JOURNAL OF BACTERIOLOGY, June 2001, p. 3436–3446 Vol. 183 No. 11
0021-9193/01/$04.00+0 DOI: 10.1128/JB.183.11.3436–3446.2001
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Regulation of Expression of the *vanD* Glycopeptide Resistance Gene Cluster from *Enterococcus faecium* BM4339

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Received 17 November 2000/Accepted 13 March 2001

A new open reading frame, encoding a putative integrase-like protein, was detected downstream from the six genes of the *vanD* glycopeptide resistance cluster in *Enterococcus faecium* BM4339 (B. Casadewall and P. Courvalin, J. Bacteriol. 181:3644–3648, 1999). In this cluster, genes coding for the VanR$_D$-VanS$_D$ two-component regulatory system were cotranscribed from the P_{R_D} promoter, whereas transcription of the *vanY$_D$*, *vanH$_D$*, *vanD*, *vanX$_D$*, and *intD* genes was initiated from the P_{Y_D} promoter located between *vanS$_D$* and *vanY$_D$* (the D subscript indicates that the gene is part of the *vanD* operon). The VanR$_D$-VanS$_D$ regulatory system is likely to activate transcription of the resistance genes from the promoter P_{Y_D}. Glycopeptide-susceptible derivatives of BM4339 were obtained by *trans* complementation of the frameshift mutation in the *ddl* gene, restoring functional D-alanine:D-alanine ligase activity in this strain. The glycopeptide-susceptible transformant BM4409, producing only D-alanyl-D-alanine-terminating peptidoglycan precursors, did not express the resistance genes encoding the VanY$_D$ D,D-carboxypeptidase, the VanH$_D$ dehydrogenase, the VanD ligase, the VanX$_D$ D,D-dipeptidase, and also the IntD integrase, although the regulatory region of the *vanD* cluster was still transcribed. In BM4409, the absence of VanR$_D$-VanS$_D$, apparently dependent, transcription from promoter P_{Y_D} correlated with the lack of D-alanyl-D-lactate-terminating precursors. The *vanX$_D$* gene was transcribed in BM4339, but detectable amounts of VanX$_D$ D,D-dipeptidase were not synthesized. However, the gene directed synthesis of an active enzyme when cloned on a multicopy plasmid in *Escherichia coli*, suggesting that the enzyme was unstable in BM4339 or that it had very low activity that was detectable only under conditions of high gene dosage. This activity is not required for glycopeptide resistance in BM4339, since this strain cannot synthesize D-alanyl-D-alanine.

Glycopeptides inhibit the late stages of peptidoglycan assembly (27, 34). Since these large, hydrophilic molecules cannot penetrate the cytoplasmic membrane of the cell, their antibacterial action results from forming complexes with the D-alanyl-D-alanine (D-Ala-D-Ala) C termini of peptidoglycan precursors on the external side of the membrane (9). The formation of these complexes prevents the cross-linking reactions catalyzed by transglycosylases, D,D-transpeptidases, and D,D-carboxypeptidases and prevents cell wall assembly.

Resistance to glycopeptides in enterococci is mediated by the synthesis of modified peptidoglycan precursors (9). Two types of precursors have been identified: (i) those ending in the depsipeptide D-alanyl-D-lactate (D-Ala-D-Lac), which exhibit a 1,000-fold-lower binding affinity for vancomycin, and (ii) precursors terminating with D-alanyl-D-serine, whose reduced affinity (7-fold) results from steric hindrance. Resistance to glycopeptides by production of D-Ala-D-Lac-terminating precursors can be categorized into three types: VanA, VanB, and VanD (9, 31). Although all three types involve genes encoding related enzymatic functions, they are distinguishable by the location of those genes, either on plasmids or on the chromosome or both, and by the different mechanisms of gene expression and regulation (9). Enterococci belonging to the VanA type are inducibly resistant to high levels of both vancomycin and teicoplanin, whereas VanB-type enterococci show inducible resistance to various levels of vancomycin only. The VanD type is unique and is characterized by constitutively (30, 31) or inducibly (29) expressed resistance to moderate levels of vancomycin (MICs, 16 to 256 mg/liter) and teicoplanin (MICs, 2 to 64 mg/liter). These three types of glycopeptide resistance are mediated by the gene clusters *vanA, vanB*, and *vanD*. Each cluster consists of five essential genes and one or two additional genes encoding functions that are not required to achieve resistance (9). In susceptible strains, the chromosomally encoded D-Ala:D-Ala ligase (Ddl) synthesizes the dipeptide incorporated into peptidoglycan precursors that are the target of glycopeptides. In resistant enterococci, synthesis of peptidoglycan precursors utilizes an alternative pathway that avoids these D-Ala-D-Ala precursors and relies instead on D-Ala:D-Lac ligases (VanA, VanB, and VanD). These enzymes are capable of synthesizing modified precursors that compete with the normal ones for their incorporation into the cell wall but escape glycopeptide binding. The dehydrogenases VanH, VanH$_B$, and VanH$_D$ convert pyruvate into D-Lac, which is in turn used by the D-Ala:D-Lac ligases (6, 12). In the VanA and VanB types, two D,D-peptidases (VanX and VanY, VanX$_B$ and VanY$_B$) increase resistance by sequentially eliminating the normal peptidoglycan precursors (1). First, the VanX and VanX$_B$ D,D-dipeptidases hydrolyze the dipeptide D-Ala-D-Ala (16, 35). This action is supplemented by the hydrolysis of the residual pentapeptide precursors by the VanY and VanY$_B$ D,D-carboxypeptidases that are not essential but increase the levels of resistance to glycopeptides (5, 16). Genes encoding a

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
E. coli		
TB1	JM83 *hsdR*(r_K^- m_K^+)	Life Technologies
DH5α	*recA1 gyrA* (Nal^r) Δ(*lacIZYA-argF*) [φ80*dlac*Δ(*lacZ*)M15]	43
E. faecalis JH2-2	Fus^r Rif^r	21
E. faecium		
BM4339	Vm^r Te^r	31
BM4409	BM4339/pAT662	14
BM4458	BM4339::pAT665	This work
BM4459	BM4339::pAT665	This work
Plasmids		
pUC18	Ap^r, *lacZ*α vector	42
pBGS18+	Km^r, *lacZ*α vector	39
pAT78	Sp^r, *oriR* from pAMβ1, *oriR* from pUC, *oriT* from RK2, *lacZ*α *cat*	7
pAT79	Sp^r, *oriR* from pAMβ1, *oriR* from pUC, *oriT* from RK2, *lacZ*α P_2*cat*	7
pAT113/Sp	Sp^r Mob$^+$ (IncP), *oriR* from pACYC184, *att* Tn*1545 lacZ*α	15
pAT145	Km^r Mob$^+$, *oriR* from pAMβ1, *oriR* from pBR322, *oriT* from RK2, *int-Tn1545*	41
pAT632	Cm^r derivative of pAT145 Δ*oriT* RK2	This work
pAT654	5.3-kb *Sau*3AI fragment ($vanS_D'vanY_DH_DDX_D$) of BM4339 cloned into pUC18	14
pAT655	2.85-kb *Xma*I-*Hin*dIII fragment ($vanX_D'int_D$) of BM4339 cloned into pUC18	This work
pAT659	0.7-kb PCR fragment ($vanX_D$) of pAT654 cloned into pUC18	This work
pAT660	1.15-kb PCR fragment ($vanY_D$) of pAT654 cloned into pBGS18+	This work
pAT662	1.2-kb *Sac*I-*Xba*I fragment (*ddl*) of BM4147 cloned into pAT79	14
pAT665	1.45-kb *Eco*RI fragment (P_2*ddl*) of pAT662 cloned into pAT113/Sp	This work
pAT666	160-bp PCR fragment (P_{Y_D}) of pAT654 cloned into pAT78	This work

