs), the membrane proteins were solubilized by heating at 98°C. The labeled membrane proteins were run on a 12% polyacrylamide gel. The gel was stained, dried, and set up for phosphorimaging overnight to detect PBPs and determine the amount of penicilloyl-protein complex. Autoradiography was carried out for 5 weeks to reveal minor PBPs.

**Nucleotide sequence accession number.** The 7,546-bp fragment containing the *vanD* gene cluster of strain 10/96A was submitted to GenBank and assigned accession no. AY082011.

## RESULTS

***E. faecium* 10/96A produces a nonfunctional D-Ala:D-Ala ligase.** *E. faecium* 10/96A produced UDP-Mur-NAc-pentadepsipeptide almost exclusively (95%) when grown in the absence of vancomycin (Table 2). To elucidate the strategy adopted by this strain to prevent the susceptible chromosomal pathway of peptidoglycan synthesis, the entire chromosomal *ddl* gene for the D-Ala:D-Ala ligase was amplified and three independent PCR products with the expected length of 1,077 bp were sequenced. Comparative analysis revealed a point mutation in codon 184 of the *ddl* gene from 10/96A relative to that of *E. faecium* BM4147 (26), leading to a Gly-to-Ser substitution (Fig. 2). This mutation was located next to the serine involved in the binding of D-Ala1, presumably leading to a nonfunctional protein (Fig. 2). Strain BM4339 has an impaired Ddl, and introduction of an intact *ddl* gene under the control of a constitutive promoter restores its susceptibility to glycopeptides (MIC = 0.5 μg/ml) (21). The decrease in glycopeptide resistance is due to synthesis of the heterologous Ddl enzyme since BM4339 possesses only very weak VanX D,D-dipeptidase

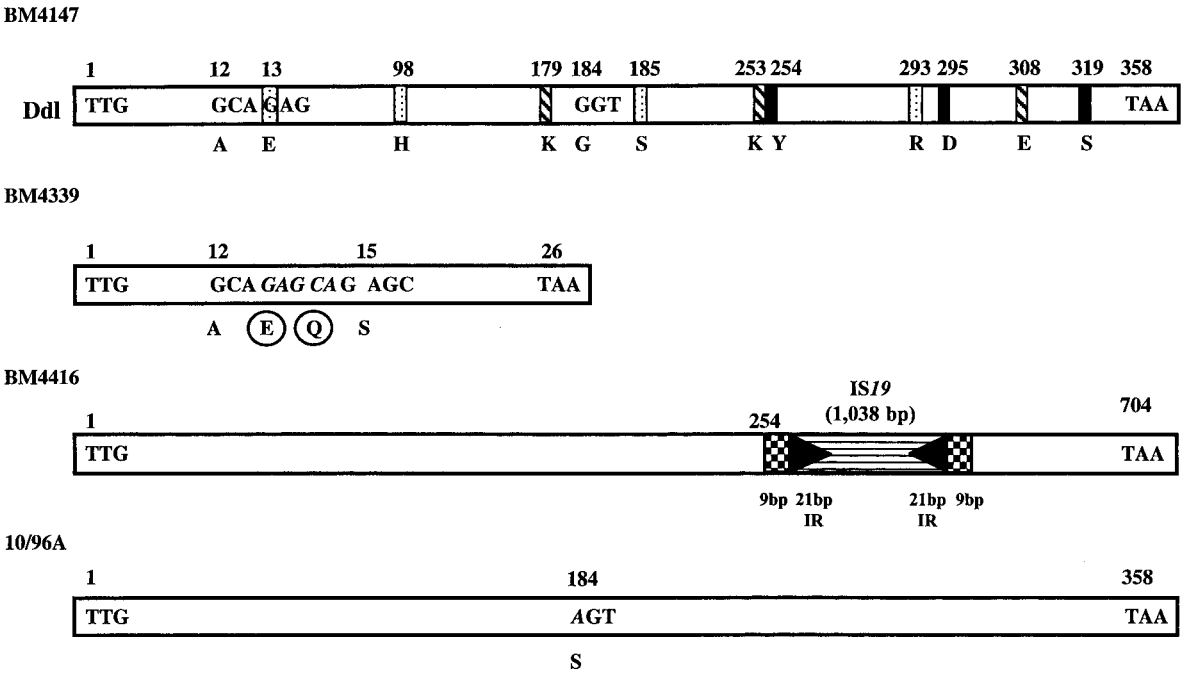


FIG. 2. Schematic representation of D-Ala:D-Ala ligases from *E. faecium* BM4147 and from VanD-type enterococci. The positions of the amino acids implicated in the binding of D-Ala1, D-Ala2, and ATP are indicated by dotted, hatched, and black bars, respectively (25, 51). In strain BM4339, a 5-bp insertion (italics) at the position corresponding to amino acid 13 is responsible for a frameshift mutation leading to the synthesis of a 26-amino-acid peptide instead of the putative 358-amino-acid Ddl (21). In BM4416, a copy of IS*19* is inserted at position 762 of the *ddl* gene. Checkerboard boxes, 9-bp duplications of target DNA; black arrowheads, 21-bp perfect inverted repeat sequences; horizontally striped box, putative transposase (17, 43). In 10/96A, the single base difference with *ddl* from *E. faecium* BM4147 leading to a Gly-to-Ser substitution at position 184 is indicated in italics.

activity (45). To test if the $G_{184}S$ mutation was responsible for impairment of Ddl in 10/96A, plasmid pAT640 containing the $ddl_{G184S}$ gene and its RBS cloned under the control of the constitutive $P_2$ promoter was electrotransformed into BM4339 (Table 1). The resulting transformant, BM4512, remained vancomycin resistant, confirming that the D-Ala:D-Ala ligase from 10/96A was not functional.

