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Regulation of VanB-Type Vancomycin Resistance Gene Expression by the $VanS_B$-$VanR_B$ Two-Component Regulatory System in *Enterococcus faecalis* V583

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Acquired VanA- and VanB-type glycopeptide resistance in enterococci is due to synthesis of modified peptidoglycan precursors terminating in D-lactate. As opposed to VanA-type strains which are resistant to both vancomycin and teicoplanin, VanB-type strains remain teicoplanin susceptible. We have determined the sequence of a 7,160-bp DNA fragment associated with VanB-type resistance in *Enterococcus faecalis* V583 that contains seven open reading frames. The distal part encoded the $VanH_B$, VanB, and $VanX_B$ proteins that are highly similar to the putative VanH, VanA, and VanX proteins responsible for VanA-type resistance. Upstream from the structural genes for these proteins were the $vanY_B$ gene encoding a D,D-carboxypeptidase and an open reading frame *vanW* with an unknown function. The proximal part of the gene cluster coded for the apparent $VanS_B$-$VanR_B$ two-component regulatory system. $VanR_B$ was related to response regulators of the OmpR subclass, and $VanS_B$ was related to membrane-associated histidine protein kinases. Analysis of transcriptional fusions with a reporter gene and promoter mapping indicated that the $VanR_B$-$VanS_B$ two-component regulatory system activates a promoter located immediately downstream from the $vanS_B$ gene. Vancomycin, but not teicoplanin, was an inducer, which explains teicoplanin susceptibility of VanB-type enterococci.

Glycopeptide antibiotics vancomycin (VM) and teicoplanin (TE) inhibit the extracellular steps of bacterial peptidoglycan synthesis by binding to the C-terminal D-alanyl-D-alanine (D-Ala-D-Ala) residues of cell wall precursors (34). The D-Ala-D-Ala target residues are synthesized intracellularly as a dipeptide by a D-Ala:D-Ala ligase and are added to UDP-*N*-acetylmuramyl-L-Ala-γ-D-Glu-L-Lys (UDP-MurNac-tripeptide) by an adding enzyme (44). VM and TE are widely used for the treatment of severe infections due to gram-positive bacteria. The emergence of glycopeptide resistance in enterococci is a serious threat to the efficacy of antibiotic therapy for enterococcal infections since there are few alternatives in cases of resistance or allergy to β-lactams.

Two types of acquired glycopeptide resistance have been described (2). High-level resistance to both VM and TE defines the VanA type, whereas VanB-type enterococci display various levels of VM resistance but remain susceptible to TE. VanA-type strains evade inhibition of cell wall assembly by glycopeptides by synthesizing the peptidoglycan precursor UDP-*N*-acetylmuramyl-L-Ala-γ-D-Glu-L-Lys-D-Ala-D-lactate (UDP-MurNAc-pentadepsipeptide) in addition to the usual UDP-*N*-acetylmuramyl-L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala (UDP-MurNAc-pentapeptide) (1, 22, 29). Replacement of D-Ala by D-lactate (D-Lac) in the C-terminal position of the precursors results in a more than 1,000-fold decreased affinity for glycopeptides, which allows bacterial growth in the presence of the antibiotics (11). VanA-type resistance is mediated by transposon Tn*1546* or related elements (2). Tn*1546* codes for nine proteins that can be assigned to the following five functional groups. (i) Tn*1546* belongs to the Tn*3* family of transposons and carries two open reading frames (ORFs), ORF1 and ORF2, that are structurally related to genes encoding transposases and resolvases, respectively (2). (ii) For synthesis of D-Ala-D-Lac, the *vanA* gene encodes the VanA ligase that is structurally related to D-Ala:D-Ala ligases but has a broader substrate specificity (11, 14). VanA preferentially catalyzes ester bond formation between D-Ala and D-2-hydroxyacids (11) and synthesizes in vivo D-Ala-D-Lac, which is added to UDP-MurNAc-tripeptide by the chromosomally encoded adding enzyme, resulting in UDP-MurNAc-pentadepsipeptide formation (11). The VanH D-2-hydroxyacid dehydrogenase encoded by *vanH* reduces pyruvate to D-Lac, the substrate for VanA (11). (iii) In addition to *vanH* and *vanA*, the *vanX* gene is also required for resistance (8). The VanX D,D-dipeptidase hydrolyzes D-Ala-D-Ala produced by the chromosomal D-Ala:D-Ala ligase (35), thereby reducing the pool of D-Ala-D-Ala, which would otherwise compete with D-Ala-D-Lac for incorporation into peptidoglycan precursors. The VanY D,D-carboxypeptidase encoded by the *vanY* gene may contribute to glycopeptide resistance at low concentrations of VanH, VanA, and VanX by hydrolysis of UDP-MurNAc-pentapeptide (6, 7). (iv) The *vanZ* gene confers low-level Te resistance by an unknown mechanism (4). (v) Genes *vanR* and *vanS* encode a two-component system that regulates expression of glycopeptide resistance genes (8). VanR is an activator required for initiation of cotranscription of *vanH*, *vanA*, and *vanX* (8, 24). Phosphorylation of VanR by VanS modulates its activity (8, 46).

UDP-MurNAc-pentadepsipeptide was also detected in enterococci with VanB-type resistance (10, 15) and is associated with the presence of *vanB* encoding the VanB protein (15). The *vanB* gene is located on large conjugative chromosomal elements (from 90 to 250 kb) (33) or on plasmids (45). Structurally, VanB is closely related to VanA (76% amino acid identity) (15) and has similar catalytic properties (30). Produc-