D,D-dipeptidase and a D,D-carboxypeptidase have also been identified in the *vanD* cluster, but their roles in resistance are unclear (14). All three clusters include genes for a two-component regulatory system, *vanR-vanS*, $vanR_B$-$vanS_B$, and $vanR_D$-$vanS_D$ (7, 14, 16). The mechanisms by which these systems regulate expression of resistance genes have been elucidated for both the VanA and VanB types. The VanS and $VanS_B$ histidine protein kinases are sensor proteins that, in the presence of both vancomycin and teicoplanin or only in the presence of vancomycin, control the phosphorylation level of their cognate VanR and $VanR_B$ response regulators, which in turn trigger transcription of the resistance genes from the promoters P_R and P_H (7, 20) and P_{R_B} and P_{Y_B} (16, 38). Additional genes have been identified in both the *vanA* and *vanB* clusters. Expression of *vanZ* is responsible for low-level teicoplanin resistance in VanA-type enterococci (3), whereas the function of the *vanW* gene product in VanB-type strains is still unknown (16).

Enterococcus faecium BM4339 was the first clinical isolate shown to harbor the *vanD* gene cluster (14, 31). The gene organization (14) and chromosomal location of the cluster (30) in this strain have been elucidated, and expression of resistance has been partially studied (31). Unlike VanA- and VanB-mediated resistance, VanD-type resistance is expressed constitutively, proposed to be the result of nonstringent control of the phosphorylation level of $VanR_D$ by the putative phosphatase activity of $VanS_D$ (14, 31). In addition, determination of the sequence of the chromosomal *ddl* gene in BM4339 revealed a frameshift mutation, likely to generate an inactive product that would be responsible for the lack of peptidoglycan precursors ending in D-Ala-D-Ala (14, 31). Constitutive expression of resistance accounts for the lack of dependence on glycopeptides for growth of BM4339. The presence of an apparently intact $vanX_D$ gene has been reported (14). However, D,D-dipeptidase activity was not detected in cytoplasmic or membrane extracts from BM4339 (31). Although such an activity is not essential in a genetic background lacking the ability to synthesize D-Ala-D-Ala, these results prompted us to probe the mechanisms responsible for expression of glycopeptide resistance in BM4339.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. The bacterial strains and plasmids used are described in Table 1. Unless specified, *Escherichia coli* TB1 (Focus, Life Technologies Inc., Gaithersburg, Md.) and *E. coli* DH5α (43) were used as host strains in cloning experiments. Bacteria were cultured in brain heart infusion broth or agar (Difco Laboratories, Detroit, Mich.) at 37°C. The method of Steers et al. (40) was used to determine the MICs of antibiotics with 10^5 CFU per spot on Mueller-Hinton agar (Bio-Rad, Marnes-la-Coquette, France) after 24 h of incubation.

Recombinant DNA techniques. Plasmid DNA isolation, cleavage of DNA with restriction endonucleases (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, England, and Gibco BRL-Life Technologies Inc.), purification of restriction fragments from agarose gel, dephosphorylation of vector DNA with calf intestinal phosphatase (Amersham Pharmacia Biotech), and ligation with T4 DNA ligase (Amersham Pharmacia Biotech) were performed by standard methods (36).

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

(i) Plasmid pAT655. To complete the sequence downstream from the $vanX_D$ gene, *E. faecium* BM4339 total DNA was digested with *Xma*I and *Hin*dIII and the size of the fragment hybridizing with a 304-bp probe corresponding to the 3′ end of the $vanX_D$ gene (Fig. 1A) was estimated (36). To identify recombinant plasmids, clones were screened by colony hybridization (36) with the same probe. Plasmid pAT655 contained a 2.85-kb *Xma*I-*Hin*dIII insert.

(ii) Plasmids pAT659 and pAT660. The $vanX_D$ and $vanY_D$ genes were amplified using primer pairs XD3-XD4 and YD4-YD5, respectively, and plasmid

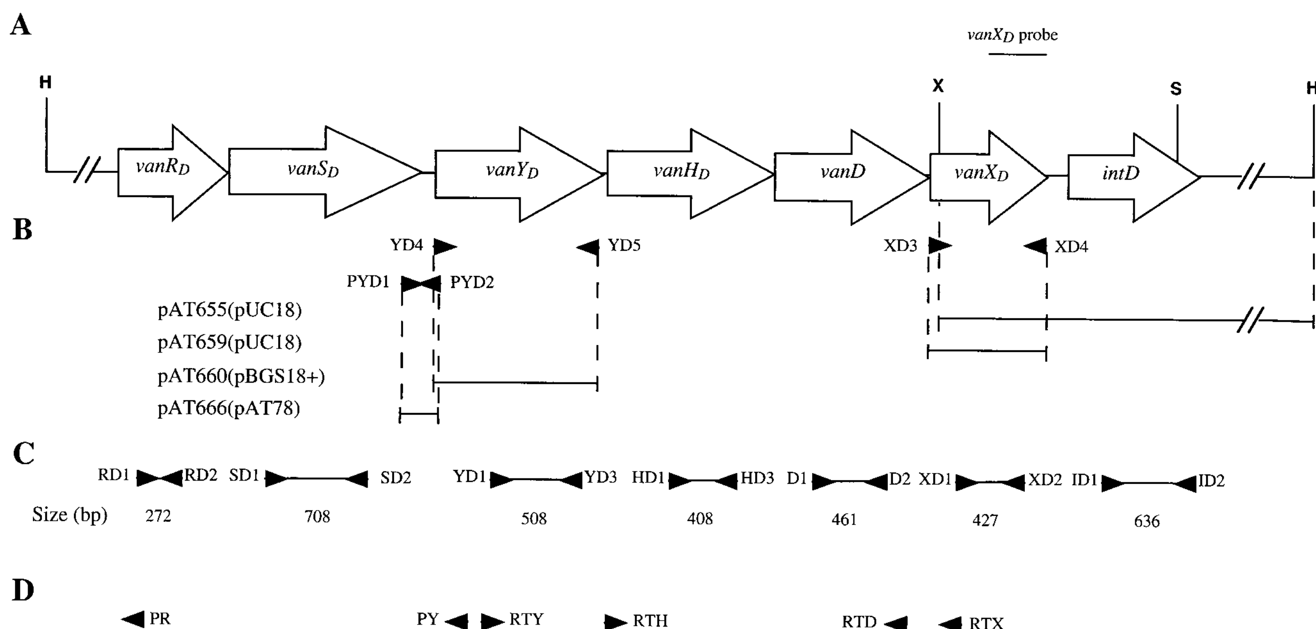

FIG. 1. Schematic representation of the *vanD* gene cluster in BM4339. (A) Map of the 8.7-kb region containing the $vanR_D$, $vanS_D$, $vanY_D$, $vanH_D$, *vanD*, $vanX_D$, and *intD* ORFs comprising the *vanD* gene cluster. Open arrows indicate sense of transcription. The PCR fragment internal to $vanX_D$ used as a probe in hybridization experiments is indicated. Abbreviations: H, *Hin*dIII; S, *Sau*3AI; X, *Xma*I. (B) Inserts in recombinant plasmids. The inserts are represented by solid lines, and the vectors are indicated in parentheses. (C) Probes used in Northern hybridization and in RT-PCR. (D) Oligodeoxynucleotides used in RT-PCR and in primer extension. Arrowheads indicate positions and orientations of primers.