The two other VanD-type *E. faecium* strains previously studied, BM4339 and BM4416, are also presumed to lack D-Ala:D-Ala ligase activity as the result of different insertion events in the chromosomal *ddl* gene (Fig. 2). In BM4339, a 5-bp insertion in the 5′ end of the *ddl* gene is responsible for a frameshift mutation leading to the synthesis of a 26-amino-acid truncated ligase, whereas in BM4416, inactivation of the gene is due to insertion of IS*19* (21, 45). Thus, in the three VanD-type strains subjected to detailed study, production of an impaired Ddl accounts for the lack of peptidoglycan precursors terminating in D-Ala–D-Ala (Table 2) (43, 45).

**Characterization of the *van* genes in *E. faecium* 10/96A and of the deduced proteins.** Plasmids pAT637($van_D'$ $vanX_D$), pAT638($vanS_D'$ $vanY_D''$) and pAT639 ($vanR_D$, $S_D'$, ORFA, ORFB, $vanS_D'$) were obtained by cloning the 632-, 1,060-, and 3,725-bp PCR fragments obtained from total DNA of 10/96A into the pCR-Blunt vector (Table 1 and Fig. 1). Sequencing of both strands of the inserts in these plasmids revealed the presence of the structural genes for the D,D-peptidases ($VanX_D$ and $VanY_D$) and also for the two-component regulatory system ($VanR_D$-$VanS_D$) which was interrupted by an insertion sequence composed of two ORFs in the same orientation (Fig. 3). The genes for the $VanH_D$ dehydrogenase and for the VanD D-Ala:D-Lac ligase have been characterized previously (23). The complete organization of the 7,546-bp *vanD* gene cluster composed of eight genes, of which six were found with the same organization as those of the *vanD* operons in *E. faecium* BM4339 and BM4416, is shown in Fig. 1 and 3.

The deduced sequences of the proteins in 10/96A were compared with those from BM4339 and BM4416 (Fig. 3). The structural similarity between the $VanH_D$ dehydrogenases, the VanD ligases, and the $VanX_D$ D,D-dipeptidases was high, between 83 and 99%. Thus, closely related counterparts of the three enzymes required for VanA- and VanB-type resistance are present in a similar organization in VanD-type *E. faecium*. The $VanX_D$ protein, despite the very low D,D-dipeptidase activity in strain 10/96A (Tables 3 and 4), displayed the amino acid motifs YA, DXXR, SXHXXGXAXD, DXM, and EXXH, corresponding to active site residues that may be involved in $Zn^{2+}$ binding and in catalysis (data not shown) (36).

Analysis of the $vanY_D$ gene and its translation product indicated that, in comparison with $VanY_D$ from BM4339 and BM4416, the protein from 10/96A was terminated prematurely after amino acid 118, resulting in a polypeptide lacking the active sites of a PBP (Fig. 3). The hydropathic profile revealed that the truncated protein had a membrane-spanning portion at the N terminus (data not shown). Further investigation of one of the three reading frames downstream from the stop