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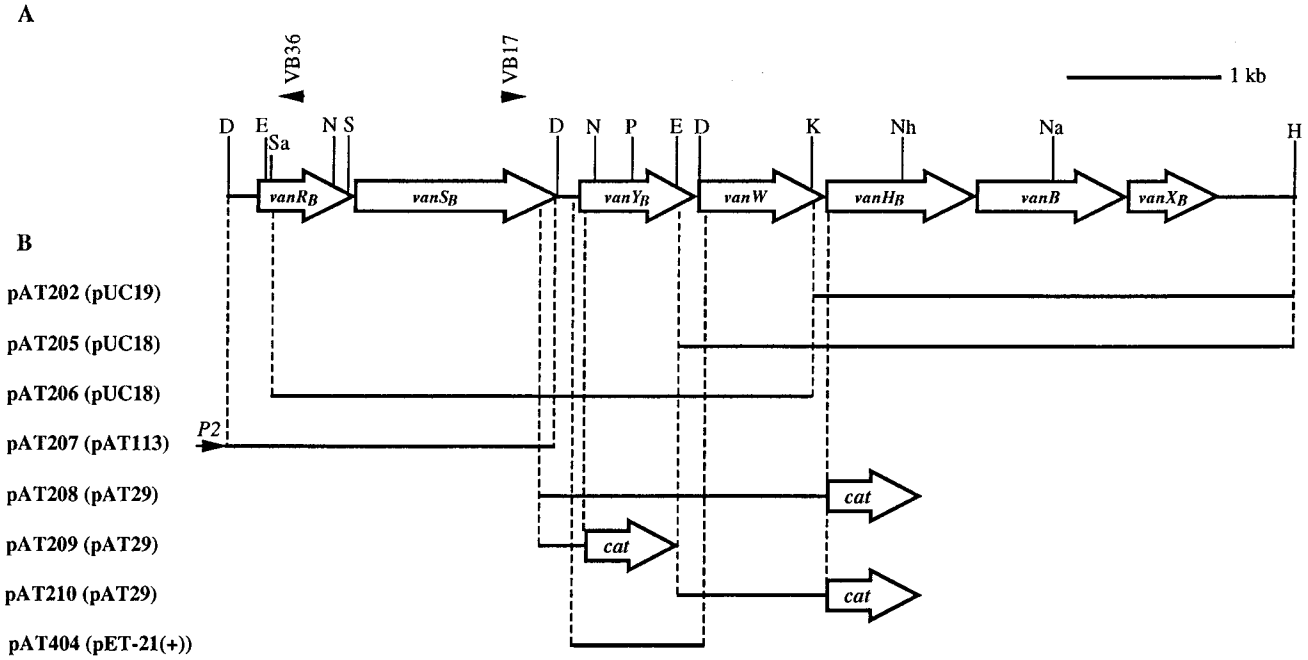

FIG. 1. Maps of the *vanB* gene cluster and of recombinant plasmids. (A) Map of the 7,160-bp *Dra*I-*Hin*dIII chromosomal region comprising the $vanR_B$, $vanS_B$, $vanY_B$, *vanW*, $vanH_B$, *vanB*, and $vanX_B$ genes. Open arrows represent coding sequences. Arrowheads indicate binding site and orientation of oligodeoxynucleotides VB36 and VB17. Restriction site abbreviations: D, *Dra*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; Na, *Nae*I; N, *Nde*I; Nh, *Nhe*I; P, *Pvu*II; S, *Sac*II; Sa, *Sau*3A [only the site that constitutes the 5′ extremity of the insert of recombinant bacteriophage λ EMBL(*vanB*) is indicated]. (B) Inserts in recombinant plasmids. The inserts are represented by solid bars, and the vectors are indicated in parentheses. The arrow labeled *P2* in pAT207 indicates the position and orientation of the *P2* promoter of the *aphA-3* gene (8).

tion of VanB and of a D,D-carboxypeptidase is induced by VM (20). It thus appears that glycopeptide resistance of both the VanA and VanB types relies on the same mechanism, which does not explain TE susceptibility of VanB-type strains. Previous experiments have shown that VanB-type strains become TE resistant upon induction with VM (20), suggesting that TE susceptibility is due to its low activity as an inducer of resistance. In addition, mutants of VanB-type strains with constitutive resistance to both VM and TE can be easily selected in vitro (20) and have also been found in vivo (23).

These observations prompted us to investigate the regulation of resistance gene expression in VanB-type *Enterococcus faecalis* V583 (36). We describe the genetic organization of the chromosomal region flanking the *vanB* gene and analysis of transcriptional activation of resistance gene expression by the $VanR_B$-$VanS_B$ two-component regulatory system.

MATERIALS AND METHODS

Strains, vectors, and growth conditions. Bacteriophage λ EMBL3 (17) was used for the construction of a genomic library of *E. faecalis* V583 (36). *Escherichia coli* P2392 [F^- e14$^-$(McrA$^-$) *hsdR514*(r_k^- m_k^+) *supE44 supF58 lacY1* or Δ(*lacIZY*)*6 galK2 galT22 metB1 trpR55 P2*] was infected with in vitro-packaged recombinant bacteriophage DNA. Restriction or PCR-amplified DNA fragments were cloned into promoter probing vector pAT78, a derivative of the gram-positive and gram-negative shuttle vector pAT29 (*oriR* pAMβ1, *oriR* pUC, *oriT* RK2 *spc lacZ*α) containing a promoterless *cat* reporter gene (8). Plasmid DNA was transformed into *E. coli* JM83 {F^- *ara* Δ(*lac-proAB*) *rpsL*(Str^r) [φ80d*lac*Δ(*lacZ*)*M15*]} (48). Plasmid DNA was prepared by a small-scale alkaline lysis procedure (9) and introduced by electrotransformation (13) into *E. faecalis* JH2-2 (Fus^r Rif^r) (25) or derivatives with a Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, Calif.).

Derivatives of integrative plasmid pAT113 (Mob$^+$ Em^r Km^r *oriR* pACYC184, *att* Tn*1545 lacZ*α) (41) were introduced into *E. faecalis* BM4138 (JH2-2::Tn*916*; our laboratory collection) by electrotransformation.

Plasmid pET-21(+) and *E. coli* BL21(DE3)/pLysS (Novagen, Madison, Wis.) were used as cloning vector and host strain, respectively, for overexpression of the VanY protein.

Cultures were performed in brain heart infusion broth or agar at 37°C. MIC values were determined by the method of Steers et al. (39) on Mueller-Hinton agar with an inoculum of 10^5 cells per spot.