pAT654 DNA (Table 1) as a template. Oligodeoxynucleotide XD3 (5′cgacgcgaattCGGTTTTACGCTTTCTGA) contained an *Eco*RI site (underlined) and 18 bases complementary to the sequence upstream from $vanX_D$ (in uppercase letters). Oligodeoxynucleotide XD4 (5′ agcggaagcTTCATTTCTTCAGGCTC) harbored a *Hin*dIII site (underlined) and 17 bases complementary to the sequence downstream from $vanX_D$ (in uppercase letters). Primer YD4 (5′ gagtgcgaattcttgATATCAGGAGGGGCGAT) contained an *Eco*RI site (underlined), a translation stop codon (italicized), and 17 bases complementary to the sequence upstream from $vanY_D$ (in uppercase letters). Primer YD5 (5′ gcagcgaagcttCTCCATTCATTTCCTCCTT) included a *Hin*dIII site (underlined) and 19 bases complementary to the sequence downstream from $vanY_D$ (in uppercase letters). The $vanX_D$ and $vanY_D$ PCR products were digested with *Eco*RI and *Hin*dIII and cloned into pUC18 and pBGS18+, respectively. Sequencing of the inserts and of the flanking regions confirmed that the two genes were under the control of the *lac* promoter of pUC18 and pBGS18+.

(iii) Plasmid pAT666. The P_{Y_D} promoter region of the *vanD* gene cluster was amplified by PCR using primers PYD1 and PYD2 and plasmid pAT654 DNA as a template. Oligodeoxynucleotide PYD1 (5′agagtcgaattcTTGAGGTTACATTGCCCG) harbored an *Eco*RI site (underlined) and 18 bases complementary to the 3′ end of $vanS_D$ (in uppercase letters). Oligodeoxynucleotide PYD2 (5′ gcactcgagctcAAAAAAATCGCCCCTCCT) contained a *Sac*I site (underlined) and 18 bases complementary to the sequence just upstream from the 5′ extremity of $vanY_D$ (in uppercase letters). After digestion of the 160-bp PCR product by *Eco*RI and *Sac*I, the fragment was cloned into similarly digested pAT78 DNA. The recombinant plasmid pAT666 was introduced into *Enterococcus faecalis* JH2-2 and *E. faecium* BM4339 by electrotransformation. Transformants selected with spectinomycin (60 μg/ml) and chloramphenicol (10 μg/ml), respectively, were screened for the presence of pAT666 DNA.

(iv) Plasmid pAT632. The *cat* gene, encoding chloramphenicol acetyltransferase (CAT), along with the enterococcal constitutive P_2 promoter, was amplified by PCR using primers 79-1 and 79-2 and plasmid pAT79 (P_2cat) DNA as a template. Oligodeoxynucleotide 79-1 (5′ cacggtatgcatGTAAAACGACGGCCAGT) contained an *Nsi*I site (underlined) and 17 bases corresponding to the universal primer −20 (in uppercase letters) (Amersham Pharmacia Biotech). Oligodeoxynucleotide 79-2 (5′ggagcgatgcatCAGGAAACAGCTATGAC) included an *Nsi*I site (underlined) and 17 bases corresponding to the universal primer Reverse (in uppercase letters) (Amersham Pharmacia Biotech). The 1-kb P_2cat PCR product was digested by *Nsi*I and cloned into the 10-kb *Pst*I fragment of pAT145, replacing the portion of pAT145 containing *oriT* from RK2. Plasmid pAT632 (Cm^r *Int-Tn*) was introduced into *E. faecium* BM4339 by electrotransformation, and transformants were selected on chloramphenicol (10 μg/ml). The presence of pAT632 was confirmed by plasmid DNA extraction (36).

(v) Plasmid pAT665. During the cloning steps for the construction of pAT662, an additional *Eco*RI site was incorporated between the 3′ end of the *ddl* gene and the *Xba*I site used to generate the plasmid (14). Plasmid pAT665 was constructed by inserting the P_2ddl fragment, obtained by digestion of pAT662 with *Eco*RI, in pAT113/Sp integrative vector DNA cleaved with *Eco*RI.

Strain construction. The integrative plasmid pAT665 (P_2ddl) was introduced into *E. faecium* BM4339/pAT632 by electrotransformation, and transformants resulting from integration of pAT665 by illegitimate recombination mediated by the integrase of Tn*1545* were selected with spectinomycin (120 μg/ml). The spontaneous loss of pAT632 (Cm^r *Int-Tn*) was obtained by subculturing transformants for ca. 30 generations in chloramphenicol-free medium. Total DNA from 12 clones was digested with *Hin*dIII, *Kpn*I, *Nde*I, *Nsi*I, and *Ssp*I and analyzed by Southern hybridization (36) using pAT113/Sp- and pAT665-labeled DNA and the P_2ddl fragment purified from pAT665 as probes. The data obtained (not shown) indicated the presence of a single chromosomal copy of pAT665 (P_2ddl) in at least two clones, BM4458 and BM4459. These transformants were shown to harbor a copy of pAT665 integrated in different loci in the chromosome and were selected for further studies.

Nucleotide sequencing. DNA sequencing was performed by the dideoxynucleotide chain termination method (37) with α-^{35}S-dATP (Amersham Pharmacia Biotech) and the T7 Sequenase Version 2.0 DNA sequencing kit (Amersham Pharmacia Biotech). The plasmid DNA used as a template was extracted with the commercial Wizard Plus Minipreps DNA Purification System (Promega, Madison, Wis.).

RNA techniques. (i) Extraction of total RNA. Enterococcal strains were grown to an optical density at 600 nm of 0.7. Suspensions were disrupted with a Mickle disintegrator using 3.5-g glass beads (106 μm) (Sigma Chemical Co., St. Louis, Mo.) in the presence of 0.25 ml of 10% sodium dodecyl sulfate, 1 ml of 2%

TABLE 2. Oligodeoxynucleotides used in RNA experiments[a]

Primer	Nucleotide sequence	Position[b]
RD2	5′-AACCGTTCACCCCATAC	575–558
YD3	5′-TTTCTCCGTCCATCACC	2940–2923
HD3	5′-AACGAAACATAGTCTGC	3611–3594
XD1	5′-AGAAGATGGCGGAGAAGCTA	5203–5223
ID1	5′-GTAAAGGCCCAGACAGT	5971–5988
ID2	5′-ATTCAAGATCCGCTCGTG	6607–6589
PR	5′-CACTTTAGTGCATCCTTGCCATTA	122–98
PY	5′-GCAAACAAAGTGTGACACATGCAGCCCTG	2064–2035
RTD	5′-AGCGGCTGTAGGAAGTAA	4958–4940
RTH	5′-TAATGGATGCCGGTGTG	3182–3199
RTX	5′-CCGTCCCATAAGAGCAAACCA	5249–5228
RTY	5′-AATCTTTGGAGTTGTGC	2077–2094

[a] Oligodeoxynucleotides were synthesized in the Unité de Chimie Organique, Institut Pasteur, Paris, France.
[b] Nucleotide numbering begins at the first base of the $vanR_D$ gene.

Macaloïd (National Lead Co., New York, N.Y.), and 3 ml of phenol (19). The mixtures were shaken three times for 1 min at 4°C and centrifuged for 15 min at 8,500 × *g*. Supernatants were extracted with phenol and chloroform. Total RNA was precipitated by addition of 0.1 volume of 3 M sodium acetate, pH 5.2, and 3 volumes of ice-cold 100% ethanol. RNA pellets were resuspended in sterile water.