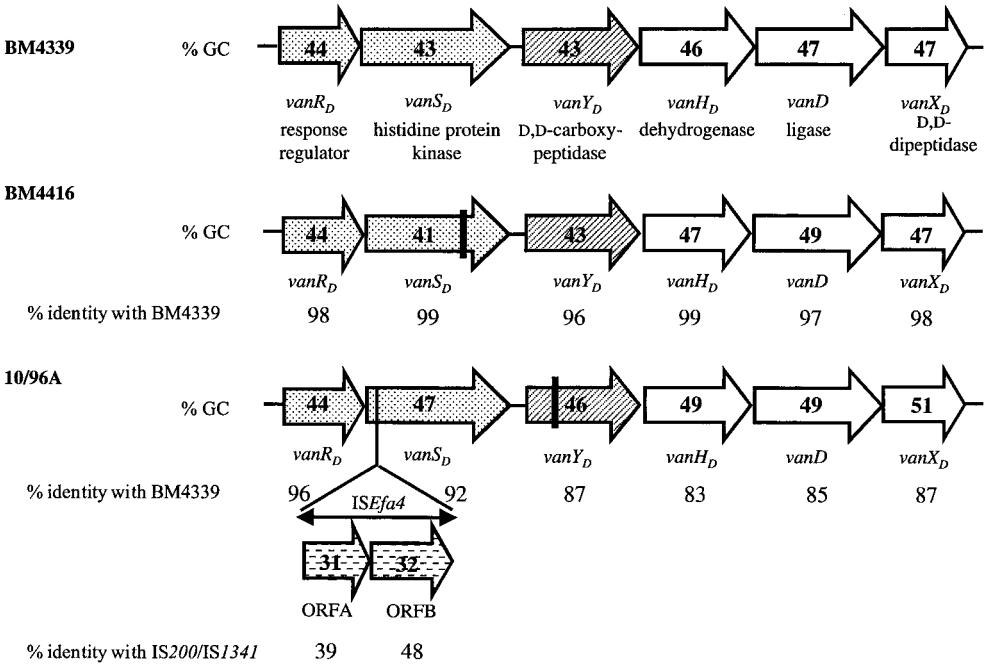


FIG. 3. Comparison of *vanD* gene clusters. Arrows represent coding sequences and indicate direction of transcription. The two-component regulatory systems are represented by dotted arrows, the D,D-carboxypeptidases are represented by hatched arrows, and the genes necessary for resistance are represented by open arrows. The guanosine-plus-cytosine content (% GC) is indicated in the arrows. The percentages of identity between the deduced proteins relative to those of BM4339 are indicated under the arrows. Insertion sequence IS*Efa4* in 10/96A is indicated by a double-headed arrow, and horizontally dashed arrows correspond to ORFA and ORFB. The vertical bars in $vanS_D$ of BM4416 and $vanY_D$ of 10/96A indicate the positions of the frameshift mutations leading to predicted truncated proteins.

codon indicated 87% identity over a length of 237 amino acids with the C-terminal portion of $VanY_D$ from BM4339 and BM4416. This stretch contained the three motifs SXXK, SG(C/N), and KTG, which are characteristic of the penicillin binding domains of PBPs (Fig. 4) (40). It was concluded that a frameshift mutation had resulted in the presence of the stop codon and thus premature termination of what would otherwise have been a full-length protein corresponding to $VanY_D$ of BM4339 and BM4416. In spite of the mutation in $vanY_D$, D,D-carboxypeptidase activity was detected in the membrane fraction of broken-cell preparations of 10/96A, and this activity was not inhibited by penicillin, as opposed to BM4339 and BM4416 (Table 3). The protein with this activity is unlikely to have been encoded by $vanY_D$ from 10/96A.

TABLE 3. D,D-Peptidase activities in extracts from *E. faecium* 10/96A[c]

| Concn of VM[a] (μg/ml) | Mean D,D-dipeptidase activity[b] ± SD (nmol $min^{-1}$ $mg^{-1}$) | Mean D,D-carboxypeptidase activity[d] ± SD (nmol $min^{-1}$ $mg^{-1}$) in fraction | |
|---|---|---|---|
| | | Membrane | Cytoplasmic |
| 0 | 1.6 ± 0.5 | 24 ± 5.9 | 3.9 ± 0.3 |
| 1 | 2.4 ± 0.6 | 23 ± 6.5 | 5.2 ± 0.6 |
| 8 | 2.3 ± 0.6 | 26 ± 5.3 | 3.9 ± 1.2 |
| 64 | 1.3 ± 0.5 | 37 ± 6.8 | 3.8 ± 0.8 |

[a] VM, vancomycin.
[b] The activity was measured in the supernatant of lysed bacteria after centrifugation at 100,000 × *g*.
[c] Results are obtained from a minimum of three independent extracts.
[d] The activities were measured in the supernatant and in the resuspended pellet fraction after centrifugation of lysed bacteria at 100,000 × *g* for 45 min.

In plasmid pAT639 ($vanR_D$, $S_D'$, ORFA, ORFB, $vanS_D'$) (Fig. 1), the deduced sequences of the $VanR_D$ and $VanS_D$ proteins exhibited structural similarity with the $VanR_D$ response regulators and $VanS_D$ histidine protein kinases of the two-component regulatory systems in strains BM4339 and BM4416 (17, 21). In addition, sequence analysis of the 3,725-bp insert in this plasmid revealed the presence of a 1,920-bp insertion sequence, designated IS*Efa4*, which was inserted in the $vanS_D$ gene of strain 10/96A (Fig. 1 and 3). Two ORFs, ORFA and ORFB, were identified in the insertion sequence that exhibited 39 and 48% identity with the genes (*tnp*) for the putative transposases of unrelated elements, IS*200* from *Helicobacter pylori* (16), and IS*1341* from the thermophilic bacterium PS3 (38), respectively (Fig. 3). IS*Efa4* belongs to the IS*605* family (32). Unlike IS*605*, ORFA and ORFB were in the same orientation and showed only 29 and 39% identity with the *tnpA* and *tnpB* genes of IS*605*, respectively.

