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Characterization of the *vanD* Glycopeptide Resistance Gene Cluster from *Enterococcus faecium* BM4339

BARBARA CASADEWALL AND PATRICE COURVALIN*

Unité des Agents Antibactériens, Institut Pasteur, 75724 Paris Cedex 15, France

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VanD-type resistance to glycopeptides in *Enterococcus faecium* BM4339 is due to constitutive synthesis of D-alanyl-D-lactate-terminating peptidoglycan precursors (B. Périchon, P. Reynolds, and P. Courvalin, Antimicrob. Agents Chemother. 41:2016–2018, 1997). The sequence of a 5,780-bp fragment was determined and revealed six open reading frames. The 3′ distal part encoded the $VanH_D$ dehydrogenase, the VanD ligase, and the $VanX_D$ DD-dipeptidase, which were highly similar to the corresponding proteins in VanA and VanB types of resistance. The deduced $VanY_D$ protein was homologous to penicillin-binding proteins that display DD-carboxypeptidase activity. The 5′ end coded for the putative $VanR_D$-$VanS_D$ two-component regulatory system. Due to a frameshift mutation in the chromosomal *ddl* gene, BM4339 produced an impaired D-alanine:D-alanine ligase. However, since expression of the resistance genes is constitutive, growth of *E. faecium* BM4339 was not dependent on the presence of glycopeptides in the culture medium.

In gram-positive bacteria, glycopeptides inhibit the last steps of peptidoglycan synthesis by binding to the C-terminal dipeptide D-alanyl–D-alanine (D-Ala–D-Ala) of peptidoglycan precursors, thus preventing their incorporation into the cell wall (23). Acquired resistance to vancomycin and teicoplanin in enterococci is due to the replacement of D-Ala–D-Ala by the depsipeptide D-alanyl–D-lactate (D-Ala–D-Lac). This substitution leads to the formation of modified peptidoglycan precursors for which glycopeptides exhibit 1,000-fold lower binding affinities (11).

Two types of acquired resistance to glycopeptides have been well characterized (for a recent review, see reference 6). VanA-type enterococci display inducible resistance to high levels of both vancomycin and teicoplanin, whereas VanB-type enterococci display inducible resistance to various levels of vancomycin but remain susceptible to teicoplanin. In addition to the host D-Ala:D-Ala ligase (Ddl), a resistance ligase, VanA or VanB, mediates formation of D-Ala–D-Lac, which competes with D-Ala–D-Ala in cell wall assembly. Two other proteins are required for resistance: VanH ($VanH_B$), a dehydrogenase which converts pyruvate into D-Lac (11), and VanX ($VanX_B$), a DD-dipeptidase which hydrolyzes D-Ala–D-Ala produced by the chromosomal Ddl (24). VanY ($VanY_B$), inessential for resistance, is a DD-carboxypeptidase which acts when significant incorporation of D-Ala–D-Ala occurs in spite of VanX hydrolysis. Under such conditions, VanY hydrolyzes pentapeptides and, to a lesser extent, pentadepsipeptides (1a). Thus, two DD-peptidases, VanX ($VanX_B$) and VanY ($VanY_B$), contribute sequentially to resistance in reducing the pool of pentapeptide precursors, favoring their replacement by pentadepsipeptides in cell wall assembly. Two proteins which belong to the family of two-component regulatory systems control the level of expression of the resistance genes in response to the presence of glycopeptides in the culture medium. The VanS ($VanS_B$) sensor governs phosphorylation of the regulator VanR ($VanR_B$), which acts as a transcriptional activator for the resistance genes. An accessory protein other than VanY, VanZ, has also been identified in VanA-type strains. VanZ confers low-level teicoplanin resistance by an unknown mechanism. The open reading frame (ORF) *vanW* is present in the *vanB* operon, but no function has yet been assigned to the corresponding protein (14).