(ii) Northern analysis. Equal amounts of total RNA (20 μg each) were separated under denaturing conditions in a 1.2% agarose-formaldehyde-morpholinepropanesulfonic acid gel, stained with ethidium bromide, and blotted onto Hybond N$^+$ membranes (Amersham Pharmacia Biotech) (11, 36). Filters were prehybridized and hybridized as described previously (11). PCR products were amplified using total DNA from *E. faecium* BM4339 as a template and primers RD1, SD1 and SD2, YD1, HD1, and XD2 (30), primers D1 and D2 (31), whose positions from the first base of the $vanR_D$ gene are 4401 to 4421 and 4862 to 4842, respectively, and primers listed in Table 2, as indicated in Fig. 1C. The PCR fragments were labeled with [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham Pharmacia Biotech) by using the Megaprime DNA Labelling System (Amersham Pharmacia Biotech) and used as probes. Washes were performed as described previously (23).

(iii) RT-PCR experiments. Total RNA samples were digested with DNase I (5 U of RNA/μg) (Amersham Pharmacia Biotech) in a final volume of 1 ml for 10 min at 37°C. Samples were treated with proteinase K (0.2 mg/ml) (Boehringer, Mannheim, Germany), extracted with phenol-chloroform, and precipitated with ethanol. Reverse transcription was carried out with 2 μg of purified RNA in a 20-μl final volume containing 1× enzyme buffer (Promega), 50 mM magnesium chloride, 0.1 mg of bovine serum albumin (New England Biolabs Inc., Beverly, Ma.)/ml, 1 mM concentrations each of four deoxyribonucleoside triphosphates (Amersham Pharmacia Biotech), 50 pmol of the primer RTX (Fig. 1D; Table 2), 20 U of RNase inhibitor (RNAguard, Amersham Pharmacia Biotech), and 200 U of Moloney murine leukemia virus reverse transcriptase RNase H$^-$ (Promega). Samples were incubated for 30 min at 37°C, and Moloney murine leukemia virus reverse transcriptase was inactivated by incubation for 5 min at 95°C. The DNA products were amplified by PCR in an 80-μl reaction volume containing the previous 20-μl samples, 50 pmol each of the RTD and RTH or RTY primers (Fig. 1D; Table 2), 1× enzyme buffer (Amersham Pharmacia Biotech), and 2 U of *Taq* DNA polymerase (Amersham Pharmacia Biotech). PCR (30 cycles) was performed in a GeneAmp PCR system 2400 (Perkin-Elmer Cetus, Norwalk, Conn.). For Southern hybridization, PCR products were transferred from agarose gel to a Hybond N$^+$ membrane (Amersham Pharmacia Biotech) (36). Hybridizations were performed using D1-D2 (31), HD1 (30)-HD3 (Table 2), and YD1 (30)-YD3 (Table 2) probes (Fig. 1C).

(iv) Primer extension analysis. The synthetic oligodeoxynucleotides PR and PY (Fig. 1D; Table 2) were 5′-end labeled with [γ-^{32}P]ATP (4,500 Ci/mmol; Amersham Pharmacia Biotech) and T4 polynucleotide kinase (Amersham Pharmacia Biotech). After phenol-chloroform extraction, labeled primers were precipitated with ethanol and redissolved in sterile water to a final concentration of 1 pmol/μl. Labeled primers (1 pmol) were annealed to 50 μg of total RNA at 65°C for 3 min, and extension was performed in a 20-μl final volume with 40 U of avian myeloblastosis virus reverse transcriptase (Boehringer) for 30 min at 42°C. After addition of 5 μl of stop solution (Amersham Pharmacia Biotech) and heat denaturation, 5-μl samples were immediately loaded onto 6% polyacrylamide–urea sequencing gels for electrophoresis. Sequencing reactions using the same primers and appropriate plasmid DNA templates were run in parallel to allow determination of the endpoints of extension products.

Analysis of peptidoglycan precursors. Extraction and analysis of peptidoglycan precursors were carried out as described previously (26, 35). Enterococci were grown in the presence of appropriate antibiotics (10 μg of chloramphenicol/ml or 100 μg of spectinomycin/ml). 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.

Enzyme assays. CAT, VanX, and VanY activities in bacterial fractions were assayed as described previously (4, 7).

For determination of CAT production, enterococcal strains JH2-2, JH2-2/pAT666, BM4339, and BM4339/pAT666 were grown in the presence of spectinomycin (60 μg/ml) or chloramphenicol (10 μg/ml), with or without vancomycin (1 or 8 μg/ml). CAT activity in S100 extracts was assayed at 37°C as described previously (7).

To determine D,D-dipeptidase and D,D-carboxypeptidase activities for *E. coli*, strains were grown in brain heart infusion broth containing ampicillin (100 μg/ml) or kanamycin (50 μg/ml). The supernatant (S100) and resuspended pellet (C100) were collected and assayed for D,D-peptidase activities by measuring the D-Ala released from substrate hydrolysis (D-Ala-D-Ala or L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala) through coupled indicator reactions using D-amino acid oxidase and horseradish peroxidase (4). 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.

Nucleotide sequence accession number. The 1,400-bp fragment containing the *intD* gene was submitted to GenBank and assigned accession no. AF288684.

RESULTS AND DISCUSSION

Identification of the *intD* gene. To clone the region flanking the 3′ end of the $vanX_D$ gene, total DNA from *E. faecium* BM4339 was digested with *Xma*I and *Hin*dIII and cloned into *E. coli*, and transformants were screened by hybridization with a probe corresponding to the 3′ portion of $vanX_D$ (Fig. 1A). The recombinant plasmid pAT655 ($vanX_D$′*intD*) carried a 2.85-kb insert (Fig. 1B), with approximately half of the insert overlapping that in pAT654 ($vanS_D$′$vanY_DH_DDX_D$) (Table 1 and Fig. 1A) (14). Analysis of the sequence revealed an open reading frame (ORF) with the same orientation as that of the genes in the *vanD* cluster (Fig. 1A). The deduced sequence displayed 28% identity with the Tn*4430* transposon-encoded TnpI integrase from *Bacillus thuringiensis* (24), 27% identity with the XerD (or XprD) recombinase from *E. coli* (22), and 23% identity with the XerC integrase of *Haemophilus influenzae* (17). The 278-amino-acid putative product of this new ORF was named IntD. IntD contained the conserved tetrad R-H-R-Y (Arg137, His224, Arg227, Tyr259), which is a hall-

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IntD     NH2---130---ICGTGIRVSELQH---74---PRKVFPHNLRHLFARS---19---IETTRIYVAASAT---13---COOH
TnpI     NH2---138---LAYTGVRISEALS---76---SNVITPHQLRHFFCTN---19---IHTTLLYTNPNQL----9---COOH
XerC     NH2---138---MYSSGLRLSELQG---79---NSHLNPHKLRHSFATH---19---LSTTQIYTHLNFQ---17---COOH
XerD     NH2---141---LYATGLRVSELVG---83---SEKLSPHVLRHAFATH---19---LSTTQIYTHVATE---13---COOH

Conserved residues           R                    H R                   Y
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FIG. 2. Partial alignment of the deduced sequences of IntD from *E. faecium* BM4339, TnpI from *B. thuringiensis* H1.1 (accession no. P10020) (24), XerC from *H. influenzae* RD/KW20 (accession no. P44818) (17), and XerD from *E. coli* K-12 (accession no. P21891) (22). Tetrads of residues conserved in all the members of the Int family of site-specific recombinases (28) are indicated in boldface. The numbers of residues separating the different segments containing the conserved amino acids are indicated for each protein.

mark for the integrase family of site-specific recombinases (Fig. 2) (28). This motif was located in the C-terminal domain of the protein (Fig. 2). The *intD* gene was not associated with another ORF in the 1.3-kb region downstream from *intD* in the pAT655 ($vanX_D$'*intD*) insert. Northern hybridization indicated that *intD* was part of the *vanD* gene cluster in BM4339 (see below).