The C-terminal portion of $VanS_D$ in strain 10/96A contained the five blocks of conserved amino acids characteristic of transmitter modules in histidine protein kinases (Fig. 5). Histidine residue 140 of $VanS_D$ from strain 10/96A was aligned with histidine residues 166 of $VanS_D$ of BM4339 and BM4416, which are the putative sites of autophosphorylation of sensors (Fig. 5). The hydropathy profile of the N-terminal putative sensor domain of $VanS_D$ from strains BM4339 and BM4416 revealed the presence of two stretches of hydrophobic amino

TABLE 4. D,D-Peptidase activities in extracts from *E. faecalis* JH2-2 derivatives[b]

| Strain/plasmid | *van* gene (strain) | Mean D,D-dipeptidase activity[a] (nmol $min^{-1}$ $mg^{-1}$) | Mean D,D-carboxypeptidase activity[c] ± SD (nmol $min^{-1}$ $mg^{-1}$) in fraction | |
|---|---|---|---|---|
| | | | Membrane | Cytoplasmic |
| JH2-2 | None | ND[d] | ND | ND |
| JH2-2/pAT635 | $vanX_D$ (BM4339) | 1.3 ± 0.5 | NA[e] | NA |
| JH2-2/pAT636 | $vanX_D$ (10/96A) | 0.8 ± 0.1 | NA | NA |
| JH2-2/pAT633 | $vanY_D$ (BM4339) | NA | 6.4 ± 1.5 | 1.9 ± 0.5 |
| JH2-2/pAT634 | $vanY_D$ (10/96A) | NA | ND | ND |

[a] The activity was measured in the supernatant of lysed bacteria after centrifugation at 100,000 × *g*.
[b] Results are obtained from a minimum of three independent extracts.
[c] The activities were measured in the supernatant and in the resuspended pellet fraction after centrifugation of lysed bacteria at 100,000 × *g* for 45 min.
[d] ND, not detectable.
[e] NA, not applicable.

acids similar to those in VanS, $VanS_B$, and EnvZ, suggesting a similar topology for these enzymes (data not shown). The IS*Efa4* copy in $vanS_D$ of strain 10/96A not only removed one of the potential membrane spanning regions of $VanS_D$, but the remaining larger portion of the protein would not have been produced. Lack of $VanS_D$ may lead to a high steady-state level of phosphorylated VanR and could thus account for the constitutive expression of the *vanD* operon in strain 10/96A.

**The *vanD* gene cluster is chromosomally located in *E. faecium* 10/96A.** The location of the *vanD* gene cluster was determined by contour-clamped homogeneous electric field gel electrophoresis after digestion of genomic DNA from 10/96A with I-*Ceu*I, an endonuclease specific for rRNA genes (34). The DNA fragments were transferred to a nitrocellulose membrane and hybridized successively with a 16S rRNA (*rrs*) probe (27) and a probe internal to *vanD* from strain 10/96A (Fig. 1). The *rrs* probe hybridized with four I-*Ceu*I fragments, and the *vanD* probe cohybridized with a 450-kb fragment (data not shown). Consequently, the *vanD* resistance operon was assigned to a chromosomal location.

As shown previously, four and five I-*Ceu*I fragments from BM4339 and BM4416, respectively, hybridized with the *rrs* probe, and a 330-kb fragment from BM4339 or a 380-kb fragment from BM4416 cohybridized with a $vanH_DDX_D$ probe (43). The comparative analysis indicated that strain 10/96A was distinct from these two isolates

**D,D-Peptidase activities in strain 10/96A.** D,D-Dipeptidase and D,D-carboxypeptidase activities in *E. faecium* 10/96A were assayed by determining the amount of D-Ala released from hydrolysis of the dipeptide D-Ala–D-Ala and of the pentapeptide UDP-Mur-NAc–L-Ala–γ-D-Glu–L-Lys–D-Ala–D-Ala, respectively (Table 3). The D,D-dipeptidase activity was measured in the supernatant of the lysed bacteria (after centrifugation at 100,000 × *g*) that had been grown in the presence of various concentrations of vancomycin (1, 8, and 64 μg/ml) as an inducer. As in BM4339, weak D,D-dipeptidase activity ($VanX_D$) was found in the cytoplasmic extracts from induced or uninduced 10/96A (Table 3) (45). Since this strain has an impaired D-Ala:D-Ala ligase, it does not require an active VanX type D,D-dipeptidase for resistance.

The level of D,D-carboxypeptidase activity in the cytoplasmic fraction of 10/96A was low. However, the membrane preparation of this strain contained substantial activity, slightly weaker than that of membrane extracts of BM4339 and BM4416, in which the activity was inhibited by low concentrations of benzylpenicillin (Table 3) (48). In 10/96A the D,D-carboxypeptidase activity was not significantly induced by vancomycin, nor was it inhibited by benzylpenicillin (Table 3).

**Comparison of D,D-peptidase activities from BM4339 and 10/96A in *E. faecalis* JH2-2.** Strains BM4339 and 10/96A do not produce D-Ala–D-Ala-containing peptidoglycan precursors following mutations in the chromosomal *ddl* gene (Table 2 and Fig. 2). Consequently, as mentioned previously no D,D-dipeptidase activity is required for glycopeptide resistance in this genetic background.