Recombinant DNA techniques. Restriction endonucleases and T4 DNA ligase were purchased from Boehringer (Mannheim, Germany) or Pharmacia LKB Biotechnology Inc. (Uppsala, Sweden). DNA was extracted from agarose gels by the QUIAEX procedure (Quiagen, Hilden, Germany). Blunting of recessed DNA termini and subsequent ligation were carried out with a commercial kit (DNA blunting kit; Amersham, Amersham, England). Sets of plasmids carrying unidirectional nested deletions were prepared with the Erase-a-Base Kit (Promega, Madison, Wis.).

Plasmid construction. The plasmids were constructed as follows (Fig. 1). Nucleotide positions refer to the numbering for the sequence submitted to GenBank under accession number U35369.

(i) Plasmids pAT202, pAT205, and pAT206. DNA from bacteriophage λ EMBL(*vanB*) (15) containing the *vanB* gene was digested with *Kpn*I-*Hin*dIII, *Eco*RI-*Hin*dIII, and *Sal*I-*Kpn*I. Vector pUC18 or pUC19 DNA (43) was digested with the same restriction enzymes. The following plasmids were constructed: pAT202 (3.3-kb *Kpn*I-*Hin*dIII fragment into pUC19), pAT205 (4.2-kb *Eco*RI-*Hin*dIII fragment into pUC18), and pAT206 (3.7-kb *Sal*I-*Kpn*I fragment into pUC18). The *Sal*I-site used for preparation of the insert of pAT206 was part of the cloning site of λ EMBL3.

(ii) Plasmid pAT207. The construction of this plasmid is outlined in Fig. 2. Plasmid pAT207 carries the $vanR_B$ and $vanS_B$ genes under the control of heterologous constitutive promoter P2 of the *aphA-3* gene from plasmid pJH1 (8).

(iii) Plasmids pAT208, pAT209, and pAT210. The PCR fragment primed by restriction-site-tagged oligodeoxynucleotides VB38 (5′-cgaggagcTCCAATATG CGCTGGAA, nucleotides 2110 to 2126) and VB39 (5′-tagatctagaGGTGATAA GGTGCGGAA, nucleotides 4080 to 4064; uppercase letters represent sequence complementary to the target and restriction sites are underlined) with total DNA of *E. faecalis* V583 as a template was digested with *Xba*I-*Sac*I and cloned into pAT78. The resulting plasmid, pAT208, contains the chromosomal region between $vanS_B$ and $vanH_B$ upstream from the promoterless *cat* gene. Plasmid pAT210 was constructed by deleting the 856-bp *Eco*RI fragment from pAT208. The DNA fragment amplified by oligodeoxynucleotides VB38 and VB41 (5′-TT CATATGCCGTTTGTGATGATTCACATTGGAATG, nucleotides 2411 to 2376) was digested with *Sac*I and cloned into pAT78 digested with *Sac*I-*Sma*I. The resulting recombinant plasmid was designated pAT209.

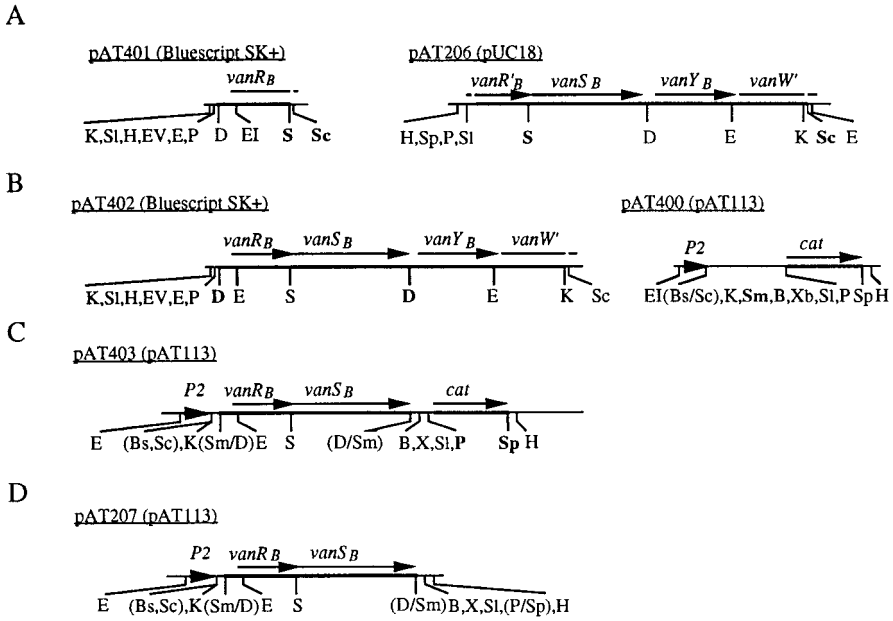

FIG. 2. Construction of plasmid pAT207. Restriction site abbreviations: B, *Bam*HI, Bs, *Bss*HII; D, *Dra*I; E, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; Sc, *Sac*I; S, *Sac*II; Sl, *Sal*I; Sm, *Sma*I; Sp, *Sph*I; X, *Xba*I. The restriction sites used for cloning are indicated in bold lettering. Thin lines represent polylinker sequences; thick lines represent plasmid inserts. Arrows indicate the extent and orientation of coding sequences. The arrowhead labeled *P2* indicates the orientation and position of the *P2* promoter of the *aphA-3* gene (8). (A) The 2.2-kb *Sac*II-*Sac*I fragment of pAT206 was cloned into the corresponding sites of pAT401. Plasmid pAT401 was constructed by cloning the amplification product generated by oligodeoxynucleotides VB37 (5′-cagcctgcagTTTAAACGGTATATTTC, nucleotides 1 to 17, *Pst*I tagged) and VB16 (5′-CAGTAACAGGACAATGA, nucleotides 899 to 883) digested with *Pst*I and *Sac*II into Bluescript SK$^+$ (Stratagene, La Jolla, Calif.). The insert of pAT401 was sequenced to screen for base misincorporations. (B) The *Dra*I fragment of pAT402 containing $vanR_B$ and $vanS_B$ was cloned into the *Sma*I site of pAT400 (3), a derivative of pAT113 (41) containing the polylinker sequence of pAT79 (8). The orientation of the insert in pAT403 was determined by restriction endonuclease digestion. (C) The *cat* gene in pAT403 was deleted by digestion with *Pst*I-*Sph*I and subsequent blunting followed by religation. (D) Plasmid pAT207.

