JOURNAL OF BACTERIOLOGY, Jan. 1997, p. 97–106 Vol. 179, No. 1
0021-9193/97/$04.00+0


# The VanS Sensor Negatively Controls VanR-Mediated Transcriptional Activation of Glycopeptide Resistance Genes of Tn*1546* and Related Elements in the Absence of Induction

MICHEL ARTHUR,[1]* FLORENCE DEPARDIEU,[1] GUY GERBAUD,[1] MARC GALIMAND,[1] ROLAND LECLERCQ,[2] AND PATRICE COURVALIN[1]

*Unité des Agents Antibactériens, Centre National de la Recherche Scientifique EP J0058, Institut Pasteur, 75724 Paris Cedex 15,[1] and Hôpital Henri Mondor, 94010 Créteil,[2] France*

Received 26 July 1996/Accepted 23 October 1996

**Transposon Tn*1546* from *Enterococcus faecium* BM4147 encodes a histidine protein kinase (VanS) and a response regulator (VanR) that regulate transcription of the *vanHAX* operon encoding a dehydrogenase (VanH), a ligase (VanA), and a D,D-dipeptidase (VanX). These last three enzymes confer resistance to glycopeptide antibiotics by production of peptidoglycan precursors ending in the depsipeptide D-alanyl-D-lactate. Transcription of *vanS* and the role of VanS in the regulation of the *vanHAX* operon were analyzed by inserting a *cat* reporter gene into *vanS*. Transcription of *cat* and *vanX* was inducible by glycopeptides in partial diploids harboring *vanS* and *vanSΩcat* but was constitutive in strains containing only *vanSΩcat*. Promoters $P_R$ and $P_H$, located upstream from *vanR* and *vanH*, respectively, were cloned into a promoter probing vector to study transactivation by chromosomally encoded VanR and VanS. The promoters were inactive in the absence of *vanR* and *vanS*, inducible by glycopeptides in the presence of both genes, and constitutively activated by VanR in the absence of VanS. Thus, induction of the *vanHAX* operon involves an amplification loop resulting from binding of phospho-VanR to the $P_R$ promoter and increased transcription of the *vanR* and *vanS* genes. Full activation of $P_R$ and $P_H$ by VanR was observed in the absence of VanS, indicating that the sensor negatively controls VanR in the absence of glycopeptides, presumably by dephosphorylation. Activation of the VanR response regulator in the absence of VanS may involve autophosphorylation of VanR with acetyl phosphate or phosphorylation by a heterologous histidine protein kinase.**

Inducible resistance to high levels of the glycopeptide antibiotics vancomycin and teicoplanin (VanA phenotype) in enterococci is mediated by Tn*1546* (Fig. 1A) or closely related transposons which are located in self-transferable plasmids (6) or in the chromosome as part of large conjugative elements (13). Resistance to glycopeptides is due to production of peptidoglycan precursors ending in the depsipeptide D-alanyl-D-lactate (D-Ala-D-Lac) instead of the dipeptide D-Ala-D-Ala found in glycopeptide-susceptible bacteria (1, 27). This substitution prevents formation of complexes between glycopeptides and peptidoglycan precursors at the cell surface that are responsible for inhibition of cell wall synthesis (9, 22). Tn*1546* encodes a dehydrogenase (VanH) and a ligase (VanA) that synthesize D-Ala-D-Lac, and a D,D-dipeptidase (VanX) that hydrolyzes D-Ala-D-Ala and thereby limits synthesis of precursors containing the target of glycopeptides (9, 23, 30). In addition to these enzymes required for resistance, Tn*1546* encodes a D,D-carboxypeptidase (VanY) which contributes to vancomycin resistance by hydrolyzing the C-terminal D-Ala residue of late peptidoglycan precursors and a protein of unknown function (VanZ) which confers low-level teicoplanin resistance (2, 4). These last two proteins are necessary for expression of resistance at a high level, since glycopeptides, in particular teicoplanin, remain partly active if the strains synthesize small amounts of D-Ala-D-Ala-ending precursors (3).

Analysis of transcriptional fusions indicated that expression of the resistance genes in Tn*1546* is activated by a two-component regulatory system (VanR-VanS) in response to the presence of glycopeptides in the culture medium (5). VanR is structurally related to response regulators of the OmpR subclass and is required for transcription initiation at promoter $P_H$, which allows cotranscription of *vanH*, *vanA*, and *vanX* (Fig. 1A) (5). DNase footprinting and gel shift experiments showed that phospho-VanR binds upstream from the transcription initiation site of $P_H$ and of a second promoter, $P_R$, located upstream from *vanR* (16). Phosphorylation increases the affinity of VanR for both promoters (16).

VanS consists of a putative membrane-associated N-terminal sensor domain and of a C-terminal cytoplasmic histidine protein kinase domain (5). The kinase domain, purified as a fusion protein with the maltose binding protein (MBP-VanS), catalyzes ATP-dependent autophosphorylation on a histidine residue (29). Phosphorylation of VanR was obtained by incubation of the protein with the phosphorylated form of MBP-VanS or with acetyl phosphate (29). Spontaneous dephosphorylation of VanR is slow in comparison to other response regulators, but MBP-VanS stimulates this reaction (29).

Previous attempts to evaluate the role of VanS in the modulation of VanR-mediated promoter activation were unsuccessful, since cloning of the regulatory genes on a multicopy plasmid vector led to constitutive expression of the *vanHAX* operon (5). In this report, regulation of the *van* gene cluster carried by natural plasmids was investigated by inserting a chloramphenicol acetyltransferase (*cat*) reporter gene into *vanS* or *vanA*. Strains of *Enterococcus faecalis* harboring a chromosomal copy of *vanR* or of *vanR* and *vanS* were also constructed to study transactivation of the $P_H$ and $P_R$ promoters located on plasmids. These complementary approaches

* Corresponding author. Mailing address: Unité des Agents Antibactériens, Institut Pasteur, 28, rue du Dr. Roux, 75724 Paris Cedex 15, France. Phone: 33 1 45 68 83 18. Fax: 33 1 45 68 83 19. Electronic mail address: Michel.Arthur@pasteur.fr.