To test whether the $vanX_D$ and $vanY_D$ genes from BM4339 and 10/96A encode functional enzymes, the genes and their RBS were cloned under the control of the constitutive $P_2$

| | |
|---|---|
| 10/96A | NH2----9--TDRIAPA**STAK**MITALTV--39--LIALMLP**SGN**DAAYTLA--106--RPEVIGL**KTG**TSSLGGA--39--COOH |
| BM4339 | NH2--120--TAKIAPA**STAK**MIMALTA--39--LIALMLP**SGN**DAAYTLA--106--RPEVIGL**KTG**TSSLGGA--39–COOH |
| BM4416 | NH2--120--TAQIAPA**STAK**MIMALTA--39--LIALMLP**SGN**DAAYTLA--106--RPEVIGL**KTG**TSSLGGA--39--COOH |
| *S.* K15 PBP | NH2---56--DTRRSTG**STTK**IMTAKVV--43--LYGLMLP**SGC**DAAYALA--100--YSGAIGV**KTG**SGPEAKY--40--COOH |
| *B.* MB24 DacF | NH2---56--NERLAPA**SMTK**IMTMLLI--42--LKGIAIA**SGN**DASVAMA---89--YPGVDGV**KTG**YTGEAKY--74--COOH |
| *E. coli* PBP6 | NH2---58--DEKLDPA**SLTK**IMTSYVV--48--NKGVIIQ**SGN**DACIALA---86--NLNVDGM**KTG**TTAGAGY--74--COOH |
| Conserved motifs | motif I motif II motif III |

FIG. 4. Partial alignment of the deduced sequences of D,D-carboxypeptidases in VanD-type *E. faecium* strains 10/96A, BM4339, and BM4416 and PBPs from *Streptomyces* sp. strain K15 (40), *Bacillus subtilis* MB24 (DacF) (56), and *E. coli* (PBP6) (18). Conserved motifs involved in the scaffolding of the active site are indicated in boldface type. The numbers of amino acids between the $NH_2$ terminus and motif I, motifs I and II, motifs II and III, and motif III and the COOH terminus are indicated.

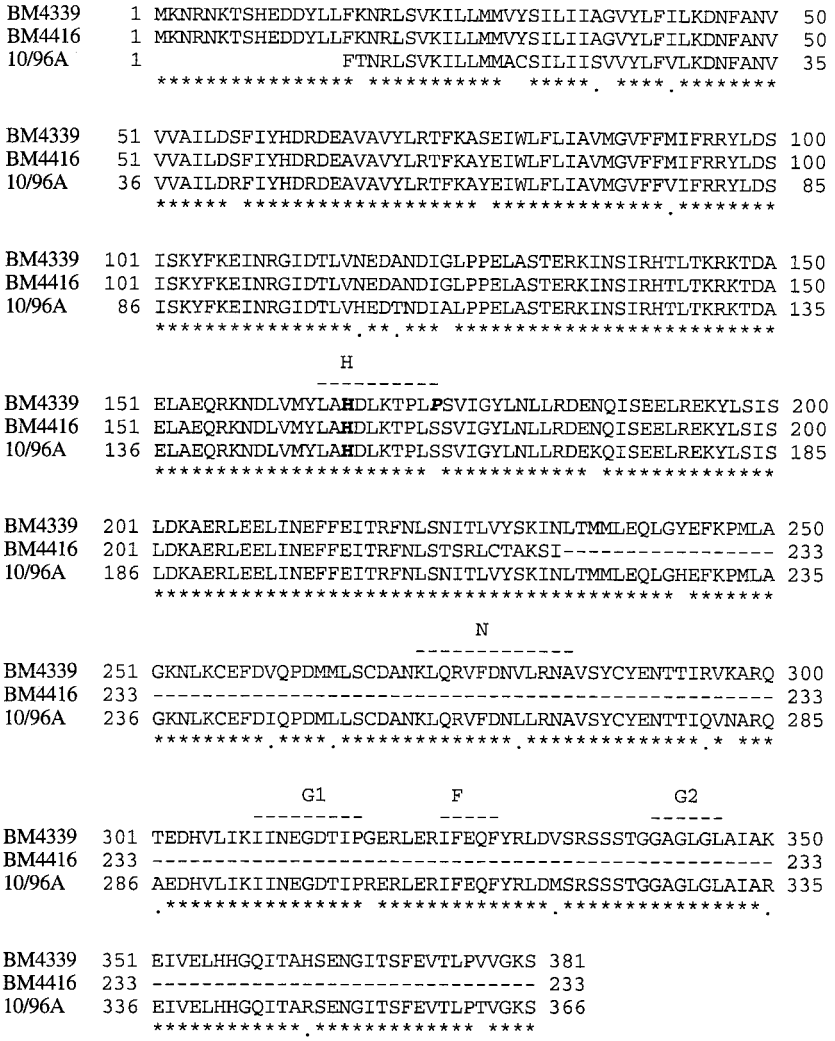


FIG. 5. Alignment of deduced amino acid sequences of $VanS_D$ sensors. Numbers at the left refer to the first amino acid in the corresponding sequence. Numbers at the right refer to the last amino acid in the corresponding line. Identical amino acids are indicated by asterisks, and the isofunctional amino acids are indicated by dots below the alignment. Conserved motifs H, N, G1, F, and G2 are indicated above the alignment by dashes lines (41). The histidine residue in boldface lettering is the putative autophosphorylation site. The proline (*P*) at position 173 putatively responsible for constitutive expression of resistance in BM4339 is indicated in boldface italic lettering. The amino acid sequence of $VanS_D$ from 10/96A starts after the insertion of IS*Efa4*.

promoter, leading to plasmids pAT635($P_2vanX_Dcat$) and pAT633($P_2vanY_Dcat$) from BM4339 and pAT636($P_2vanX_Dcat$) and pAT634($P_2vanY_Dcat$) from 10/96A, which were all electrotransformed into *E. faecalis* JH2-2 (Fig. 1). Although the deduced sequences of the two $VanX_D$ proteins do not contain mutations in the conserved residues known to be involved in zinc binding and catalysis (Fig. 4), only very weak hydrolysis of D-Ala–D-Ala was detected in cytoplasmic extracts from *E. faecalis* JH2-2 harboring pAT635($P_2vanX_Dcat$) and pAT636($P_2vanX_Dcat$) (Table 4). These results are in agreement with those obtained with crude extracts of BM4339 and 10/96A (Table 3).