Plasmid pAT404. The PCR fragment primed by oligodeoxynucleotides VB49 (5′-tgacgagctCAAAGGAGGCATTCAAT, nucleotides 2339 to 2355) and VB45 (5′-GCCGTCAAATCTCATTC, nucleotides 3281 to 3265) was digested with *Sac*I and cloned into *Sac*I-*Sma*I-digested pUC19. Plasmid pAT404 was constructed by subcloning the *Sac*I-*Hin*dIII fragment of this plasmid into expression vector pET-21(+).

Nucleotide sequencing. Nucleotide sequencing was performed by the dideoxy-chain termination method (37) with T7 DNA polymerase (T7 sequencing kit; Pharmacia) and [α-^{35}S]dATP (Amersham) as described in the manufacturers' recommendations. For direct sequencing of PCR-amplified DNA, this protocol was modified as follows. Template and oligodeoxynucleotides were heat denatured and rapidly cooled on ice. Annealing buffer, labeling buffer, [α-^{35}S]dATP, and T7 DNA polymerase were added, and the resulting solution was immediately distributed into the dideoxynucleotide mixtures and incubated for 5 min at 37°C. The products of the sequencing reactions were resolved on denaturing 6% polyacrylamide gels.

Computer analysis of sequence data. Sequence data were analyzed with the GCG Sequence Analysis Software Package version 7 (Genetics Computer Group, Madison, Wis.). The GenBank, EMBL, and SwissProt databases were screened for sequence similarities by the algorithm developed by Lipman and Pearson (26).

GenBank accession number. The 7,160-bp sequenced chromosomal fragment was submitted to GenBank and assigned accession number U35369.

S1 nuclease and primer extension mapping of RNA. Total RNA was prepared from enterococcal strains grown to an optical density at 600 nm (OD_{600}) of 0.7 (19) in the presence of 5 μg of VM per ml for strain V583 or in the absence of antibiotic for strains V583 and JH2-2. Single-stranded probes for S1 nuclease mapping were prepared by extension of end-labeled primers (9) with double-stranded pAT208 DNA as a template (see Fig. 6A). Probe A (1,129 bp) was generated by extension of primer VB39 and subsequent digestion with *Eco*RI. Probe B (984 bp) was prepared with primer VB25 (5′-ATATCGGTAATGC CACG, nucleotides 3090 to 3074) followed by *Sac*I digestion. Primer VB41 was used for preparation of probe C (297 bp), and the elongation product was digested with *Sac*I. Denatured probes (2×10^5 cpm) were hybridized with 50 μg of total RNA overnight at 35°C. Samples were diluted in ice-cold S1 nuclease buffer and digested with 100 U of S1 nuclease (Pharmacia) as described previously (9). For primer extension analysis, oligonucleotide VB41 was 5′ end labeled, 2×10^5 cpm were incubated with 50 μg of RNA overnight at 30°C, and extension was performed with 40 U of avian myeloblastosis virus reverse transcriptase (Promega) for 90 min at 42°C as described previously (9). The S1 nuclease-protected fragments or primer elongation products were analyzed by electrophoresis on 4 or 6% denaturing polyacrylamide gels.

Enzyme assays. For determination of chloramphenicol acetyltransferase (CAT) production, *E. faecalis* JH2-2 derivatives were grown to an OD_{600} of 0.7 in brain heart infusion broth containing spectinomycin (60 μg/ml). VM (1 μg/ml) or TE (0.5 μg/ml) was added to the cultures at an OD_{600} of 0.2. The cells were harvested, washed in 0.1 M phosphate buffer (pH 7.0), resuspended in the same buffer, treated with lysozyme, and lysed by sonication. The lysates were centrifuged for 45 min at 100,000 × *g*, and the CAT activity (nanomoles per minute per milligram of protein) was measured as described previously (8, 38).

E. coli BL21(DE3)/pLysS/pAT404 and BL21(DE3)/pLysS/pET-21(+) were grown in brain heart infusion broth containing 34 μg of chloramphenicol per ml and 50 μg of carbenicillin per ml to an OD_{600} of 0.6. For induction of gene expression, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and incubation was continued for 2 h. The cells were harvested, resuspended in 0.1 M phosphate buffer, and lysed by sonication. The lysate was centrifuged (100,000 × *g* for 45 min at 4°C), the supernatants (fraction S100) were collected, and the pellet was resuspended in 0.1 M phosphate buffer (pH 7.0; fraction C100). To estimate D,D-carboxypeptidase activity, D-Ala release from the pentapeptide L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala was determined by using D-amino acid oxidase coupled to peroxidase as indicator reactions as described previously (5).

RESULTS AND DISCUSSION

Cloning and sequencing of *van* genes. Bacteriophage λ EMBL(*vanB*) contains the *vanB* gene of *E. faecalis* V583 (15). Plasmids pAT202, pAT205, and pAT206 were obtained by subcloning the 3.3-kb *Kpn*I-*Hin*dIII, the 4.2-kb *Eco*RI-*Hin*dIII, and the 3.7-kb *Kpn*I-*Sal*I fragments of the recombinant bacteriophage into pUC vectors (43) (Fig. 1). Sequence determination of the inserts of these plasmids on both strands revealed the presence of seven ORFs in the same orientation (Fig. 1). Since the first ORF was interrupted, inverted PCR (42) was carried out with *Dra*I-digested, religated total DNA of *E. faecalis* V583 as a template with oligodeoxynucleotide primers