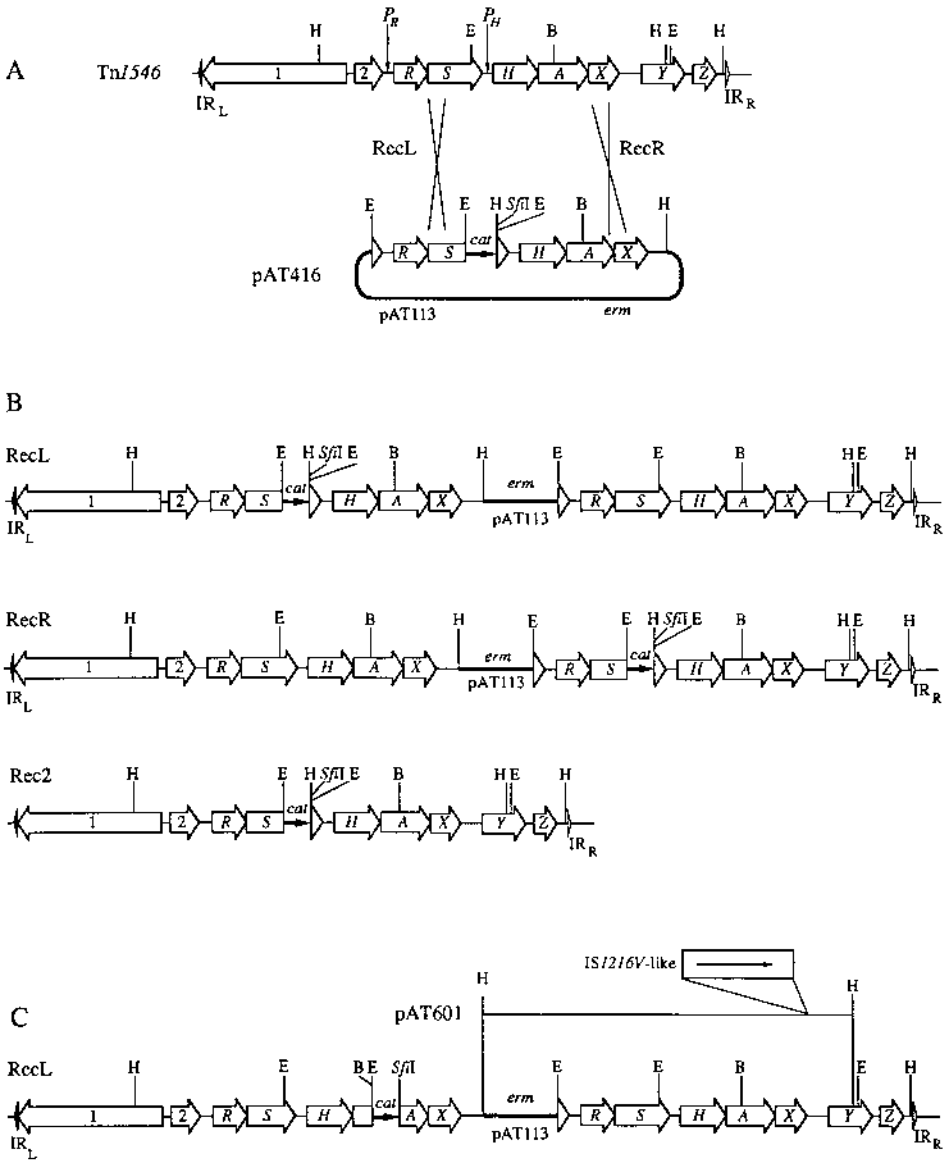


FIG. 1. Insertional inactivation of *vanS* and *vanA* by homologous recombination. (A) Maps of Tn*1546* and pAT416. Tn*1546* carries nine ORFs (indicated by arrows). 1, ORF1 (transposase); 2, ORF2 (resolvase); *R*, *vanR* (response regulator); *S*, *vanS* (histidine protein kinase); *H*, *vanH* (dehydrogenase); *A*, *vanA* (ligase); *X*, *vanX* (D,D-dipeptidase); *Y*, *vanY* (D,D-carboxypeptidase); and *Z*, *vanZ* (unknown function). The closed and open arrowheads (labeled $IR_L$ and $IR_R$) indicate the positions of the left and right terminal inverted repeats of Tn*1546*, respectively. The positions of the $P_R$ and $P_H$ promoters are indicated by vertical arrows. Plasmid pAT416 was obtained by inserting the $P_R$*vanRSP*$_H$*HAX* cluster into the suicide vector pAT113 conferring resistance to erythromycin (*erm* gene) and then by insertion of the *Eco*RI *cat* cassette into *vanS*. Homologous recombination between pAT416 and Tn*1546* can occur on either side of *cat*, as indicated by crosses labeled RecL and RecR. (B) Structures resulting from single (RecL or RecR) and double (Rec2) homologous recombination events. (C) Structure generated by RecL-type integration of pAT417($P_R$*vanRSP*$_H$*HA*Ω*catX*). The *Hin*dIII fragment of BM4315/pAT417RecL that was circularized to generate pAT601 is indicated by a thin line above the map. This fragment carries an IS*1216V*-like element (represented by an open box containing an arrow indicating the direction of transcription of the putative transposase gene). Restriction sites, B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII. The figure is not drawn to scale.

were used to study the regulation of *vanR* and *vanS* and the modulation of VanR-mediated promoter activation by VanS.

Since insertional inactivation of *vanS* and *vanA* by homologous recombination could not be obtained with plasmid pIP816-1 carrying Tn*1546*, the experiments were performed with Tn*1546*-related elements from four clinical isolates of *Enterococcus faecium* that were introduced into *E. faecalis* by conjugation. The structure and regulation of these elements were compared to evaluate if *van* gene clusters from clinical isolates of enterococci are conserved. The strategy used for gene inactivation could be of general interest, since it allows the regulation of target genes to be analyzed in their natural environment, determination of their copy number and localization, and cloning of flanking sequences.

## MATERIALS AND METHODS

**Strains, plasmids, and growth conditions.** The bacterial strains and plasmids used are described in Table 1. Strains were grown in brain heart infusion broth and agar (Difco Laboratories, Detroit, Mich.) at 37°C. The MICs of vancomycin and teicoplanin were determined by the method of Steers et al. (24) with $10^5$ CFU per spot on agar after 24 h of incubation.

**Analysis of enterococcal DNA.** Analysis of restriction profiles was performed on genomic DNA prepared in solution (19) or embedded in agarose plugs (21).

TABLE 1. Strains and plasmids

| Strain or plasmid | Relevant properties | Reference or source |
|---|---|---|
| *E. coli* | | |
| JM83 | *ara* Δ(*lac-pro*) *strA thi* ϕ*80* (*lacZΔM15*) | 31 |
| *E. faecalis* | | |
| JH2-2 | JH2 Fus$^r$ Rif$^r$ | 17 |
| BM4311 | pAT89($P_R$*vanRS*) inserted into the chromosome of JH2-2::Tn*916* | 5 |
| BM4312 | pAT420($P_2$*vanR*) inserted into the chromosome of JH2-2 | This study |
| BM4332 | pAT616($P_R$*vanRP$_H$HAXcat*) inserted into the chromosome of JH2-2 | This study |
| BM4300 | pAT394($P_R$*vanRSP$_H$HAXcat*) inserted into the chromosome of JH2-2 | 3 |
| BM4110/pIP816-1 | JH2 Str$^r$ harboring Tn*1546* on natural plasmid pIP816-1 | 3 |
| Plasmids | | |
| pAT80 | *Bgl*II-*Xba*I $P_R$*vanRSP$_H$HAXcat* fragment of Tn*1546* cloned into pAT78 | 5 |
| pAT78 | *oriR*pAMβ1 *oriR*pUC *oriT*RK2 *spc lacZ*α *cat* | 5 |
| pAT90 | $P_H$ promoter cloned into pAT78 | 5 |
| pAT418 | $P_R$ promoter cloned into pAT78 | This study |
| pAT87 | $P_H$ promoter and *vanHAX* operon cloned into pAT78 | 5 |
| pAT145 | *oriR*pAMβ1 *oriR*pUC *oriT*RK2 *int-Tn* Km$^r$ | 26 |
| pAT113 | *oriR*pACYC184 *oriT*RK2 *att-Tn* Em$^r$ Km$^r$ *lacZ*α | 26 |
| pAT394 | *Kpn*I-*Hin*dIII fragment ($P_R$*vanRSP$_H$HAXcat*) of pAT80 cloned into pAT113 | 4 |
| pAT414 | In vitro *Xba*I-*Sph*I deletion of the *cat* gene of pAT394 | This study |
| pUC1813 | Ap$^r$ *lacZ*α vector | 18 |
| pAT415 | *Eco*RI *cat* cassette cloned into pUC1813 | This study |
| pAT416 | Insertion of the *Eco*RI *cat* cassette into *vanS* of pAT414 | This study |
| pAT417 | Insertion of the *Bam*HI *cat* cassette into *vanA* of pAT414 | This study |
| pAT79 | *oriR*pAMβ1 *oriR*pUC *oriT*RK2 *spc lacZ*α $P_2$*cat* | 5 |
| pAT400 | *Eco*RI-*Hin*dIII fragment ($P_2$*cat*) of pAT79 cloned into pAT113 | This study |
| pUC19 | Ap$^r$ *lacZ*α vector | 31 |
| pAT419 | Amplified *vanR* gene cloned into pUC19 | This study |
| pAT420 | *Sac*I-*Hin*dIII fragment (*vanR*) of pAT419 cloned into pAT400 | This study |
| pAT614 | Amplified $P_R$*vanR* fragment cloned into pUC19 | This study |
| pAT615 | *Eco*RI-*Xba*I fragment ($P_R$*vanR*) of pAT614 cloned into pAT113 | This study |
| pAT616 | *Eco*RI-*Hin*dIII fragment ($P_H$*vanHAXcat*) of pAT80 cloned into pAT615 | This study |
| pAT601 | Circular *Hin*dIII fragment of BM4315/pAT417 | This study |
| pAT617 | Amplified $P_R$*vanRSP$_H$* fragment of BM4316 cloned into pUC19 | This study |
| pKK232-8 | *oriR*pBR Ap$^r$ Cm$^r$ Tc$^r$ | 8 |
| pAT600 | $P_R$*vanRSP$_H$HAXcat* insert of pAT80 cloned into pKK232-8 | This study |

Large restriction fragments were separated by pulsed-field gel electrophoresis according to the recommendations of the supplier of the Autobase system for zero integrated-field gel electrophoresis (TechGen, les Ulis, France). For Southern blot hybridization, DNA was transferred by vacuum onto a Nytran membrane (Schleicher and Schuell, Dassel, Germany) with a Trans Vac TE80 apparatus (Hoefer Scientific Instruments, San Francisco, Calif.). Hybridization was performed under stringent conditions (6) with plasmid DNA labeled with [α-$^{32}$P]dATP (3,000 Ci/mmol; Amersham Radiochemical Center, Amersham, England) by nick translation with a commercially available kit (Amersham International, Little Chalfont, Buckinghamshire, England).