No D,D-carboxypeptidase activity was detected in extracts from membrane or cytoplasmic fractions from JH2-2/pAT634($P_2vanY_Dcat$) harboring $vanY_D$ of 10/96A, whereas some activity was present in JH2-2/pAT633($P_2vanY_Dcat$) harboring $vanY_D$ of BM4339 (Table 4). Compared with the other VanD-type strains, *E. faecium* 10/96A produced almost exclusively UDP-MurNAc-pentadepsipeptide (95%), whereas UDP-MurNAc-tetrapeptide (2%) and UDP-MurNAc-tripeptide were present in insignificant amounts (Table 2). Despite the presence of the three conserved motifs in the same ORF corresponding to the cytoplasmic portion (Fig. 4), the frameshift mutation in the $vanY_D$ gene of strain 10/96A accounted for the lack of D,D-carboxypeptidase activity (Table 4). These results suggest that the truncated $VanY_D$ from 10/96A was not active due to loss of the domain containing the active site.

**Analysis of PBPs from *E. faecium* 10/96A.** D,D-Carboxypeptidases from BM4339 and BM4416 belong to the PBP family of catalytic serine enzymes but are susceptible to benzylpenicillin (43, 45, 48). We have studied the binding of benzyl[$^{14}$C]penicillin to membrane preparations of *E. faecium* 10/96A in comparison with the other VanD-type strains, BM4339 and BM4416 (Fig. 6). A PBP which migrated as a doublet on

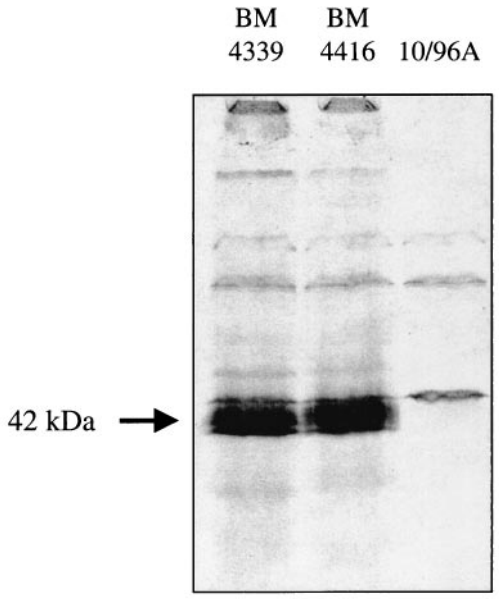


FIG. 6. Binding of benzyl[$^{14}$C]penicillin to membrane proteins of VanD-type *E. faecium*. Membrane preparations of the strains indicated at the top were incubated with benzyl[$^{14}$C]penicillin (1 μg/ml) for 5 min at 37°C; the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 12% polyacrylamide gel; the gel was stained with Coomassie blue, destained, and dried; and the PBPs were revealed by autoradiography.

sodium dodecyl sulfate gel with an apparent molecular mass of 40 to 42 kDa was present in strains BM4339 and BM4416 but absent from membranes of *E. faecium* 10/96A (Fig. 6). This result supported the hypothesis that the defect in the $vanY_D$ gene in the latter strain results in a lack of inhibition by benzylpenicillin of the D,D-carboxypeptidase present, which is presumably encoded by a different gene.

## DISCUSSION

VanD-type resistance to glycopeptides is conferred on *E. faecium* 10/96A by the chromosomal *vanD* gene cluster, which includes at least eight ORFs (Fig. 3). The 5′ portion contains the $vanR_D$ and $vanS_D$ genes, which encode a two-component regulatory system although $vanS_D$ is disrupted by a copy of insertion sequence IS*Efa4* (Fig. 1 and 3). The downstream $vanY_D$ gene encodes a polypeptide of only 118 amino acids rather than the expected 355 amino acids due to a mutation that disrupted the reading frame of the protein; if the mutation had not occurred, $vanY_D$ would have encoded a D,D-carboxypeptidase related to PBPs (Fig. 1 and 3). The 3′ portion of the *vanD* gene cluster contains the $vanH_D$, *vanD*, and $vanX_D$ genes which encode a dehydrogenase, a D-Ala:D-Lac ligase, and a D,D-dipeptidase, respectively. Comparison of the *vanD* operon in 10/96A with those of BM4339 and BM4416 revealed 83 to 96% identity, and the deduced proteins are homologous to the corresponding enzymes in VanA- and VanB-type strains (Fig. 3). However, no genes homologous to *vanZ* and *vanW* from the *vanA* and *vanB* operons, respectively, were found. Strains of the VanD-type of acquired glycopeptide resistance share other characteristics that distinguish them from VanA- and VanB-type enterococci; in particular, resistance is constitutively expressed and is not transferable by conjugation to other enterococci (44).