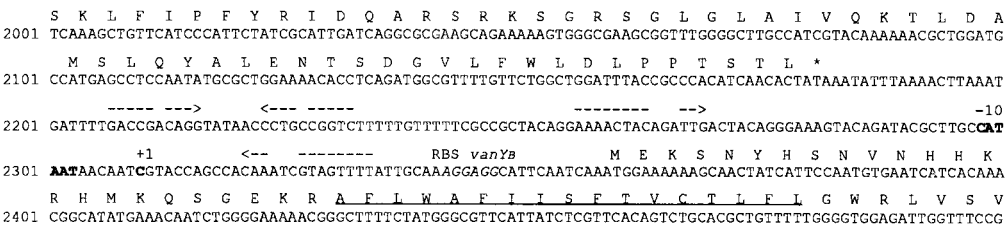

FIG. 3. Sequence of nucleotides 2101 to 2499 of the 7,160-bp *Dra*I-*Hin*dIII fragment carrying the *vanB* gene cluster from *E. faecalis* V583. The deduced amino acids of the C-terminal portion of $VanS_B$ and of the N-terminal portion of $VanY_B$ are shown above the nucleotide sequence and are aligned with the first nucleotide of each codon. Putative ribosomal binding sites (RBS) are printed in italics and indicated above the nucleotide sequence. Clusters of hydrophobic amino acids are underlined. Horizontal arrows indicate regions of dyad symmetry. The hexanucleotide similar to −10 consensus promoter sequences and the putative transcription initiation site (+1), as determined by reverse transcriptase mapping, are indicated in boldface lettering.

VB17 (5′-TGATGATACTGCACTTT, nucleotides 1985 to 2001) and VB36 (5′-ACCAGTTGATAGGTGTT, nucleotides 326 to 310) (Fig. 1). The resulting 527-bp DNA fragment was sequenced directly, completing the sequence of the first ORF. All seven ORFs were preceded by sequences complementary to the 3′ extremity of *Bacillus subtilis* 16S rRNA (3′-UCUUU CCUCC-5′) (31) that could constitute ribosomal binding sites.

Sequence comparisons. The deduced sequences of the putative proteins encoded by the *vanB* gene cluster were compared with those coded for by the *vanA* operon from Tn*1546*. The structural similarity between $VanH_B$, VanB, and $VanX_B$ and VanH, VanA, and VanX, respectively, was very high (67, 76, and 71% identical amino acids, respectively). Thus, closely related counterparts of the three genes required for VanA-type resistance are present, in a similar organization, in *E. faecalis* V583. By contrast, proteins $VanR_B$ and VanR and $VanS_B$ and VanS displayed a much lower degree of similarity (34 and 23% amino acid identities, respectively). The primary sequences of $VanY_B$ and VanY were only distantly related (30% amino acid identity). ORF *vanW* did not reveal significant similarity to known sequences by screening of the SwissProt, GenBank, and EMBL databases. No homolog to TE resistance gene *vanZ* was present in the DNA region examined.

Structural similarity between $VanS_B$-$VanR_B$ and two-component regulatory systems. The deduced sequences of the $VanS_B$ and $VanR_B$ proteins exhibited structural similarity with histidine protein kinases and response regulators (RRs) of two-component regulatory systems, respectively (32, 40). The C-terminal portion of $VanS_B$ (shown in part in Fig. 3) contained the five blocks of conserved amino acids characteristic for transmitter modules of histidine protein kinases (32) (Fig. 4). Histidine residue 226 was aligned with histidine residues 164 of VanS, 214 of PhoR, and 211 of EnvZ, which are the sites of autophosphorylation of the latter two proteins. The hydropathy profile of the N-terminal putative sensory domain revealed the presence of two stretches of hydrophobic amino acids (data not shown) similar to those in VanS and EnvZ, suggesting a similar membrane topology for these enzymes (16). The N-terminal half of the deduced amino acid sequence of $VanR_B$ showed structural similarity with the receiver domains of RRs (Fig. 5). The characteristic conserved lysine and aspartate residues were present in $VanR_B$. On the basis of sequence similarity, the $VanR_B$ protein could be assigned to the OmpR subclass of RRs (40). Proteins belonging to this subclass are thought to bind to promoter sequences that are recognized by the major form of RNA polymerase holoenzyme, corresponding to *E. coli* $E\sigma^{70}$ (40). This structural homology suggested that $VanS_B$ and $VanR_B$ could function as a signal-transducing system similar to the EnvZ-OmpR two-component regulatory system.

***trans*-activation of $vanY_B$, *vanW*, and $vanH_B$ transcription by $VanR_B$ and $VanS_B$.** To allow constitutive expression, the $vanR_B$ and $vanS_B$ genes were cloned into expression vector pAT400 carrying the constitutive P2 promoter (3), and the resulting plasmid, pAT207 (Fig. 2), was integrated into the chromosome