**Plasmid construction.** Plasmid DNA isolation, digestion with restriction endonucleases (Boehringer, Mannheim, Germany), amplification of DNA by PCR with *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.), ligation of DNA fragments with T4 DNA ligase (Pharmacia Biotech, Saint-Quentin-en-Yvelines, France), nucleotide sequencing with T7 DNA polymerase (Pharmacia), and transformation of *Escherichia coli* JM83 with recombinant plasmids were performed by standard methods (7).

Plasmids pAT416 (Fig. 1A) and pAT417 were derivatives of integrative vector pAT113 (Em$^r$ Km$^r$ *att-Tn*) that carried the $P_R$*vanRSP$_H$HAX* gene cluster with a *cat* reporter gene inserted into *vanS* or *vanA*, respectively. To construct these plasmids, pAT394(pAT113Ω$P_R$*vanRSP$_H$HAXcat*) DNA was digested with *Xba*I and *Sph*I, treated with a DNA blunting kit (Amersham), and self-ligated to remove the *cat* gene downstream from *vanX*. The resulting plasmid, pAT414($P_R$*vanRSP$_H$HAX*), was independently digested with *Eco*RI or *Bam*HI and ligated with an *Eco*RI or a *Bam*HI-*Bgl*II *cat* cassette to generate pAT416($P_R$*vanRS*Ω*catP$_H$HAX*) or pAT417($P_R$*vanRSP$_H$HA*Ω*catX*), respectively. For construction of the *Eco*RI cassette, the *cat* gene of pAT80($P_R$*vanRSP$_H$HAXcat*) was amplified with oligodeoxyribonucleotides C1 and C2 (Unité de Chimie Organique, Institut Pasteur, Paris, France), digested with *Eco*RI, and cloned into the *Eco*RI site of pUC1813, generating pAT415. Primer C1 (5′ AGATGAATTCTAAGATAAAAATTT*AGGAGG*) contained an *Eco*RI site (underlined) and the ribosome binding site (RBS) of *cat* (italicized). Primer C2 (5′ AGTAGAATTC GGCCTTTTTGGCC AAGCTTTA*TTA* TAAAAGCCAGTC) comprised recognition sites for *Eco*RI, *Sfi*I, and *Hin*dIII (underlined) and the stop codon of *cat* (italicized). The *Bam*HI-*Bgl*II *cat* cassette was obtained by amplification of the *cat* gene of pAT415 with the reverse sequencing primer (Pharmacia) and oligodeoxyribonucleotide C3 (5′ AGTAA GATCT GGCCTTTTTGGCCTA*TTA*TAAAAGCCAGTC), which contained *Bgl*II and *Sfi*I sites (underlined) and the stop codon of *cat* (italicized).

For construction of pAT418 ($P_R$*cat*), the open reading frame 2 (ORF2)-*vanR* intergenic region of Tn*1546* was amplified by using oligodeoxyribonucleotides R1 and R2 as primers and DNA of plasmid pAT80($P_R$*vanRSP$_H$HAXcat*) as a template. Primers R1 (5′ CAAAGAATTCATACAGGAAATTATCAG) and R2 (5′ CAAAGAGCTCTCATCATCCACAATAAG) contained, respectively, *Eco*RI and *Sac*I restriction sites (underlined) that allowed directional cloning of $P_R$ upstream from the *cat* reporter gene of the shuttle promoter probing vector pAT78(*cat*). The 276-bp insert of the resulting plasmid, pAT418($P_R$*cat*), corresponded to nucleotides 3732 to 4007 of Tn*1546* and included the entire ORF2-*vanR* intergenic region (216 bp), a 3′ portion of ORF2 (28 bp), and a 5′ portion of *vanR* (32 bp). The 197-bp fragment of Tn*1546* (positions 3765 to 3961), previously used in gel shift experiments (16), was internal to the insert of pAT418($P_R$*cat*).

For construction of pAT420(pAT113Ω$P_2$*vanR*), the *vanR* gene of pAT80($P_R$*vanRSP$_H$HAXcat*) was amplified by PCR with oligodeoxyribonucleotides R3 (5′ CTAAGAGCTCCAAACTTATGTGAAAGG) and R4 (5′ GTAAT CTAGAATTATTTTTCAATTTTAT), which contained *Sac*I and *Xba*I restriction sites, respectively (underlined). The 722-bp amplified portion of Tn*1546* corresponded to nucleotides 3950 to 4671 of Tn*1546* and consisted of the *vanR* coding sequence, the translation stop codon, and 26 bp upstream from the initiation codon including the RBS. The *Sac*I-*Xba*I restriction fragment carrying *vanR* was cloned into pUC19 to generate pAT419(pUC19Ω*vanR*). Plasmid pAT400(pAT113Ω$P_2$*cat*) was constructed by cloning the *Eco*RI-*Hin*dIII fragment of pAT79 carrying $P_2$ and *cat* into pAT113 digested with the same enzymes. Plasmid pAT420(pAT113Ω$P_2$*vanR*) was obtained by replacing the *cat* gene of pAT400(pAT113Ω$P_2$*cat*) with the *vanR* gene of pAT419(pUC19Ω*vanR*) by using *Sac*I and *Hin*dIII.

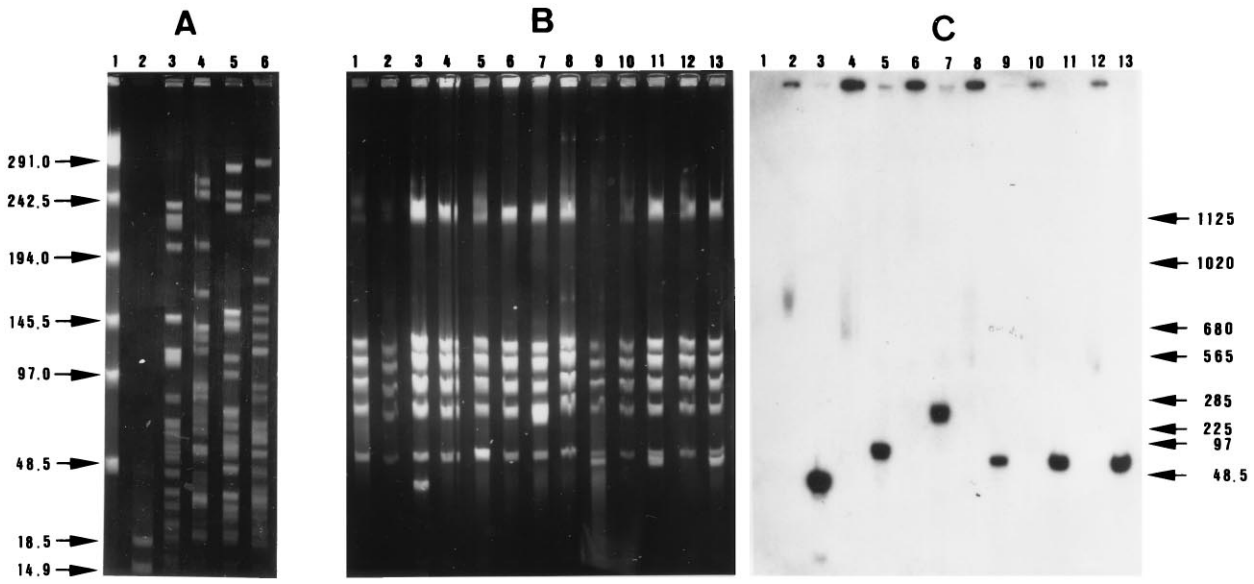


FIG. 2. Analysis of genomic DNA of enterococci by zero integrated-field gel electrophoresis. (A) *Sma*I restriction profiles of clinical isolates of *E. faecium*. Lanes: 1, concatemers of bacteriophage lambda cI857 Sam7 DNA (Bio-Rad, Hercules, Calif.); 2, Raoul molecular weight markers (Appligene, Illkirch, France); 3, HM1071; 4, HM1072; 5, HM1073; 6, HM1074. (B) *Sfi*I restriction profiles of *E. faecalis* transconjugants and their derivatives obtained by insertion of a *Sfi*I-tagged *cat* cassette into *vanS* or *vanA*. Lanes: 1, JH2-2 recipient; 2, BM4318; 3, BM4318(*vanA*Ω*cat*); 4, BM4319; 5, BM4319(*vanA*Ω*cat*); 6, BM4313; 7, BM4313(*vanS*Ω*cat*); 8, BM4314; 9, BM4314(*vanA*Ω*cat*); 10, BM4315; 11, BM4315(*vanA*Ω*cat*); 12, BM4316; 13, BM4316(*vanS*Ω*cat*). BM4318 and BM4319 are JH2-2 transconjugants not described in the text. (C) Southern blot hybridization of the gel in panel B with $^{32}$P-labeled DNA of pAT600($P_R$*vanRSP$_H$HAXcat*) as a probe. The sizes of the markers are indicated to the sides.