Compared with the VanA- and VanB-type strains, VanD-type strains BM4339, BM4416 (also designated N97-330), and 10/96A have negligible D,D-dipeptidase activity, encoded by $vanX_D$ alleles, despite the presence of critical residues implicated in the binding of $Zn^{2+}$ and in catalysis (Tables 3 and 4) (36, 43, 45). Lack of such an activity should result in a glycopeptide-susceptible phenotype, since bacteria are unable to remove peptidoglycan precursors ending in D-Ala–D-Ala, the target for glycopeptides. However, the chromosomal *ddl* gene is disrupted by a 5-bp insertion in BM4339; by insertion of an IS*19* (also called IS*Efm1*) element in BM4416; and by a single mutation, $G_{184}S$, next to the serine involved in the binding of D-Ala1 in strain 10/96A (Fig. 2) (17, 21, 43). Both insertions and the mutation lead to production of quasi exclusively peptidoglycan precursors terminating in D-lactate (Table 2). Enterococci containing a vancomycin resistance cluster but with an impaired D-Ala:D-Ala ligase can only grow in the presence of vancomycin if these strains are inducible for vancomycin resistance. Such strains rely entirely for growth on synthesis of peptidoglycan precursors containing D-Ala–D-Lac instead of D-Ala–D-Ala (7, 15, 50, 53). In the VanD-type strains studied, there were no qualitative differences between the peptidoglycan precursors produced by uninduced or induced cells, indicating that the *vanD* clusters were expressed constitutively, thus bypassing the requirement for glycopeptides (Table 2).

In VanA- and VanB-type strains, VanS or $VanS_B$ sensors act as a kinase in the presence of glycopeptides. VanS and $VanS_B$ also negatively control promoters $P_H$, $P_{YB}$ and $P_R$, $P_{RB}$ that mediate transcription of the resistance (*vanHAX* and $vanH_BBX_B$) and regulatory (*vanRS* and $vanR_BS_B$) genes, respectively, in the absence of glycopeptides (4, 5). Under noninducing conditions, the wild-type sensors are therefore considered to act as phosphatases preventing accumulation of the phosphorylated form of the response regulators. According to this model, a constitutive phenotype is associated with loss of the phosphatase activity of the kinase and expression of the resistance genes remains unaltered under noninducing or inducing conditions (4, 5, 15). In VanA-type strains, in the absence of VanS, dephosphorylation of VanR phosphorylated by an heterologous kinase is extremely slow compared to that of the related response regulators, leading to a high level of phospho-VanR and thus to constitutive high-level transcription of the resistance genes (5, 55). In VanB-type strains, constitutive expression of glycopeptide resistance is most probably due to an impaired dephosphorylation of $VanR_B$ by $VanS_B$, since substitutions affecting homologous residues in related sensor kinases result in a defect of the phosphatase, but not of the kinase activity, of the proteins (1, 57). Constitutive expression of the *vanB* cluster is due to amino acid substitutions at two specific positions on either side of histidine 233, which corresponds to the putative autophosphorylation site of $VanS_B$ (15).

Alignment of the deduced amino acid sequences of the $VanS_D$ sensors from *E. faecium* BM4339, BM4416, and 10/96A revealed a mutation at position 173 in the sensor of BM4339, leading to a Pro-to-Ser substitution (Fig. 5). This substitution is in a critical region, since it alters a residue close to histidine

166, corresponding to the putative autophosphorylation site of $VanS_D$, an observation which could account for the constitutive expression of the *vanD* cluster in BM4339 (Fig. 5). Previous comparison of the $vanS_D$ genes from BM4339 and N-97-330 (so called BM4416) showed that the latter strain had suffered a 1-bp deletion at position 670 (BM4339 numbering), which results in a frameshift mutation leading presumably to the synthesis of a 233-amino-acid truncated and nonfunctional sensor instead of a protein containing 381 amino acids as in BM4339 (17). Strain 10/96A contains a different type of mutational event which bypasses the requirement of glycopeptide for constitutive expression of the resistance genes. Insertion sequence IS*Efa4* was found 45 bp downstream from the start site of the $vanS_D$ gene of strain 10/96A and it possessed the characteristics of the IS*605*-family (Fig. 3). IS*605*, detected in *H. pylori*, is unusual in that it contains, in opposite orientation, homologs of genes for the putative transposases of two other unrelated insertion sequence elements, IS*200* from *H. pylori* and IS*1341* from the thermophilic bacterium PS3 (32). IS*Efa4* is characterized by (i) the absence of terminal inverted repeats, (ii) lack of duplication of target sequences, (iii) inserting with its left end next to 5′-TTTAAC, and (iv) two ORFs encoding putative transposases but in the same orientation.