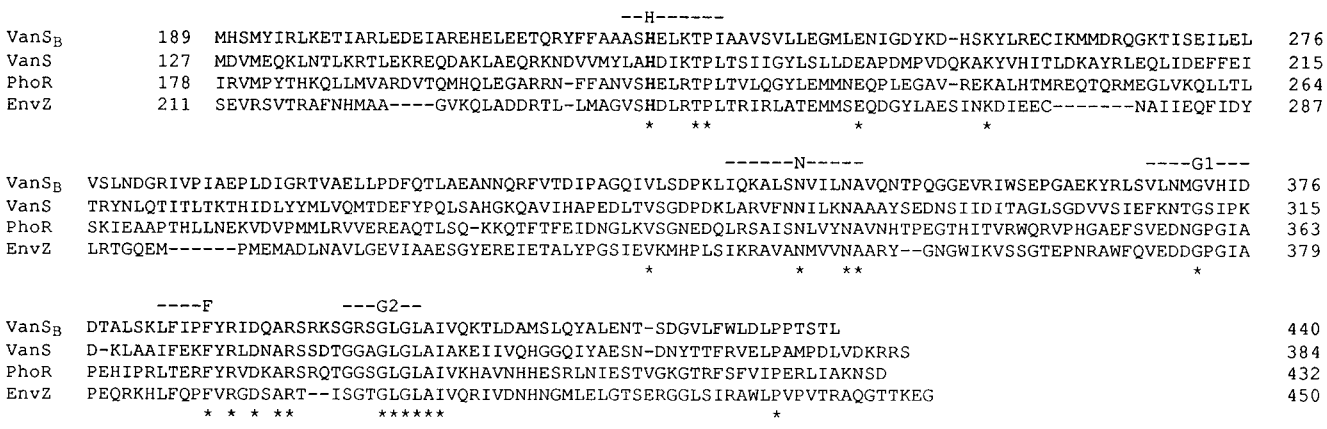

FIG. 4. Alignment of the deduced amino acid sequences of $VanS_B$ from *E. faecalis* V583, VanS from Tn*1546*, and PhoR (28) and EnvZ (12) from *E. coli*. Numbers at the left refer to the first amino acid of the corresponding sequence. Numbers at the right refer to the last amino acid in the corresponding line. Identical amino acids are indicated by asterisks below the alignment. Dashes indicate gaps introduced to optimize similarity. Conserved sequence motifs H, N, G1, F, and G2 (32) are indicated above the alignment by dashed lines. The histidine residue in bold lettering is the putative autophosphorylation site. The percentages of identical amino acids between $VanS_B$ and the other proteins in the aligned portion were 23% for VanS, 26% for PhoR, and 22% for EnvZ.

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VanR_B          MSIRILLVEDDDHICNTVRAFLAEARYEVDACTDGNEAHTKFYENTYQLVILDIMLPGMNGHELLREFRAQNDT---PILMMTALSDDENQI    89
VanR            MSDKILIVDDEHEIADLVELYLKNENYTVFKYYTAKEALECIDKSEIDLAILDIMLPGTSGLTICQKIR-DKHT--YPIIMLTGKDTEVDKI    89
OmpR          MQENYKNLVVDDDMRLRALLERYLTEQGFQVRSVANAEQMDRLLTRESFHLMVLDLMLPGEDGLSICRRLRSQSNP--MPIIMVTAKGEEVDRI    92
PhoB            MARRILVVEDEAPIREMVCFVLEQNGFQPVEAEDYDSAVNQLNEPWPDLILLDWMLPGGSGIQFIKHLKRESMTRDIPVVMLTARGEEEDRV    92
                     * * *           *                                 *  ** ****  *                 *  * *

VanR_B  RAFDAEADDYVTKPFKMRILLKRVEALLR--------GSGALAKEFRVGRLTLLPEDFRVLCDGTELPLTRKEFEILLLLVQNKGRTLTHEIILSRIWGY   181
VanR    TGLTIGADDYITKPFRPLELIARVKAQLRR-YKKFSGVKEQNENVIVHSGLVINVNTHECYLNEKQLSLTPTEFSILRILCENKGNVVSSELLFHEIWGD   188
OmpR    VGLEIGADDYIPKPFNPRELLARIRAVLRRQANELPGAPSQEEAVIAFGKFKLNLGTREMFREDEPMPLTSGEFAVLKALVSHPREPLSRDKLMNLARGR   192
PhoB    RGLETGADDYITKPFSPKELVARIKAVMRRI------SPMAVEEVIEMQGLSLDPTSHRVMAGEEPLEMGPTEFKLLHFFMTHPERVYSREQLLNHVWGT   186
             ****  ***    *  *  *  *                                                  *  *

VanR_B  DFDGD-GSTVHTHIKNLRAKLPENI-----IKTIRGVGYRLEESL        220
VanR    EYFSKSNNTITVHIRHLREKMNDTIDNPKYIKTVWGVGYKIEK          231
OmpR    EY-SAMERSIDVQISRLRRMVEEDPAHPRYIQTVWGLGYVFVPDGSKA     239
PhoB    NVYVE-DRTVDVHIRRLRKALEPG-GHDRMVQTVRGTGYRFSTRF        229
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FIG. 5. Alignment of the deduced amino acid sequences of $VanR_B$ from *E. faecalis* V583, VanR from Tn*1546*, OmpR (12), and PhoB (27) from *E. coli*. Numbers at the right refer to the last amino acid in the corresponding line. Identical amino acids are indicated by asterisks below the alignment. Dashes indicate gaps introduced to optimize similarity. Residues in boldface lettering correspond to highly conserved amino acids in the effector domain of RRs (32). Aspartate residue 53 of $VanR_B$, VanR, and PhoB and residue 55 of OmpR are the putative sites of phosphorylation. The percentages of identical amino acids between $VanR_B$ and the other sequences were 34% for VanR, 27% for OmpR, and 31% for PhoB.

of *E. faecalis* BM4138. To investigate the role of $VanR_B$ and $VanS_B$ in the regulation of VanB-type resistance gene expression, transcriptional fusions with a promoterless *cat* reporter gene were used. Plasmids pAT208, pAT209, and pAT210, constructed by cloning PCR or restriction fragments into promoter probing vector pAT78 (Fig. 1) (8), were introduced by electroporation into BM4138::pAT207; pAT208 was also introduced into BM4138. Transcriptional activation in *trans* by $VanR_B$ and $VanS_B$ was tested without or after induction with VM or TE by determination of CAT activity of S100 cell extracts (Table 1). In the absence of $VanR_B$ and $VanS_B$ and in extracts from uninduced cultures or cultures grown in the presence of TE, no significant CAT activity was detected. By contrast, extracts from BM4138::pAT207/pAT208 and BM4138::pAT207/pAT209 cultures induced with VM displayed CAT activity, indicating that the chromosomally encoded $VanR_B$-$VanS_B$ two-component regulatory system *trans*-activates transcription of at least $vanY_B$, *vanW*, and $vanH_B$ in response to VM. Since $vanR_B$ and $vanS_B$ are constitutively expressed, transcriptional activation appears to be due to functional activation of histidine protein kinase $VanS_B$ by VM, leading to phosphorylation and activation of RR $VanR_B$. This confirms that inefficiency of TE as an inducer of transcription of resistance genes is responsible for TE susceptibility of VanB-type strains. Expression of VanA-type resistance genes is inducible by VM, TE, and moenomycin, a transglycosylase inhibitor (21). The possibility that accumulation of cell wall precursors may be the signal that triggers expression of resistance genes in these strains has been evoked (21). The finding that transcriptional activation by $VanR_B$-$VanS_B$ is VM specific suggests that the activating signal in these strains may be the antibiotic itself or part of it.