Transposition is known to play an important role in the dissemination of acquired glycopeptide resistance in enterococci. The *vanA* gene cluster is included in Tn*1546*, a Tn*3*-related transposon (8), and the *vanB* operon is part of transposons Tn*1547* (32) and Tn*1549*-Tn*5382* (13, 18). Whether the IntD integrase is involved in the mobility of the *vanD* resistance gene cluster remains unknown.

Transcriptional analysis of the *vanD* gene cluster. Studies of expression of the *vanA* and *vanB* clusters have shown that *vanH, vanA*, and *vanX* and at least $vanY_B$, *vanW*, and $vanH_B$ are cotranscribed from their respective promoters, P_H and P_{Y_B}, located between the *vanS* and *vanH* genes and between the $vanS_B$ and $vanY_B$ genes, respectively (7, 16). The promoters P_R and P_{R_B}, controlling transcription of the genes for the two-component regulatory system, have been identified by characterizing the activity of VanRS (2, 20) and of $VanR_BS_B$ (38) on expression of the resistance genes, respectively. The similarity between the *vanA, vanB*, and *vanD* clusters, particularly in gene organization (14), suggests that the transcriptional start points in the *vanD* cluster would be located in the same regions as those in the *vanA* and *vanB* operons. Northern hybridization and reverse transcription-PCR (RT-PCR) were carried out to test this hypothesis, and primer extension was done to locate the *vanD* promoters precisely. Glycopeptide-susceptible *E. faecalis* JH2-2 was used as a negative control in Northern hybridization. Total RNA was extracted from exponentially growing JH2-2 and BM4339 and analyzed by Northern hybridization with probes internal to the seven genes in the *vanD* cluster, including *intD* (Fig. 1C). Hybridization was performed in duplicate in two independent experiments. As expected, RNA from JH2-2 failed to hybridize with the probes (data not shown). Three major transcripts were detected in BM4339. A transcript with the expected size of ca. 2,100 nucleotides hybridized with the $vanR_D$ and the $vanS_D$ probes (Fig. 3A, lanes 1 and 3), indicating that the genes for the two-component system are cotranscribed from a start point upstream from $vanR_D$. Two transcripts were detected with the $vanY_D$, $vanH_D$,

FIG. 3. Analysis of the *vanD* gene cluster transcription by Northern hybridization. Total RNA from BM4339 (lanes 1, 3, and 5) and BM4409 (lanes 2, 4, and 6) was hybridized with the $vanR_D$ (lanes 1 and 2) and the $vanS_D$ (lanes 3 and 4) probes (A) and the *vanD* (lanes 1 and 2), the $vanX_D$ (lanes 3 and 4), and the *intD* (lanes 5 and 6) probes (B) (see Fig. 1C). The size of the transcripts was determined according to RNA molecular weight marker I (Boehringer) (not shown).

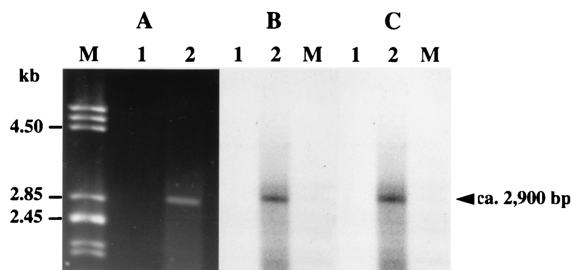


FIG. 4. Analysis of the transcription of the $vanY_D$ and *vanD* genes. Electrophoresis of the products obtained by RT-PCR using the primers RTY and RTD (see Fig. 1D and Table 2) (A) and corresponding Southern hybridization (B and C). Incubations were carried out in the absence (lanes 1) or presence (lanes 2) of reverse transcriptase. Lanes M contained DNA from bacteriophage lambda digested with *Pst*I as a marker. (B) Hybridization with a *vanD* probe (see Fig. 1C). (C) Hybridization with a $vanY_D$ probe (see Fig. 1C).

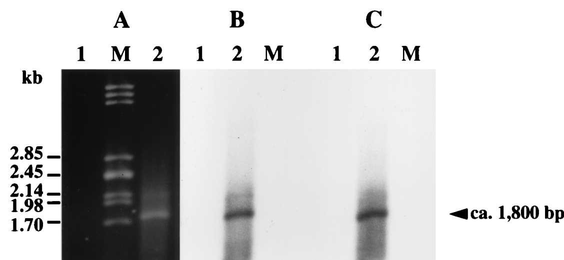


FIG. 5. Analysis of the transcription of the $vanH_D$ and *vanD* genes. Electrophoresis of the products obtained by RT-PCR using the primers RTH and RTD (see Fig. 1D and Table 2) (A) and corresponding Southern hybridization (B and C). Incubations were carried out in the absence (lanes 1) or presence (lanes 2) of reverse transcriptase. Lanes M contained DNA from bacteriophage lambda digested with *Pst*I as a marker. (B) Hybridization with a *vanD* probe (see Fig. 1C). (C) Hybridization with a $vanH_D$ probe (see Fig. 1C).

van_D, and $vanX_D$ probes, one of ca. 3,800 nucleotides and the other of ca. 5,000 nucleotides (Fig. 3B, lanes 1 and 3, and data not shown). The presence of degradation products did not prevent observation of these two transcripts with the probes internal to $vanY_D$, $vanH_D$, and *vanD* (Fig. 3B, lane 1, and data not shown). The hybridizing degradation products increased going from *vanD* to $vanY_D$ (data not shown), suggesting that they result from a contaminating 3′ to 5′ exonuclease (33). The ca. 5,000-bp product was also observed using the *intD* probe (Fig. 3B, lane 5), and the size of this transcript was in agreement with that predicted for a single mRNA, including $vanY_D$, $vanH_D$, *vanD*, $vanX_D$, *and intD*. The size of the ca. 3,800-bp transcript correlated with a mRNA that would include the $vanY_D$, $vanH_D$, *vanD*, and $vanX_D$ genes. No inverted repeats, likely to form a hairpin structure for termination of transcription, were identified between the $vanX_D$ and *intD* genes.

To confirm that the $vanY_D$, $vanH_D$, *vanD*, and $vanX_D$ genes were contranscribed, reverse transcription with purified total RNA from BM4339 and primer RTX internal to $vanX_D$ was performed (Fig. 1D). The complementary DNA was amplified by PCR using primer pairs internal to *vanD*, $vanY_D$, and $vanH_D$ (Fig. 1D). Amplification resulted in the expected 2.9- and 1.8-kb products that cohybridized with probes specific for *vanD* and $vanY_D$ (Fig. 1C and 4) and for *vanD* and $vanH_D$ (Fig. 1C and 5), respectively. These results, along with those obtained by Northern hybridization, indicate that the $vanY_D$, $vanH_D$, *vanD*, and $vanX_D$ genes are cotranscribed and that the approximately 3,800-nucleotide resulting messenger starts upstream from $vanY_D$.

Primer extension was used to explore the region upstream from $vanR_D$ and the $vanS_D$-$vanY_D$ intergenic region for transcriptional start sites using the oligodeoxynucleotides PR and PY (Fig. 1D) complementary to the 5′ end of $vanR_D$ and $vanY_D$ respectively, as primers. This allowed the positioning of transcriptional start sites P_{R_D} (Fig. 6) and P_{Y_D} (Fig. 7). These results were confirmed by using two other oligodeoxynucleotides complementary to sequences close to PR and PY but located closer to the mapped start sites (data not shown). Promoters P_{R_D} *and* P_{Y_D} contained the −35 and −10 regions corresponding to the σ^{70} recognition sequences which were separated by 17 bp.