To our knowledge, *E. faecium* 10/96A is only the third glycopeptide-resistant *Enterococcus* in which an insertion has been identified within a *van* gene but is the first in VanD-type strains. Disruption of *vanY* by IS*1476* and insertion of IS*1216V* located towards the 3′ end of *vanS* have been reported in VanA-type strains (35; A. L. Darini, M. F. Palepou, D. James, and N. Woodford, Letter, Antimicrob Agents Chemother. **43:** 995-996, 1999). The latter insertion leads to the loss of 11 amino acids from the C terminus of the VanS sensor and their possible replacement by 10 amino acids resulting from readthrough of the inserted IS*1216V* (Darini et al., letter). According to the authors, this change would not affect the function of the VanS sensor, because the critical residues remain intact. The disruption of *vanY* leads to a decrease of its activity but has no phenotypic consequence, since in VanA-type strains, VanY is not necessary for vancomycin resistance (3, 9, 35). In the case of IS*Efa4*, the insertion led to the production of a truncated $VanS_D$, allowing strain 10/96A to grow in the absence of glycopeptide in the medium.

The frameshift mutation in the $vanY_D$ gene of strain 10/96A results in a truncated polypeptide of 118 amino acids lacking the active site of a D,D-carboxypeptidase as indicated by the lack of activity of the protein after cloning the complete gene in *E. faecalis* JH2-2 (Table 4). The mutation disrupted the reading frame of the $VanY_D$ protein of 10/96A, which would otherwise have contained the active site motifs of a PBP (Fig. 4). These motifs are present in $VanY_D$ of BM4339 and BM4416, the proteins bind benzylpenicillin, and the D,D-carboxypeptidase activity is inhibited by benzylpenicillin (Fig. 4) (48). The truncated product of the $vanY_D$ gene of 10/96A did not bind penicillin (Fig. 6), nor was there any D,D-carboxypeptidase activity in the cytoplasmic fraction, implying that reinitiation of the C-terminal portion of the protein was unlikely to have occurred, particularly as no potential RBS was identified upstream from possible start sites. Surprisingly, substantial D,D-carboxypeptidase activity was detected in the membrane fraction (Table 3). This activity was not inhibited by benzylpenicillin, was not induced by vancomycin, and was presumed to be catalyzed by a totally different protein. Further investigation will indicate whether strain 10/96A has acquired a gene, not present in the *vanD* gene cluster, which encodes a VanY- or $VanY_B$ type protein.

Consideration of the peptidoglycan precursors of the three VanD strains supports this hypothesis. When peptidoglycan synthesis was blocked by ramoplanin in BM4339 and BM4416, UDP-MurNAc-tetrapeptide was present (21 and 24%, respectively) in addition to UDP-MurNAc-pentadepsipeptide (Table 2). As the D-Ala:D-Ala ligase of both these strains is inactive, little if any UDP-MurNAc-pentapeptide would have been present; consequently, tetrapeptide is likely to have resulted from removal of D-lactate from UDP-MurNAc-pentadepsipeptide. PBPs that function as D,D-carboxypeptidases hydrolyze esters in addition to peptides (46); therefore, it is presumed that $VanY_D$ of BM4339 and BM4416, which are PBPs and whose activity was inhibited by low concentrations of benzylpenicillin, will hydrolyze UDP-MurNAc-pentadepsipeptide with the production of tetrapeptide. The peptidoglycan precursors accumulated in strain 10/96A were almost exclusively UDP-MurNAc-pentadepsipeptide (95%), and only 2% UDP-MurNAc-tetrapeptide was present. The D,D-carboxypeptidase activity present in VanA- and VanB-type strains and catalyzed by $VanY_{\Delta 1\text{-}45}$ preferentially hydrolyzes peptidoglycan precursors terminating in acyl-D-Ala–D-Ala (3). If the D,D-carboxypeptidase in membranes of 10/96A has the same specificity as VanY or $VanY_B$, it would account for the lack of UDP-MurNAc-tetrapeptide in the precursors accumulated in this strain, as virtually no UDP-MurNAc-pentapeptide was available as substrate (Table 2). The D,D-carboxypeptidase activity is not required for resistance, but it is possible that the gene encoding this enzyme was acquired prior to the mutation, leading to a defective D-Ala:D-Ala ligase.

We have shown previously that, in VanA-type strains, increased transcription of the *vanHAX* operon is associated with increased incorporation of D-Ala–D-Lac into peptidoglycan precursors, to the detriment of D-Ala–D-Ala, and with a gradual increase in vancomycin resistance levels (8). More-complete elimination of D-Ala–D-Ala-containing precursors is required for teicoplanin resistance (8). An unusual feature of the VanD-type strains is their susceptibility to teicoplanin (MIC = 4 μg/ml), despite constitutive production of peptidoglycan precursors that terminate essentially only in D-Ala–D-Lac. The small amount of pentapeptide could have been synthesized by the VanD ligase. Teicoplanin susceptibility has been associated with mutations in the VanS sensor of some VanA-type strains (29).

The nucleotide divergence between the *vanD* alleles (BM4339, BM4416, and 10/96A) and the geographical dispersion of the isolates (Canada, United States, and Brazil) lead to the hypothesis that the VanD-type strains represent independent introductions in enterococci of gene clusters from undefined donor species.

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