Localization of a target promoter for $VanR_B$-$VanS_B$. Previous transcriptional analysis of the *vanA* gene cluster has shown that *vanH*, *vanA*, and *vanX* are cotranscribed from a single start site located between the *vanS* and the *vanH* genes. We therefore screened the $vanS_B$-$vanH_B$ intergenic region for transcriptional start sites by S1 nuclease protection and primer extension. The ability of the insert in pAT209 to be a target for transcriptional activation suggested that a promoter activated by $VanR_B$ and $VanS_B$ was located between $vanS_B$ and $vanY_B$. The 5′ extremity of the mRNA transcript of $vanY_B$ was mapped by primer extension analysis and S1 nuclease protection with probe C (Fig. 6B). Primer extension identified a 5′ end at nucleotide 2310. The longest S1 nuclease-protected fragments corresponded to start sites at positions 2308 and 2309, 1 and 2 nucleotides upstream, respectively, from the site mapped by primer extension. In addition, shorter products that could correspond to partially degraded mRNA were generated. The region between the 5′ end of $vanH_B$ and the 3′ end of $vanS_B$ was screened by S1 nuclease mapping (Fig. 6C) to test for possible additional start sites upstream from $vanH_B$, *vanB*, and $vanX_B$, which are homologous to the three genes strictly required for VanA-type resistance. The S1 nuclease-protected fragment obtained with probe A corresponded to the length of the undigested probe. A faint band of approximately 250 bp that would map immediately upstream from $vanH_B$ was seen. However, this product was not detected with a different RNA preparation or by primer extension (data not shown), and no CAT activity was detected in extracts of induced BM4138::

TABLE 1. Activation of *cat* transcription by $VanR_B$ and $VanS_B$

E. faecalis (relevant characteristics)	Plasmid (relevant characteristics)	Sp act of CAT[a]		
		Not induced	Induced with VM	Induced with TE
BM4138	pAT208 (*′vanS_B vanY_B vanW vanH_B′cat*)	ND[b]	ND	ND
BM4138	pAT209 (*′vanS_B vanY_B′cat*)	ND	ND	ND
BM4138::pAT207 (*P2 vanR_B vanS_B*)	pAT208 (*′vanS_B vanY_B vanW vanH_B′cat*)	ND	1,715 ± 968	ND
BM4138::pAT207 (*P2 vanR_B vanS_B*)	pAT78 (*cat*)	ND	ND	ND
BM4138::pAT207 (*P2 vanR_B vanS_B*)	pAT209 (*′vanS_B vanY_B′cat*)	ND	13,228 ± 1,369	ND
BM4138::pAT207 (*P2 vanR_B vanS_B*)	pAT210 (*′vanY_B vanW vanH_B′cat*)	ND	ND	ND

[a] CAT specific activity is expressed in nanomoles per minute per milligram of protein. Values are the means ± standard deviations of a minimum of three independent experiments. Induction was performed with 1 or 0.5 μg of VM or TE per ml, respectively.

[b] ND, not detectable. OD variations in the presence or absence of the substrate were not different.