For construction of pAT616(pAT113Ω$P_R$*vanRP$_H$vanHAXcat*), the $P_R$ promoter and the *vanR* coding sequence of pAT80($P_R$*vanRSP$_H$HAXcat*) was amplified by PCR with oligodeoxyribonucleotides R1 and R4 (nucleotides 3732 to 4671 of Tn*1546*). The amplified fragment was digested with *Eco*RI and *Xba*I and cloned into pUC19 to generate pAT614(pUC19Ω$P_R$*vanR*). Plasmid pAT615(pAT113Ω$P_R$*vanR*) was constructed by cloning the *Eco*RI-*Xba*I fragment of pAT614 carrying $P_R$*vanR* into pAT113. Plasmid pAT616(pAT113Ω$P_R$*vanRP$_H$vanHAXcat*) was obtained by cloning the *Eco*RI-*Hin*dIII fragment of pAT80 carrying $P_H$*vanHAXcat* between the *Xba*I and *Hin*dIII sites of pAT615(pAT113Ω$P_R$*vanR*) after treatment of the protruding ends of restriction fragments with a DNA blunting kit (Amersham).

The nucleotide sequences of the inserts in pAT418 ($P_R$*cat*), pAT419 (*vanR*), and pAT614($P_R$*vanR*) were redetermined.

Plasmid pAT600($P_R$*vanRSP$_H$HAXcat*), which was used as a probe, was generated by cloning the *Eco*RI-*Hin*dIII fragment of pAT80($P_R$*vanRSP$_H$HAXcat*) into the pKK232-8 vector that does not contain DNA from a gram-positive bacterium.

**Strain construction.** Glycopeptide resistance was transferred from *E. faecium* clinical isolates to *E. faecalis* JH2-2 by filter mating with selection on rifampin (20 μg/ml), fusidic acid (10 μg/ml), and vancomycin (10 μg/ml). Plasmids pAT416($P_R$*vanRS*Ω*catP$_H$HAX*) and pAT417($P_R$*vanRSP$_H$HA*Ω*catX*) were introduced into the transconjugants by electrotransformation (10), and transformants resulting from a single recombination event (Fig. 1) were selected on erythromycin (10 μg/ml). The transformants were isolated on chloramphenicol (10 μg/ml), and individual colonies were screened for erythromycin susceptibility resulting from loss of pAT113 by a second recombination event (Fig. 1).

Integrative plasmid pAT420($P_2$*vanR*) was introduced into *E. faecalis* JH2-2/pAT145 (Km$^r$ *Int-Tn*) by electrotransformation, and clones resulting from chromosomal integration of pAT420 mediated by the Int-Tn integrase were selected on agar containing erythromycin (10 μg/ml). Spontaneous loss of pAT145 (Km$^r$ *Int-Tn*) was obtained by subculturing transformants for ca. 30 generations in antibiotic-free medium. Total DNA of a clone, designated BM4312($P_2$*vanR*), was digested with *Eco*RI and *Hin*dIII and analyzed by Southern hybridization with pAT420-labeled DNA as a probe. The data obtained (not shown) indicated the presence of a single chromosomal copy of pAT420($P_2$*vanR*). A similar approach was used to construct BM4332($P_R$*vanRP$_H$HAXcat*), which contained a single chromosomal copy of integrative plasmid pAT616. Southern blot analysis of the *Eco*RI and *Sac*II restriction profiles of genomic DNA of BM4332 was performed with pAT616-labeled DNA as a probe.

Plasmids pAT418($P_R$*cat*) and pAT90($P_H$*cat*) were introduced by electrotransformation into BM4311($P_R$*vanRS*) and BM4312($P_2$*vanR*). Plasmid DNA of clones selected on spectinomycin (60 μg/ml) was digested with *Eco*RI plus *Hin*dIII and compared to the restriction profiles of pAT418 and pAT90 purified from *E. coli* JM83 to screen for DNA rearrangements.

**Nucleotide sequence of a portion of the *van* gene cluster of BM4316.** Total DNA of BM4316 was amplified with oligodeoxyribonucleotides R5 (5′ CAAA GAGCTCATACAGGAAATTATCAG) and R6 (5′ ATGATCTAGA GGATC CACAGTAATGCCGATGTTATT), which contained *Sac*I and *Xba*I-*Bam*HI restriction sites, respectively (underlined). The amplified fragment was digested with *Sac*I and *Xba*I and cloned into pUC19 to generate pAT617. The sequence of the insert in pAT617 was determined and found to be identical to the corresponding portion of Tn*1546* (nucleotides 3732 to 6040) that includes the *vanR* and *vanS* genes and the $P_R$ and $P_H$ promoters.

**Enzyme assays.** For determination of D,D-dipeptidase and CAT specific activities, strains were grown in broth until the optical density at 600 nm reached 0.7. The culture medium contained vancomycin or teicoplanin at various concentrations for induction and spectinomycin (30 μg/ml) to counterselect loss of pAT78 and its derivatives. Bacteria were harvested by centrifugation (5,000 × *g* for 10 min at 4°C), washed in 0.1 M phosphate buffer (pH 7.0), treated with lysozyme, and lysed by sonication. The lysate was centrifuged (100,000 × *g* for 45 min at 4°C), and the supernatant (S100 extract) was assayed for VanX and CAT activity at 37°C, as described elsewhere (3). To measure VanX D,D-dipeptidase activity, the amount of D-Ala released from D-Ala-D-Ala (Bâle Biochimie, Voisins-le-Bretonneux, France) was determined with D-amino acid oxidase coupled to peroxidase as indicator reactions. Protein concentrations were estimated by the method of Bradford by the Bio-Rad (Richmond, Calif.) protein assay, with bovine serum albumin as a standard. Specific activity was defined as the number of nanomoles of product formed per minute per milligram of protein contained in S100 extracts.

## RESULTS

**Properties of the clinical isolates.** Genomic DNAs of the four strains of *E. faecium* displayed distinct *Sma*I restriction profiles (Fig. 2A), indicating that these clinical isolates were not clonally related. Glycopeptide resistance was transferable from each strain to *E. faecalis* JH2-2 by filter mating at frequencies ranging from $10^{-3}$ to $10^{-6}$ per donor (Table 2). The clinical isolates were resistant to various antibiotics, but glycopeptide resistance was transferred alone for three of the four isolates and was associated with tetracycline resistance for the remaining isolate.