Regulation of the P_{Y_D} promoter by $VanR_D$ and $VanS_D$. To investigate the role of the $VanR_D$-$VanS_D$ two-component regulatory system in the control of transcription of the $vanY_D$, $vanH_D$, *vanD*, and $vanX_D$ genes, a DNA fragment containing the P_{Y_D} promoter was cloned upstream from the *cat* reporter gene of the shuttle promoter probing vector pAT78(*cat*) to generate plasmid pAT666 (P_{Y_D} *cat*) (Fig. 1B). *E. faecalis* JH2-2 was used as a control for expression of the *cat* gene under the control of the P_{Y_D} promoter in the absence of $vanR_D$-$vanS_D$. No significant CAT activity was detected in *E. faecalis* JH2-2/pAT666(P_{Y_D} *cat*) (Fig. 8); the *cat* gene carried by pAT666 (P_{Y_D} *cat*) was transcribed in this strain at a low level similar to that detected with the promoterless pAT78 (*cat*) (data not shown). This basal level of transcription was not modified by the presence of vancomycin at 1 μg/ml (Fig. 8). In contrast, when pAT666 (P_{Y_D} *cat*) was introduced into *E. faecium* BM4339, significant production of CAT was detected (Fig. 8), at a ca. 2,800-fold-higher level than that displayed by BM4339 or BM4339/pAT78 (*cat*) (data not shown).

These observations indicate that the P_{Y_D} promoter did not direct constitutive transcription but required a signal to promote gene expression. Promoter P_{Y_D} was not 1066016.dhp active in *E. faecalis* JH2-2, owing to the absence of an inducing signal, which could be transmitted by the $VanR_D$ and $VanS_D$ regulatory partners or by some other regulatory factors specific for *E. faecium* strains.

According to the homology between the *vanA, vanB*, and *vanD* gene clusters, the chromosomally encoded $VanR_D$-$VanS_D$ system could be responsible for *trans* activation of transcription from P_{Y_D} in BM4339/pAT666 (P_{Y_D} *cat*). If so, constitutive expression of the *vanD* gene cluster in BM4339 suggests that the $VanR_D$ protein is present in its phosphorylated form, thus activating transcription from the P_{Y_D} promoter. This could be due to alteration in signal recognition or of the phosphatase activity of the cognate $VanS_D$ sensor, or to phosphorylation of $VanR_D$ by a nonpartner protein kinase (14).

The presence of vancomycin at a concentration of 8 μg/ml doubled transcription from P_{Y_D} in BM4339 (Fig. 8). However, the construction used may not reflect the in vivo conditions, since the high-copy-number plasmid pAT666 (P_{Y_D} *cat*) allows for high-level expression from the P_{Y_D} promoter. Nevertheless, an inducing effect of vancomycin on the level of transcription

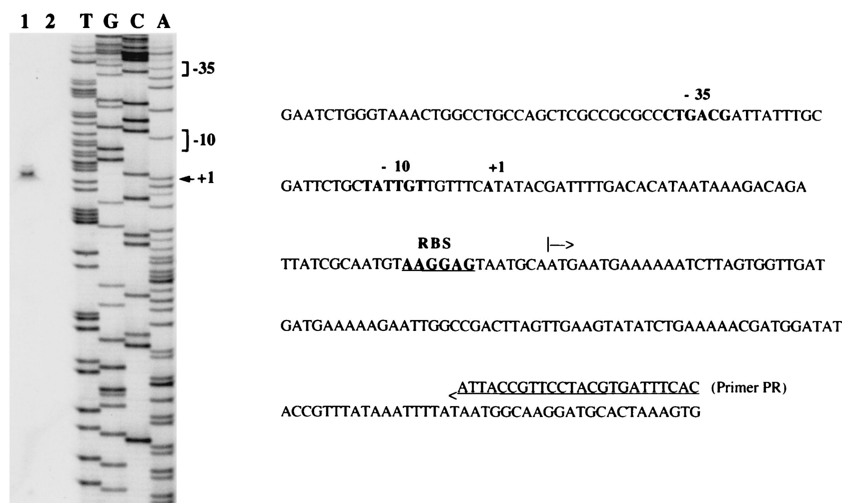


FIG. 6. Identification of the transcriptional start site for the *vanR*$_D$ and *vanS*$_D$ genes in BM4339 by primer extension analysis. Left panel, lane 1, primer elongation product obtained with oligodeoxynucleotide PR and 50 μg of total RNA from BM4339 (arrowhead); lane 2, control without RNA; lanes T, G, C, and A, results of sequencing reactions performed with the same primer. Right panel, sequence from nucleotide position −128 to +122 (numbering from the A of the ATG start codon of *vanR*$_D$, negative in the 3′ to 5′ direction and positive in the 5′ to 3′ direction). The +1 transcriptional start site for the *vanR*$_D$ and *vanS*$_D$ mRNA in BM4339 and the −35 and −10 promoter sequences located upstream are in boldface. The ATG start codon of *vanR*$_D$ is indicated by an arrow, and the RBS is in boldface and underlined.

of the chromosomal resistance genes cannot be excluded, since this would not necessarily make an impact on the level of translation or of protein activity in BM4339.

VanX$_D$ and VanY$_D$ D,D-peptidase activity in *E. coli*. Strain BM4339 does not produce D-Ala-D-Ala-containing peptidoglycan precursors because of a frameshift mutation in the chromosomal *ddl* gene (14). Thus, no D,D-dipeptidase activity is required for glycopeptide resistance in this genetic background. In fact, BM4339 does not produce active VanX$_D$ (31), although *vanX*$_D$ is transcribed (Fig. 3B, lane 3) and the deduced sequence of VanX$_D$ does not contain mutations in the conserved residues known to be involved in zinc binding and catalysis (25). To test if *vanX*$_D$ and *vanY*$_D$ encode functional enzymes, the genes and their ribosome binding sites (RBS)

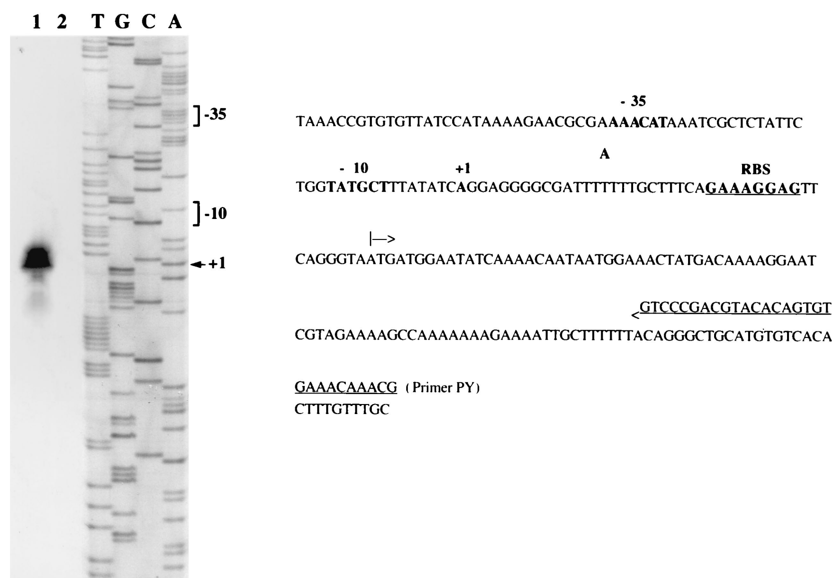


FIG. 7. Identification of the transcriptional start site for the *vanY*$_D$, *vanH*$_D$, *vanD, vanX*$_D$, and *intD* genes in BM4339 by primer extension analysis. Left panel, lane 1, primer elongation product obtained with oligodeoxynucleotide PY and 50 μg of total RNA from BM4339 (arrowhead); lane 2, control without RNA; lanes T, G, C, and A, results of sequencing reactions performed with the same primer. Right panel, sequence from nucleotide position −110 to +109 (numbering from the A of the ATG start codon of *vanY*$_D$, negative in the 3′-to-5′ direction and positive in the 5′-to-3′ direction). The +1 transcriptional start site for the *vanY*$_D$, *vanH*$_D$, *vanD, vanX*$_D$, and *intD* mRNA in BM4339 and the −35 and −10 promoter sequences located upstream are in boldface. The ATG start codon of *vanY*$_D$ is indicated by an arrow, and the RBS is in boldface and underlined.