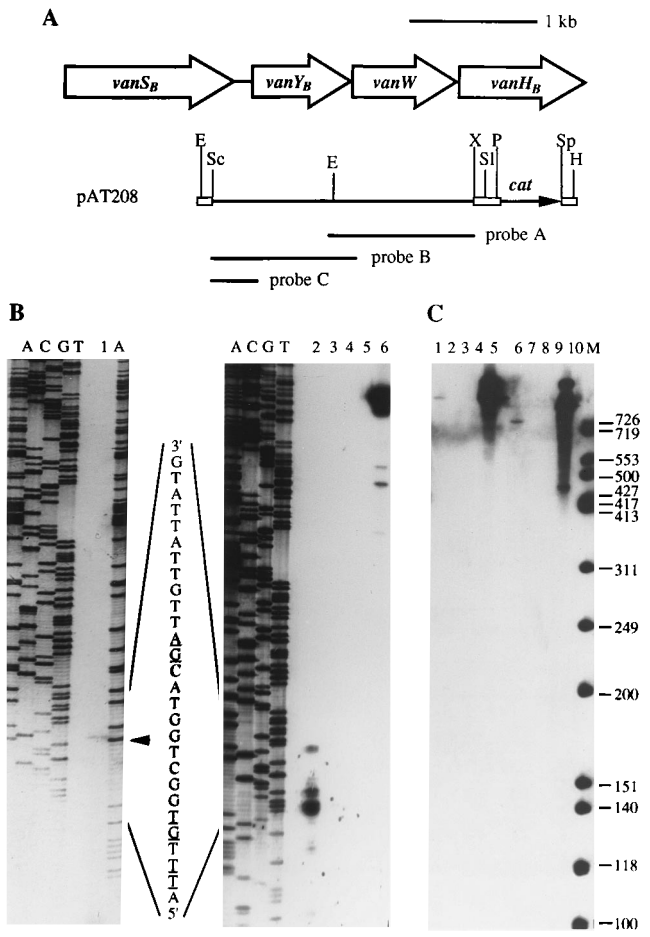

FIG. 6. Mapping of the 5′ terminus of mRNAs. (A) Construction of probes. Probes were prepared by primer extension from oligodeoxynucleotides VB39, VB25 (5′-ATATCGGTAATGCCACG, nucleotides 3090 to 3074), and VB41, with pAT28 DNA as a template. The probes were digested with *Eco*RI or *Sac*I. Restriction site abbreviations: E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; Sc, *Sac*I; Sl, *Sal*I; Sp, *Sph*I; X, *Xba*I. Open arrows represent coding sequences. Open boxes indicate polylinker sequences and are not drawn to scale. The arrow labeled *cat* represents the *cat* gene of pAT78. (B) S1 nuclease protection and primer extension mapping. The primer elongation product obtained with oligodeoxynucleotide VB41 and 50 μg of RNA from VM-induced *E. faecalis* V583 is shown in lane 1 and indicated by an arrowhead. RNA (50 μg) from VM-induced (lane 2) and uninduced (lane 3) *E. faecalis* V583 and JH2-2 (lane 4) hybridized with probe C. Control without RNA (lane 5) and undigested probe (lane 6) are also shown. Lanes A, C, G, and T indicate sequencing reactions with pAT206 DNA as a template and VB41 as a primer. The sequence indicated between the two panels is complementary to positions 2298 to 2325 of the sequence shown in Fig. 3. The 5′ extremities identified by S1 nuclease mapping are underlined. The 5′ extremity found by primer extension is indicated by bold lettering. (C) S1 nuclease protection mapping. RNA (50 μg) extracted from VM-induced (lanes 1 and 6) and uninduced (lanes 2 and 7) *E. faecalis* V583 and JH2-2 (lanes 3 and 8) hybridized with probes A (lanes 1, 2, and 3) and B (lanes 5, 6, and 7), respectively. Negative controls without RNA (lanes 4 and 9) and undigested probes A and B (lanes 5 and 10, respectively). The sizes of the radiolabeled size marker fragments (ϕX174/*Hin*fI; Promega) (lane M) are indicated on the right.

pAT207/pAT210, indicating that this band does not correspond to a transcript from a target promoter for $VanR_B$-$VanS_B$. Hybridization with probe B protected a fragment of approximately 750 bp. This would place the 5′ terminus of the mRNA into the region already identified by primer extension and S1 nuclease protection with probe C. Taken together, these results indicate that the transcriptional start site identified corresponds to the major site of transcription initiation for at least $vanY_B$, *vanW*, and $vanH_B$. A putative −10 sequence (TATAAT) was present 7 bp upstream from the 5′ mRNA extremity identified by primer extension (Fig. 3). No −35 sequence was found at a suitable position upstream from the −10 site. A GC-rich sequence with dyad symmetry followed by a stretch of T nucleotides that could constitute a ρ-independent terminator was found upstream from this putative promoter, suggesting that no readthrough takes place from the $vanR_B$ and $vanS_B$ genes into the downstream region. This possibility is supported by the S1 nuclease mapping results. No mRNA complementary to probes A, B, and C was detected by S1 nuclease protection in uninduced *E. faecalis* V583, confirming that transcription of resistance genes is VM dependent.

D,D-Carboxypeptidase activity of $VanY_B$. Plasmid pAT404 was constructed by cloning the structural gene for $VanY_B$ into expression vector pET-21(+) (Fig. 1) and was introduced into *E. coli* BL21(D3)/pLysS. The resulting strain allows IPTG-inducible $vanY_B$ expression. Induced or uninduced *E. coli* BL21(D3)/pLysS/pET-21(+) and uninduced BL21(D3)/pLysS/pAT404 produced low amounts of D,D-carboxypeptidase (Table 2). Upon induction with IPTG, the D,D-carboxypeptidase activity of the extracts from BL21(D3)/pLysS/pAT404 was readily detectable, indicating that $vanY_B$ encodes a VM-inducible D,D-carboxypeptidase that probably accounts for the previously described VM-inducible D,D-carboxypeptidase activity in VanB-type enterococci (20). As seen for VanY (47), $VanY_B$ enzymatic activity was mostly associated with the insoluble cell fraction and was released by solubilization with Triton X-100 (data not shown), suggesting that $VanY_B$ is membrane associated. The hydrophobicity profile (data not shown) revealed the presence of a stretch of hydrophobic amino acids near the N terminus that could constitute a membrane-associated region. Carboxypeptidase activity was not affected by penicillin G or ampicillin at concentrations of 10 mM and was inhibited only by very high concentrations of cefalotin (80% inhibition at 10 mM, 70% at 1 mM, and <20% at 0.1 mM) and cefoxitin (70% at 10 mM and <20% at 1 mM), confirming that $VanY_B$, like VanY, is insensitive to β-lactams. No significant similarity between $VanY_B$ and other carboxypeptidases could be detected, although motifs SXXK (amino acids 20 to 23), S(Y)XN (amino acids 263 to 265) and K(H/R)T(S)G (amino acids 61 to 63) present in all penicillin-interactive proteins (18) were also found in $VanY_B$, albeit not in the typical order.

Origin of the *van* genes. The high degree of similarity between the genes involved in VanA- and VanB-type resistance is confined to $vanH_B$, *vanB*, $vanX_B$, and their counterparts in the *vanA* gene cluster. Although both resistance types are regulated by two-component systems and are associated with D,D-carboxypeptidase production, the genes encoding these functions display only little structural similarity. Furthermore, no homolog of *vanW* is present in Tn*1546*, there is apparently

TABLE 2. D,D-Carboxypeptidase activity of extracts of *E. coli* BL21(D3)/pLysS harboring pET-21(+) or pAT404

Plasmid content (relevant characteristics)	Induction	Sp act of D,D-carboxypeptidase[a]	
		S100	C100
pET-21(+)	−	0.4	0.4
	+	ND[b]	0.2
pAT404 [pET-21(+)Ω$vanY_B$]	−	ND	0.4
	+	25	362

[a] Specific D,D-carboxypeptidase activity is expressed in nanomoles per minute per milligram of protein. Activities of the soluble (S100) and insoluble (C100) fractions of cell lysates centrifuged at 100,000 × *g* were determined.

[b] ND, not detectable (i.e., values lower than 0.2 nmol/min/mg of protein).

no gene corresponding to *vanZ* in *E. faecalis* V583, and *vanY* and $vanY_B$ are found in different locations relative to the other resistance genes. Although both clusters are carried by transposons (2, 33), these elements are structurally and functionally dissimilar. It thus appears that VM resistance determinants have not been acquired en bloc but result from the assembly of genes from various origins. These findings do not favor recent acquisition of glycopeptide resistance by enterococci from a naturally resistant organism. The complexity of gene organization and differences in gene order and degree of similarity rather hint to a longer evolutionary process in which various regulatory and structural genes were assembled in a modular fashion.

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