**Insertional inactivation of the *vanS* and *vanA* genes.** Transformants resulting from integration of suicide plasmid pAT416

TABLE 2. MICs of glycopeptides

| Strain[a] | MIC (μg/ml) | |
|---|---|---|
| | Vancomycin | Teicoplanin |
| JH2-2 (recipient) | 2 | 1 |
| Clinical isolate HM1071 (KM $MLS_B$ P SM TC VanA) | 1,024 | 512 |
| Transconjugant BM4313 (240 kb, Tn*1546*-like, $10^{-6}$, TC)[b] | 512 | 64 |
| BM4313/pAT416RecR(*vanS*/*vanS*Ω*cat*) | 1,024 | 64 |
| BM4313(*vanS*Ω*cat*) | 1,024 | 128 |
| BM4313/pAT417RecL(*vanA*/*vanA*Ω*cat*) | 1,024 | 64 |
| BM4313(*vanA*Ω*cat*) | 2 | 1 |
| Clinical isolate HM1072 (CIP FOS KM $MLS_B$ P SM TC VanA) | 1,024 | 512 |
| Transconjugant BM4314 (50 kb, Tn*1546*-like, $10^{-3}$) | 256 | 64 |
| BM4314/pAT416RecL(*vanS*/*vanS*Ω*cat*) | 512 | 64 |
| BM4314(*vanS*Ω*cat*) | 512 | 64 |
| BM4314/pAT417RecL(*vanA*/*vanA*Ω*cat*) | 128 | 8 |
| BM4314(*vanA*Ω*cat*) | 2 | 1 |
| Clinical isolate HM1073 (KM P SM TC VanA) | 512 | 64 |
| Transconjugant BM4315 (50 kb, Tn*1546*::IS*1216V*-like, $10^{-3}$) | 64 | 8 |
| BM4315/pAT416RecR(*vanS*/*vanS*Ω*cat*) | 64 | 8 |
| BM4315(*vanS*Ω*cat*) | 64 | 8 |
| BM4315/pAT417RecL(*vanA*/*vanA*Ω*cat*) | 16 | 4 |
| BM4315(*vanA*Ω*cat*) | 2 | 1 |
| Clinical isolate HM1074 (CIP CM FOS KM P SM TC VanA) | 1,024 | 512 |
| Transconjugant BM4316 (50 kb, Tn*1546*-like, $10^{-3}$) | 256 | 64 |
| BM4316/pAT416RecR(*vanS*/*vanS*Ω*cat*) | 256 | 64 |
| BM4316(*vanS*Ω*cat*) | 256 | 64 |
| BM4316/pAT417RecL(*vanA*/*vanA*Ω*cat*) | 32 | 8 |
| BM4316(*vanA*Ω*cat*) | 2 | 1 |

[a] CIP, ciprofloxacin; CM, chloramphenicol; FOS, fosfomycin; KM, kanamycin; $MLS_B$, macrolide, lincosamide, and streptogramin B-type antibiotics; P, penicillin G; SM, streptomycin; TC, tetracycline; VanA, high-level resistance to glycopeptides.

[b] Characteristics of the plasmids in the transconjugants (size, type of Tn*1546*-related element, frequency of transfer per donor, and resistance marker associated with VanA).

($P_R$*vanRS*Ω*cat*$P_H$*HAX*) by homologous recombination into the *van* gene clusters of the four transconjugants were selected on erythromycin. Recombination can occur in two regions of the clusters generating the RecL and RecR structures depicted in Fig. 1B. In both cases, integration of pAT416 was expected to result in a partial duplication of the *van* gene cluster, which included a functional copy and an inactivated copy of the *vanS* gene (*vanS*/*vanS*Ω*cat*). Accordingly, integration of pAT416 ($P_R$*vanRS*Ω*cat*$P_H$*HAX*) did not significantly alter the MICs of vancomycin or teicoplanin (Table 2).

Clones resulting from a second intramolecular recombination event generating a single copy of the *van* gene cluster that carried *vanS* inactivated by *cat* (*vanS*Ω*cat*) were obtained by screening for resistance to chloramphenicol ($Cm^r$) and susceptibility to erythromycin ($Em^s$) (structure Rec2; Fig. 1B). The *vanS* gene was not required for resistance, since the $Cm^r$ $Em^s$ clones were resistant to glycopeptides (Table 2) and harbored a single insertionally inactivated copy of the gene (see below).

A similar approach was used to construct derivatives of the four transconjugants that harbored a functional copy and an inactivated copy of *vanA* following integration of pAT417 ($P_R$*vanRSP*$_H$*HA*Ω*catX*), or a single copy of the *van* gene cluster containing *vanA* inactivated by *cat* following a second recombination event. Insertional inactivation of *vanA* abolished glycopeptide resistance, confirming that *vanA* is required for resistance (Table 2).

**Localization of Tn*1546*-like elements in the transconjugants.** The *Sfi*I restriction profiles of genomic DNAs of the *E. faecalis* JH2-2 recipient (Fig. 2B; lane 1) and the four transconjugants (Fig. 2B, lanes 6, 8, 10, and 12) resolved by pulsed-field gel electrophoresis were indistinguishable. A probe made from pAT600($P_R$*vanRSP*$_H$*HAXcat*) DNA produced a strong hybridization signal close to the origin of migration (Fig. 2C, lanes 6, 8, 10, and 12). Thus, transfer of VanA-type resistance was not associated with any chromosome rearrangement, and plasmid DNA was not resolved. Insertion of the *cat* cassette, which contains an *Sfi*I restriction site, resulted in an additional DNA band which hybridized with the pAT600 probe (Fig. 2B and 2C, lanes 7, 9, 11, and 13). Since the probe contained sequences complementary to DNA on either side of the *Sfi*I site, hybridization to a single fragment indicated that the *van* gene clusters of the four transconjugants were carried by circular plasmids that did not contain an *Sfi*I site. Thus, introduction of the *Sfi*I-tagged *cat* gene cassette allowed selective linearization of the plasmids and direct estimation of their size (Table 2). This technique is more versatile than conventional analysis based on plasmid DNA purification that is often complicated by the presence of several plasmids in enterococci. The relative intensities of the plasmid and chromosomal DNA bands (Fig. 2B) suggest that the plasmids were present at low copy numbers per chromosome.

**Structure of Tn*1546*-related elements in the transconjugants and their derivatives.** The *Eco*RI and *Hin*dIII restriction profiles of genomic DNA of the clinical isolates and of the transconjugants were analyzed by Southern hybridization with labeled pAT600($P_R$*vanRSP*$_H$*HAXcat*) DNA as a probe (data not shown). No differences were observed between the donors and the corresponding transconjugants, indicating that transfer of glycopeptide resistance was not associated with DNA rearrangements in the *van*-related gene clusters. For three of the