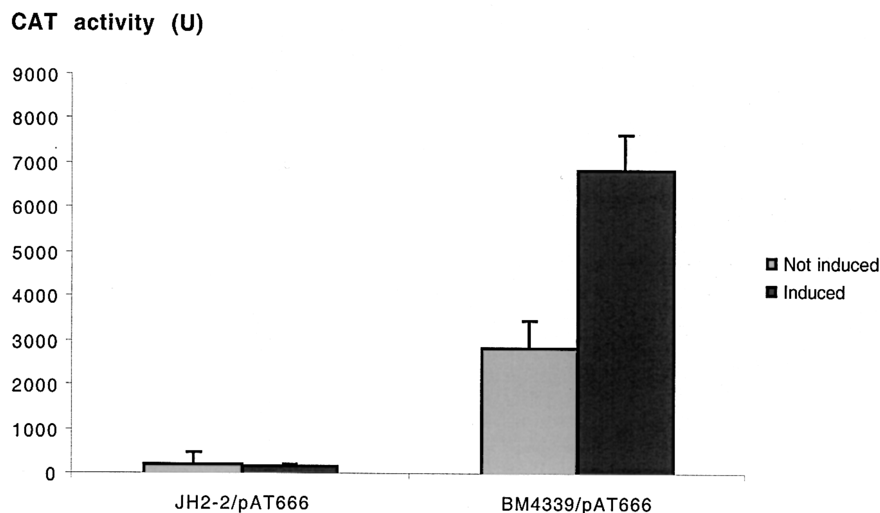


FIG. 8. CAT activity in cytoplasmic extracts from *E. faecalis* JH2-2 and from *E. faecium* BM4339 harboring plasmid pAT666 (P_{Y_D} *cat*). Controls were performed without addition of vancomycin to the culture medium ("not induced" bars), and induction was achieved by adding 1 μg of vancomycin/ml to cultures of JH2-2/pAT666 and 8 μg of vancomycin/ml to cultures of BM4339/pAT666 ("induced" bars). Enzymatic activity was expressed as nanomoles of product formed per minute per milligram of protein in S100 extracts. Results are means ± standard deviations obtained from three independent extracts.

were cloned into *E. coli* under the control of the P_{lac} promoter of pUC18 and pBGS18+, respectively. Hydrolysis of D-Ala-D-Ala was detected in cytoplasmic extracts from *E. coli* harboring pAT659 (*vanX$_D$*) but not pUC18 (Table 3), indicating that *vanX$_D$* encodes a functional enzyme. It is possible that VanX$_D$ is translated in BM4339 but is unstable and is degraded. Alternatively, its activity may be too low to be measured in BM4339, in which there is a single copy of the vanX$_D$ gene, but is detectable when it is cloned in pUC18 in *E. coli*, which can harbor up to 700 copies of the plasmid.

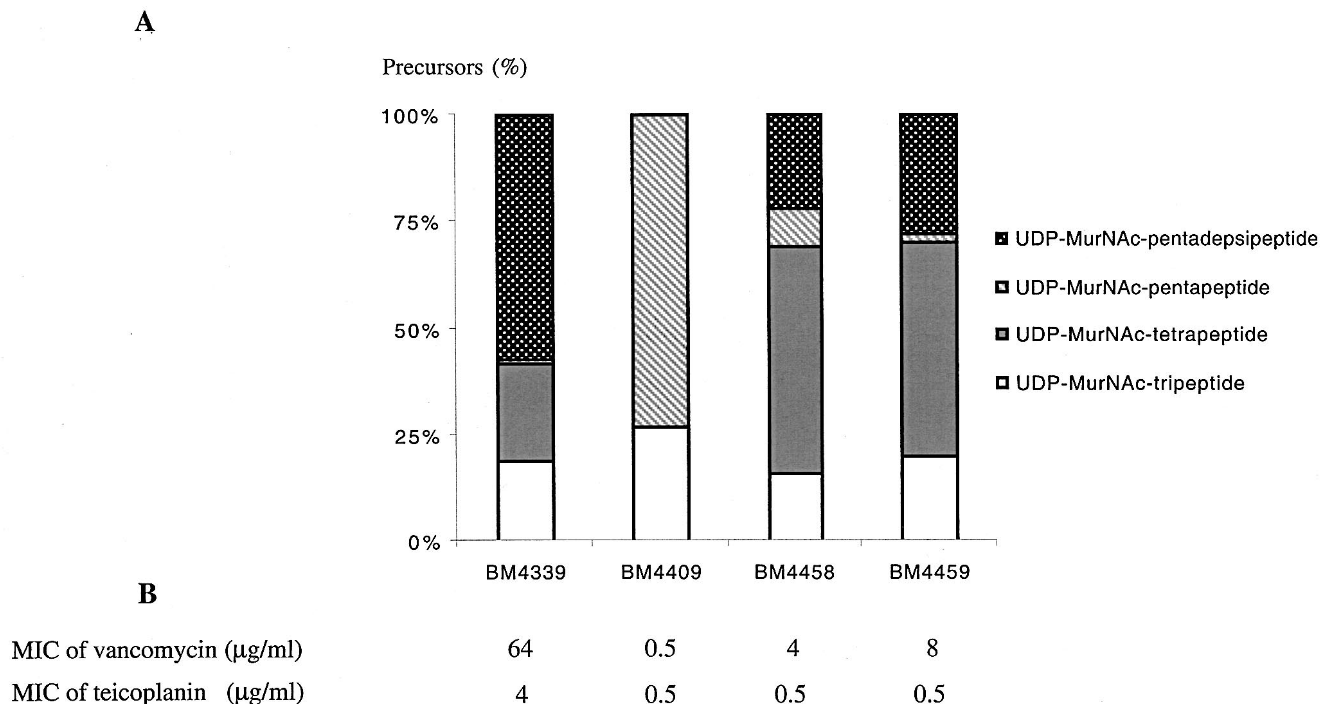
D,D-carboxypeptidase activity was detected in membrane preparations from *E. coli* harboring pAT660 (*vanY$_D$*), which was completely inhibited by the presence of 10 mM penicillin G (Table 3), as shown for BM4339 (31). The sequence of VanY$_D$ is homologous to those of penicillin-binding proteins (14), and the expression of the BM4339 *vanY$_D$* gene in *E. coli* generates an enzymatically active D,D-carboxypeptidase. The mechanism by which this enzyme affects resistance remains to be elucidated, since, although VanY$_D$ presumably binds penicillins on the external surface of the cytoplasmic membrane, the tetrapeptide product formed by the D,D-carboxypeptidase is located in the cytoplasm. The location and properties of the VanY$_D$ protein are currently under investigation (P. E. Reynolds, B. Casadewall, and P. Courvalin, unpublished data).