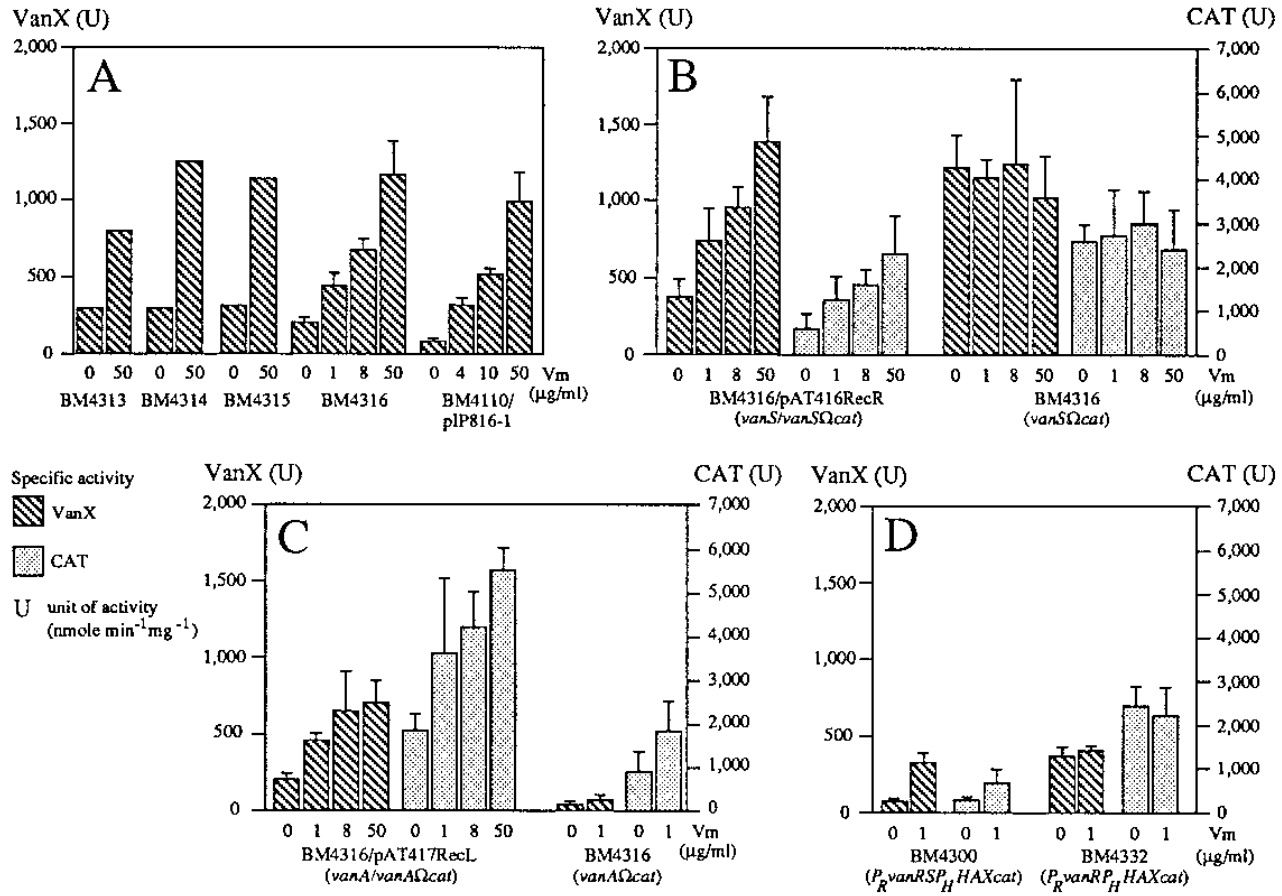


FIG. 3. VanX D,D-dipeptidase and CAT specific activities in transconjugants (A), derivatives of transconjugant BM4316 (B and C), and in BM4332 (D). Induction was performed with various concentrations of vancomycin (Vm), as indicated at the bottom of each panel. Enzymatic activity was expressed in nanomoles of product formed per minute and per milligram of protein in S100 extracts. Results are means ± standard deviations obtained from a minimum of three independent extracts.

four transconjugants, the profiles were indistinguishable from those expected for the presence of Tn*1546* in different sequence environments. The profiles of the remaining transconjugants (BM4315) revealed the presence of an ca. 0.8-kb insertion into the 4.1-kb *Eco*RI fragment of Tn*1546*. To characterize the insertion, genomic DNA of the BM4315 derivative obtained by integration of pAT417 (structure RecL; Fig. 1C) was digested with *Hin*dIII, ligated with T4 DNA ligase, and transformed into *E. coli* JM83. One of the resulting transformants harbored a plasmid, designated pAT601, that consisted of the pAT113 vector and adjacent Tn*1546*-related sequences. Nucleotide sequencing revealed an 808-bp insertion sequence (GenBank accession number U49512) that displayed 99.8% identity with IS*1216V* (13), an element already detected upstream from the truncated ORF1 of a Tn*1546*-related element in a clinical isolate of *E. faecium* (13). In BM4315, transposition of the IS*1216V*-like element generated an 8-bp duplication of the target sequence GTATTGAA, corresponding to nucleotides 8,724 to 8,731 of the *vanX-vanY* intergenic region of Tn*1546*. Occurrence of this IS*1216V*-like element provides an additional example of the invasion of *van* gene clusters of clinical isolates of enterococci by insertion sequences (13, 14). Acquisition of these genetic elements is the only type of polymorphism that has been detected in *van*-related gene clusters in spite of extensive restriction mapping and DNA sequencing (6, 12–14), suggesting that the spread of VanA-type resistance in enterococci is recent.

Southern blot analysis with the probe made from pAT600 ($P_R$*vanRSP*$_H$*HAXcat*) DNA was also performed on derivatives of the transconjugants obtained by integration of pAT416($P_R$*vanRSΩcatP*$_H$*HAX*) or pAT417($P_R$*vanRSP*$_H$*HAΩcatX*) or by insertion of *cat* into *vanS* or *vanA* following a second recombination event. The *Eco*RI and *Hin*dIII restriction profiles were consistent with the maps of the RecL, RecR, or Rec2 structures depicted in Fig. 1 (data not shown and Table 2). The analysis confirmed the presence of two alleles of *vanS* or *vanA* in strains resulting from integration of pAT416(*vanS*/*vanSΩcat*) or of pAT417(*vanA*/*vanAΩcat*). Strains resulting from a double recombination event contained only one inactivated copy of the genes (*vanSΩcat* or *vanAΩcat*).

**Regulation of gene expression in the transconjugants and their derivatives.** Induction by vancomycin led to similar levels of D,D-dipeptidase synthesis in the four transconjugants and in *E. faecalis* BM4110/pIP816-1 carrying Tn*1546* on the natural plasmid pIP816-1 (Fig. 3A). The nucleotide sequences of the *vanR* and *vanS* genes and of the $P_R$ and $P_H$ promoters of one representative transconsjugant (BM4316) were determined and were found to be identical to the sequence of Tn*1546* (see Materials and Methods).

Integration of pAT416($P_R$*vanRSΩcatP*$_H$*HAX*) into the *van* gene cluster of BM4316 (Fig. 1B) led to a moderate increase in D,D-dipeptidase specific activity (Fig. 3B). Synthesis of D,D-dipeptidase was inducible by vancomycin. Induction was similarly dependent on the concentration of the inducer in

TABLE 3. *Trans*-activation of the $P_H$ and $P_R$ promoters by VanR

| Host | Plasmid | CAT sp act (nmol $min^{-1}$ $mg^{-1}$) under the following inducing conditions[a] | | |
|---|---|---|---|---|
| | | Uninduced | Vancomycin | Teicoplanin |
| JH2-2 | pAT78(*cat*) | 230 ± 110 | 220 ± 79 | 310 ± 130 |
| | pAT79($P_2$*cat*) | 3,100 ± 1,000 | 3,300 ± 970 | 4,200 ± 900 |
| | pAT418($P_R$*cat*) | 830 ± 520 | 380 ± 160 | 550 ± 340 |
| | pAT90($P_H$*cat*) | 150 ± 49 | 150 ± 22 | 360 ± 170 |
| | pAT87($P_H$*HAXcat*) | 230 ± 35 | 390 ± 140 | 380 ± 260 |
| BM4311($P_R$*vanRS*) | pAT78(*cat*) | 450 ± 240 | 290 ± 70 | 230 ± 120 |
| | pAT418($P_R$*cat*) | 370 ± 97 | 3,700 ± 1,000 | 4,300 ± 1,400 |
| | pAT90($P_H$*cat*) | 160 ± 95 | 2,000 ± 620 | 7,900 ± 2,600 |
| | pAT87($P_H$*HAXcat*) | 390 ± 210 | 1,500 ± 340 | 2,900 ± 1,100 |
| BM4312($P_2$*vanR*) | pAT78(*cat*) | 120 ± 66 | 100 ± 19 | 29 ± 140 |
| | pAT418($P_R$*cat*) | 4,400 ± 1,200 | 5,300 ± 1,500 | 10,000 ± 1,300 |
| | pAT90($P_H$*cat*) | 10,000 ± 1,600 | 9,900 ± 2,700 | 15,000 ± 560 |
| | pAT87($P_H$*HAXcat*) | 9,700 ± 2,300 | 9,000 ± 2,400 | 10,000 ± 1,900 |

[a] Induction was performed with vancomycin (0.5 μg/ml) or teicoplanin (0.25 μg/ml). Results are the means ± standard deviations obtained from a minimum of three independent extracts.

BM4316/pAT416RecR(*vanS*/*vanS*Ω*cat*) (Fig. 3B) and in the transconjugant BM4316 (Fig. 3A). This was expected, since BM4316/pAT416RecR(*vanS*/*vanS*Ω*cat*) contained two copies of the gene encoding the D,D-dipeptidase and both a functional and a *cat*-inactivated copy of the regulatory gene *vanS* (Fig. 1B).

BM4316(*vanS*Ω*cat*) harbored a single copy of the *van* gene cluster with *cat* inserted into *vanS* (Fig. 1B; Rec2 structure). In this strain, the D,D-dipeptidase was constitutively synthesized at a high level (Fig. 3B) similar to that obtained for BM4316 after induction with the highest concentration of vancomycin tested (Fig. 3A). Thus, VanS prevents synthesis of VanX in the absence of glycopeptides.

Transcription of the *cat* reporter gene located within *vanS* in BM4316/pAT416RecR(*vanS*/*vanS*Ω*cat*) was inducible by vancomycin (Fig. 3B). The *cat* gene of BM4316(*vanS*Ω*cat*) was constitutively transcribed at a high level corresponding to the maximum induction in BM4316/pAT416RecR(*vanS*/*vanS*Ω*cat*). These results indicate that transcription of *vanS* is inducible by vancomycin and negatively regulated by VanS in the absence of glycopeptides.

Synthesis of CAT and of D,D-dipeptidase was inducible by vancomycin in strain BM4316/pAT417RecL(*vanA*/*vanA*Ω*cat*) (Fig. 3C), which resulted from RecL-type integration of pAT417 (Fig. 1C). The transcription level of *cat* inserted into *vanA* in this strain was ca. twofold higher than that of *cat* inserted into *vanS* in BM4316/pAT416RecR(*vanS*/*vanS*Ω*cat*). Thus, the *vanS* regulatory gene is not transcribed at a level much lower than that of the *vanHAX* operon.

Inducible synthesis of CAT and of D,D-dipeptidase was also detected in BM4316(*vanA*Ω*cat*) (Fig. 3C), which carries a single copy of *vanA* inactivated by *cat*. However, the level of synthesis of the enzymes was low, probably because of partial growth inhibition of this susceptible strain by the inducer. In addition, insertion of *cat* into *vanA* may have a polar effect on transcription of *vanX*, as observed for other transcriptional fusions (1a), although the *cat* cassette does not contain any known transcriptional terminator.

To determine if the glycopeptide resistance genes were regulated in a similar fashion in the other transconjugants, CAT and D,D-dipeptidase specific activities were determined for derivatives of BM4313, BM4314, and BM4315 obtained by integration of pAT416(*vanRS*Ω*catHAX*). As found for BM4316/pAT416RecR(*vanS*/*vanS*Ω*cat*) (Fig. 3B), synthesis of CAT and of D,D-dipeptidase was inducible by vancomycin for both RecL and RecR structures (data not shown). Likewise, insertion of *cat* into the *vanS* genes following a second recombination led to constitutive high-level synthesis of CAT and VanX in the four transconjugants. Thus, the regulation of *van* gene clusters from unrelated clinical isolates of *E. faecium* appears similar.

**Regulation of the *vanHAX* operon of Tn*1546* in the absence of VanS.** To confirm that transcription of the *vanHAX* operon of Tn*1546* is also constitutive in the absence of VanS, integrative plasmid pAT616(pAT113Ω$P_R$*vanRP*$_H$*vanHAXcat*) was inserted into the chromosome of *E. faecalis* JH2-2. The resulting strain, BM4332, carried a single copy of the *van* gene cluster containing a large deletion in *vanS* located between the stop codon of *vanR* and the *Eco*RI site 228 bases upstream from the *vanS* stop codon. Expression of the *vanX* and *cat* genes of BM4332($P_R$*vanRP*$_H$*vanHAXcat*) was constitutive (Fig. 3D). Production of VanX and CAT was inducible by vancomycin in BM4300($P_R$*vanRSP*$_H$*vanHAXcat*), which harbors a chromosomal copy of the same cluster with an intact *vanS* sensor gene. Comparison of the levels of expression of the reporter genes in BM4332(Δ*vanS*) and BM4300(*vanS*$^+$) (Fig. 3D) indicated that VanS negatively controls the *vanHAX* operon in the absence of glycopeptides, as was found for insertion inactivation of the *vanS* gene of BM4316 (Fig. 3B).

**Regulation of the $P_H$ and $P_R$ promoters by VanR and VanS.** DNA fragments containing the $P_R$ and $P_H$ promoters were cloned upstream from the *cat* reporter gene of the shuttle promoter probing vector pAT78(*cat*) to generate plasmids pAT418($P_R$*cat*) and pAT90($P_H$*cat*), respectively. The *cat* gene carried by these plasmids was transcribed at similar low levels in *E. faecalis* JH2-2, indicating that neither $P_H$ nor $P_R$ was active in the absence of VanR and VanS (Table 3).

Synthesis of CAT encoded by pAT418($P_R$*cat*) was inducible by glycopeptides in BM4311($P_R$*vanRS*), which is a derivative of JH2-2 harboring a chromosomal copy of the *vanR* and *vanS* genes expressed under the control of the native $P_R$ promoter. Thus, the $P_R$ promoter is regulated by the VanR-VanS two-component regulatory system leading to inducible transcription of *vanR*. As previously described (5), activation of the $P_H$ promoter in BM4311($P_R$*vanRS*) led to inducible transcription of *cat* downstream from $P_H$ in pAT90($P_H$*cat*) or from the *vanHAX* operon in pAT87($P_H$*vanHAXcat*). Transactivation of

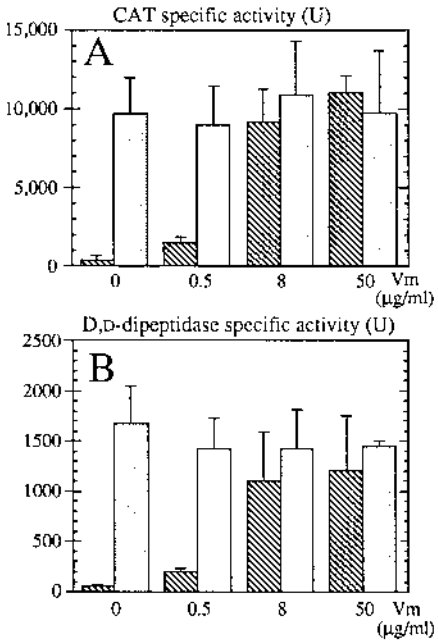


FIG. 4. CAT (A) and VanX D,D-dipeptidase (B) specific activities in extracts of BM4311($P_R$*vanRS*) and BM4312($P_2$*vanR*) harboring pAT87($P_H$*vanHAXcat*) (▨ and ▩, respectively). Induction was performed with various concentrations of vancomycin (Vm) as indicated at the bottom of each panel. Enzymatic activity was expressed as nanomoles of product formed per minute and per milligram of protein in S100 extracts. Results are the means ± standard deviations obtained from a minimum of three independent extracts.

$P_R$ and $P_H$ by the VanR-VanS system led to similar levels of CAT synthesis, suggesting that the strengths of the two promoters were comparable.

In order to analyze the regulation of the $P_R$ and $P_H$ promoters in the absence of *vanS*, the *vanR* coding sequence and RBS were cloned under the control of the $P_2$ promoter of the *aphA-3* kanamycin resistance gene and were introduced into the chromosome of *E. faecalis* JH2-2. The expression of *vanR* from the $P_2$ promoter was estimated to be equivalent to expression from the $P_R$ promoter under inducing conditions, based on comparison of CAT activities in strains JH2-2/pAT79($P_2$*cat*) and BM4311($P_R$*vanRS*)/pAT418($P_R$*cat*) (Table 3). In the resulting host strain used to analyze transcription from the $P_R$ and $P_H$ promoters, BM4312($P_2$*vanR*), the *cat* genes of plasmids pAT418($P_R$*cat*), pAT90($P_H$*cat*), and pAT87($P_H$*vanHAXcat*) were transcribed constitutively and at similarly high levels (Table 3). Thus, VanS was responsible for negative control of VanR-mediated activation of the $P_R$ and $P_H$ promoters in the absence of glycopeptides.

Analysis of transactivation of the *cat* reporter gene of pAT87($P_H$*vanHAXcat*) was performed in the presence of high concentrations of vancomycin (Fig. 4A). Similar levels of CAT specific activity were detected for constitutive or maximally induced transcription of the *cat* reporter gene of pAT87 in BM4312($P_2$*vanR*) and in BM4311($P_R$*vanRS*), respectively. Thus, VanR was able to fully activate the $P_H$ promoter in the absence of VanS. Determination of D,D-dipeptidase specific activity confirmed that the level of synthesis of VanX (Fig. 4B) increases with the level of transcription of the *vanHAX* operon, indicating that this activity can be used as a reporter of the activation of the $P_H$ promoter.