Characterization of glycopeptide-susceptible derivatives of *E. faecium* BM4339. Complementation of the frameshift mutation in the BM4339 chromosomal *ddl* gene (14) was studied in two systems. Transformation of BM4339 with the high-copy-number plasmid pAT662 (*P$_2$ddl*) restored glycopeptide susceptibility in *E. faecium* BM4409 by *trans* complementation (Fig. 9B). The effect of a single copy of the heterologous *ddl* gene in the chromosome of BM4339 was investigated, since *cis* complementation was anticipated to be more stable and more analogous to the natural situation. Transformants were obtained following integration of the suicide plasmid pAT665 (*P$_2$ddl*) into the BM4339 chromosome. Glycopeptide-susceptible BM4458 and BM4459 [BM4339::pAT665 (*P$_2$ddl*)] differed in their sites of insertion (data not shown), and the MICs

TABLE 3. D,D-dipeptidase and D,D-carboxypeptidase activities in *E. coli* TB1 harboring the indicated plasmids

Plasmid harbored	Activity (nmol min^{-1} mg^{-1})[a]		
	D,D-dipeptidase[b]	D,D-carboxypeptidase[c]	
		Without penicillin G	With penicillin G (10 mM)
pUC18	ND[d]	ND	NT[e]
pAT659 (pUC18Ω*vanX$_D$*)	810 ± 65	ND	NT
pBGS18+	NT	ND	ND
pAT660 (pBGS18+Ω*vanY$_D$*)	NT	3.39 ± 0.11	ND

[a] Enzymatic activity was assayed on protein extracts from *E. coli* harboring the vectors pUC18 and pBGS18+ or plasmids pAT659 and pAT660 which carry the *vanX$_D$* and the *vanY$_D$* genes, respectively.
[b] Activity in cytoplasmic extracts.
[c] Activity in membrane extracts.
[d] ND, not detectable.
[e] NT, not tested.



	BM4339	BM4409	BM4458	BM4459
MIC of vancomycin (μg/ml)	64	0.5	4	8
MIC of teicoplanin (μg/ml)	4	0.5	0.5	0.5

FIG. 9. Analysis of derivatives of *E. faecium* BM4339 harboring a wild-type *ddl* gene on a high-copy-number plasmid (BM4409) or the same gene integrated as a single copy in the chromosome (BM4458 and BM4459). (A) Proportions of late soluble cytoplasmic peptidoglycan precursors accumulated in the presence of ramoplanin. (B) Levels of resistance to vancomycin and teicoplanin.

of vancomycin for these strains were significantly, but unequally, decreased (Fig. 9B). The MICs of teicoplanin were also altered (Fig. 9B).

The amounts of late peptidoglycan precursors were analyzed in the BM4339 derivatives BM4409 [BM4339/pAT662 (P_2ddl)], BM4458, and BM4459. Transformants BM4458 and BM4459 [BM4339::pAT665 (P_2ddl)] contained different amounts of pentapeptides, consistent with the vancomycin MICs (Fig. 9A and B). This observation indicates that the chromosomal sequences flanking the heterologous *ddl* gene are likely to affect its expression. Although a larger proportion of pentapeptide was present in the peptidoglycan precursor pool of BM4458 and BM4459 in comparison with that in BM4339, tetrapeptide was the main component, being present at similar and high levels in both strains (Fig. 9A). The ratio of pentadepsipeptide to tetrapeptide in the two transformants was inverted in comparison with that in BM4339 (Fig. 9A). In BM4339, tetrapeptide originated mainly from pentadepsipeptide, with possibly a small amount generated from pentapeptide synthesized by the Ddl activity of VanD. However, the Ddl in BM4458 and BM4459, encoded by the single chromosomal copy of *ddl*, synthesized a greater amount of pentapeptide, and tetrapeptide results from hydrolysis of both pentapeptide and pentadepsipeptide. As already shown for VanY (1), further investigations have demonstrated that the D,D-carboxypeptidase of BM4339 has greater activity against pentapeptide than pentadepsipeptide (P. E. Reynolds, B. Casadewall, and P. Courvalin, unpublished data).

In addition to tripeptide precursors, BM4409 [BM4339/pAT662 (P_2ddl)] contains only pentapeptide (Fig. 9A). No pentadepsipeptide was detected, as if the *vanD* gene cluster was no longer expressed (Fig. 9A). This result is in agreement with the high-level expression of a functional Ddl and with the low MICs of glycopeptides for BM4409 (Fig. 9B). To investigate the effect of a wild-type Ddl on the expression of the *vanD* gene cluster, Northern hybridization was performed with BM4409 and BM4339 total RNA, the latter being a control for transcription of the *vanD* cluster. Surprisingly, the $vanR_D$-$vanS_D$ regulatory region was transcribed in BM4409 (Fig. 3A), whereas the $vanY_D$, $vanH_D$, *vanD*, $vanX_D$, and *intD* genes were not (Fig. 3B and data not shown). Cell wall biosynthesis in BM4409 has apparently been switched from the production of D-Ala-D-Lac-ending precursors, which occurs constitutively in BM4339, to that of D-Ala-D-Ala-containing precursors. The lack of transcription of the genes controlled by P_{Y_D} indicated an absence of activation of the promoter. This is consistent with the existence of a signal-transducing pathway in *E. faecium* BM4339, probably involving the $VanR_D$-$VanS_D$ two-component system. One explanation for the silencing of transcription of $vanY_D$, $vanH_D$, *vanD*, $vanX_D$, *and intD* could be that the high levels of D-Ala-D-Ala, synthesized by the heterologous Ddl in BM4409, disrupt transduction of the signal by preventing $VanR_D$-phosphate from accumulating and activating transcription at the P_{Y_D} promoter. To test this possibility, the effect of D-Ala-D-Ala in the culture medium on the level of vancomycin resistance in BM4339 was determined (6). If high levels of intracellular D-Ala-D-Ala prevent expression of the *vanD* resistance genes from P_{Y_D}, BM4339 would be expected to become susceptible to vancomycin. The level of vancomycin resistance of BM4339 was unaffected by D-Ala-D-Ala added at

final concentrations ranging from 0 to 40 mM (data not shown). Since the uptake of dipeptides is mediated by peptide permeases with broad specificity (10), the lack of an effect of D-Ala-D-Ala is unlikely to result from inefficient transport. Alternatively, the absence of transcription of the resistance genes in BM4409 may result from the lack of a signal by $VanR_D$-$VanS_D$, thus preventing transcription from the P_{Y_D} promoter.

In conclusion, the *vanD* glycopeptide resistance gene cluster from *E. faecium* BM4339 comprises seven genes which are transcribed from two promoters, P_{R_D} for the $vanR_D$ and $vanS_D$ regulatory genes and P_{Y_D} for the $vanY_D$, $vanH_D$, *vanD*, $van\ X_D$, and *intD* genes. Expression of the latter five genes is likely to result from activation of transcription from P_{Y_D} by the $VanR_D$ response regulator. The signal responsible for constitutive expression of the resistance genes remains to be established.

ACKNOWLEDGMENTS

We thank T. Msadek for help with RNA preparation and helpful discussions and J. Blanchard for critical reading of the manuscript. B.C. is grateful to F. Depardieu for construction of pAT632 and to S. Goussard and B. Périchon for constant technical advice.

This work was supported in part by a Bristol-Myers Squibb unrestricted biomedical research grant in infectious diseases and by the Programme de Recherche Fondamentale en Microbiologie, Maladies Infectieuses et Parasitaires from the Ministère de l'Education Nationale, de la Recherche et de la Technologie. B.C. was the recipient of a grant from the Fondation pour la Recherche Médicale.

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