## DISCUSSION

Analysis of the regulation of glycopeptide resistance genes by the VanR-VanS two-component regulatory system revealed that the VanS sensor negatively controls VanR-mediated promoter activation in the absence of glycopeptides. The negative control was required for inducible expression of the vancomycin resistance genes, since high-level constitutive activation of the $P_R$ and $P_H$ promoters by VanR was obtained in the absence of VanS. We propose that the negative control is due to dephosphorylation of VanR by VanS. This hypothesis implies that VanR is activated by phosphorylation independently from VanS in the mutants carrying an inactivated copy of the *vanS* gene. Expression of the *vanHAX* operon was also found to be controlled by the level of synthesis of the regulatory proteins in addition to the expected modulation of VanR activity by VanS. Induction of resistance appears, therefore, to involve a cascade of events that includes increased synthesis of VanR, activation of the $P_H$ promoter, production of the resistance proteins, synthesis of pentadepsipeptide peptidoglycan precursors, and elimination of pentapeptide precursors (3). This sequence of events may require several hours, as estimated by the length of the lag phase separating the addition of the inducer from the capacity of enterococci to grow in the presence of glycopeptides (20).

Two complementary approaches were used to analyze the transcription of glycopeptide resistance genes carried by Tn*1546* and related elements. In the first approach, a *cat* reporter gene was inserted into *vanS* or *vanA* to inactivate these genes and to estimate their levels of transcription. The transcriptional fusions were introduced in *van*-related gene clusters of natural plasmids (Table 2; Fig. 2) by homologous recombination in order to avoid artifacts resulting from modification of the copy number or of the environment of the resistance genes. A partial duplication of the *van* gene cluster containing both a functional copy and an inactivated copy of *vanS* was generated by integration of the suicide plasmid pAT416($P_R$*vanRS*Ω*catP*$_H$*HAX*) (Fig. 1). A second homologous recombination event produced a single copy of the cluster containing *vanS* inactivated by *cat*. These constructs allowed the study of the regulation of *vanS* in the presence or in the absence of a functional copy of the gene (Fig. 3). D,D-Dipeptidase specific activity was determined to estimate the levels of transcription of the *vanHAX* operon in these strains. In the second approach, transactivation of the isolated $P_R$ and $P_H$ promoters by chromosomally encoded VanR or VanR and VanS was analyzed (Table 3; Fig. 4). The two strategies resulted in the same finding, indicating that modulation by VanS of VanR-mediated promoter activation accounts for transcriptional control of the *vanR* and *vanS* regulatory genes and of the *vanHAX* operon. In addition, comparison of *van* gene clusters from unrelated clinical isolates of *E. faecium* revealed a high degree of conservation at both the structural and the functional levels.

Transcription of the *cat* reporter gene located in *vanS* was inducible by vancomycin in BM4316/pAT416RecR(*vanS*/*vanS*Ω*cat*), which carries both an inactivated copy and an intact copy of *vanS* (Fig. 3B). In the presence of glycopeptides, the VanR-VanS two-component regulatory system encoded by the chromosome of BM4311($P_R$*vanRS*) transactivated the $P_R$ promoter of pAT418($P_R$*cat*) (Table 3). Thus, transcription of the *vanR* and *vanS* genes was inducible by glycopeptides and coordinately regulated. An amplification loop results from activation of the $P_R$ promoter and increased synthesis of VanR. As was found for other two-component regulatory systems (25), regulation of the *van* gene cluster involves modulation of both the absolute concentration of the response regulator and the

relative concentrations of its phosphorylated and nonphosphorylated forms.

Constitutive synthesis of CAT and of D,D-dipeptidase was detected in BM4316(*vanSΩcat*), which harbored a single copy of the *vanS* gene inactivated by *cat* (Fig. 3B), and in BM4332($P_R$*vanRP*$_H$*vanHAXcat*), which contained a large deletion in the *vanS* gene (Fig. 3D). Likewise, production of VanR encoded by the chromosome of BM4312($P_2$*vanR*) led to constitutive transactivation of the $P_R$ and $P_H$ promoters carried by plasmids pAT418($P_R$*cat*), pAT90($P_H$*cat*), and pAT87($P_H$ *vanHAXcat*) (Table 3). In both analyses, the constitutive levels of transcription of the reporter gene were similar to those obtained after maximal induction in strains producing VanS (Fig. 3 and 4). These results indicate that VanS negatively controls VanR-mediated activation of the $P_R$ and $P_H$ promoters in the absence of glycopeptides and that VanS is not required for full activation of the promoters. Dephosphorylation of VanR by VanS is likely to be responsible for this negative control, since VanS catalyzes this reaction in vitro (29). Although direct evidence that phospho-VanR is the actual activator has not yet been obtained, response regulators of the OmpR subclass are thought to be activated by phosphorylation (15), and phosphorylation of VanR increases the affinity of the regulator for the $P_R$ and $P_H$ promoter regions (16). Phosphorylation of VanR by acetyl phosphate or by a histidine protein kinase encoded by the *E. faecalis* chromosome may provide alternative pathways for VanR activation in the absence of VanS (5, 11, 29). In *E. coli*, both modes of activation of the PhoB response regulator allow expression of the *pho* regulon in the absence of the PhoR histidine protein kinase (28). Although these alternative pathways are not expected to be very efficient (11, 15), two factors may contribute to the accumulation of phospho-VanR in the absence of VanS. Spontaneous hydrolysis of phospho-VanR is remarkably slow (half-life, 10 to 12 h) in comparison to other response regulators (29). In addition, the amplification loop resulting from activation of the $P_R$ promoter by VanR and increased synthesis of VanR may compensate for inefficient phosphorylation of the regulator. Taken together, these observations indicate that the phosphorylated form of VanR may accumulate in significant amounts in the absence of VanS, leading to constitutive activation of the $P_R$ and $P_H$ promoters at a high level in *vanS* mutants constructed in vitro. However, VanR may be predominently phosphorylated by VanS in strains harboring the intact *van* gene cluster. This mode of activation could be required in certain environmental conditions if the intracellular concentration of acetyl phosphate is low and the putative heterologous histidine protein kinase responsible for cross-talk is not active because the corresponding pathway is not turned on. The VanR-VanS two-component regulatory system potentially faces various cross-talk conditions in different hosts, since the *van* gene cluster is carried by conjugative plasmids. Efficient phosphorylation of VanR by VanS in the presence of glycopeptides coupled with efficient dephosphorylation in the absence of the antibiotics is expected to allow inducible expression of the cluster in any host.

In vitro analysis revealed striking differences between the $P_R$ and $P_H$ promoters (16). S1 nuclease protection experiments indicated that VanR and phospho-VanR bind to a similar 80-bp region of the $P_H$ promoter that contains two putative 12-bp VanR binding sites (16). In contrast, the $P_R$ promoter contains a single 12-bp binding site, and phosphorylation of VanR enlarges the size of the protected region from 20 to 40 bp (16). Gel shift experiments showed that the effective concentrations of VanR and phospho-VanR able to saturate a DNA fragment carrying the $P_H$ promoter at 50% were ca. 20 and 0.04 μM, respectively (16). Higher effective concentrations able to saturate at 50% were obtained for the binding of VanR (100 μM) and phospho-VanR (1.6 μM) to the $P_R$ promoter. In spite of these differences, regulation of the $P_R$ and $P_H$ promoters by VanR and VanS appeared similar in vivo (Table 3). Likewise, transcription of the *cat* gene inserted into *vanS* or *vanA* in derivatives of BM4316 was induced at comparable levels by various concentrations of vancomycin (Fig. 3). Thus, there was no indication that the VanR-VanS system might regulate the $P_R$ and $P_H$ promoters differently. Of note, these observations imply that the *vanR* and *vanS* regulatory genes and the *vanHAX* operon are transcribed at similar high levels.

It has been proposed that binding of phospho-VanR to the $P_R$ promoter may result in repression rather than in activation, since the region protected from S1 nuclease digestion by phospho-VanR overlaps the putative −35 (TTATGT) RNA polymerase binding site and since this putative −35 sequence and the −10 (TATAAT) sequence are close to the consensus sequence for $\sigma^{70}$ promoters (16). On the contrary, our analysis indicated that the $P_R$ promoter was activated by VanR. It remains to be evaluated whether differential gene expression from the $P_R$ and $P_H$ promoters occurs in certain conditions.

## ACKNOWLEDGMENTS

We thank R. Quintiliani Jr. for helpful discussions and V. Loncle-Provot and the students of the course Bactériologie-1995 for their contribution to the experiments described in this report, in particular H. Bermudes, C. Carvalho, B. Perichon, and L. Schlegel for sequencing the IS*1216V*-related element.

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