A new type of acquired resistance to glycopeptides, VanD, was recently found in *Enterococcus faecium* BM4339 (22) and in other *E. faecium* strains (19). Clinical isolate BM4339 is resistant to intermediate levels of vancomycin (MIC = 64 μg/ml) and to low levels of teicoplanin (MIC = 4 μg/ml) and does not harbor the *vanA* or *vanB* operon. Glycopeptide resistance in this strain is constitutively expressed and mediated by synthesis of pentadepsipeptide precursors ending in D-Ala–D-Lac, which represent the main components of cell wall cytoplasmic precursors (22).

In this work, we describe the genetic organization of the *vanD* gene cluster in *E. faecium* BM4339. We also show that a frameshift mutation in the chromosomal *ddl* gene accounts for the lack of precursors terminating in D-Ala–D-Ala in this strain.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. The bacterial strains and plasmids used are described in Table 1. Unless specified, *Escherichia coli* JM83 (35) and *E. coli* TB1 (Focus, Life Technologies Inc., Gaithersburg, Md.) were used as the hosts 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. (30) was used to determine the MICs of glycopeptides with 10^5 CFU per spot on Mueller-Hinton agar (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France) after 24 h of incubation.

Recombinant DNA techniques. Plasmid DNA isolation, cleavage of DNA with restriction endonucleases (Amersham, Little Chalfont, Buckinghamshire, England; Gibco BRL-Life Technologies Inc.; and Pharmacia, Uppsala, Sweden), purification of restriction fragments from agarose gel, dephosphorylation of vector DNA with calf intestinal phosphatase (Pharmacia), and ligation with T4 DNA ligase (Pharmacia) were performed by standard methods (26).

Plasmid construction. The plasmids were constructed as explained below (Fig. 1).

(i) Plasmid pAT654. *E. faecium* BM4339 total DNA was partially digested with *Sau*3AI and ligated with pUC18 DNA cleaved by *Bam*HI. To identify recombinant plasmids, clones were screened by colony hybridization (26) with the 605-bp fragment internal to *vanD* purified from pAT656 (22) as a probe (Fig. 1).

(ii) Plasmid pAT657. To amplify a fragment internal to the $vanR_D$ and the $vanS_D$ genes, the degenerate oligodeoxynucleotide VR (1) was used in combination with the specific primer SD (5′ GTTCTTCCAGACGCTCA), complementary to the 5′ end of the insert in pAT654. Oligodeoxynucleotide VR [5′ GGIGCIGA(T/C)GA(T/C)TA(T/C)ITIIIIAA(A/G)CCITT, where I is deoxyinosine] was deduced from the sequences of conserved motifs located in the C

* Corresponding author. Mailing address: Unité des Agents Antibactériens, Institut Pasteur, 28 rue du Docteur Roux, 75724 Paris Cedex 15, France. Phone: (33) 1 45 68 83 20. Fax: (33) 1 45 68 83 19. E-mail: pcourval@pasteur.fr.

TABLE 1. Strains and plasmids

Strain or plasmid	Relevant properties[a]	Reference or source
Strains		
E. coli		
JM83	F$^-$ *ara* Δ(*lac-proAB*) *rpsL* (Str^r)[ϕ*80*d*lac*Δ(*lacZ*)M15]	35
TB1	JM83 *hsdR*(r_K^- m_K^+)	Life Technologies Inc.
INVαF′	F′ *endA1 recA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 gyrA96 relA1* ϕ80*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169*λ$^-$	Invitrogen
E. faecium		
BM4339	Vm^r Te^r (VanD type)	22
BM4147	Vm^r Te^r (VanA type)	16
BM4409	BM4339/pAT662	This work
Plasmids		
pUC18	Ap^r, *lacZ*α vector	33
pGB2	Sm^r Sp^r derivative of pSC101	12
pCR2.1	Ap^r Km^r, *oriR* from ColE1, *lacZ*α vector	Invitrogen
pAT79	*oriR* from pAMβ1, *oriR* from pUC, *oriT* from RK2; Sp^r *lacZ*α P_2 *cat*	5
pAT654	5.3-kb *Sau*3AI fragment ($vanS_D'$ $vanY_DH_DDX_D$) of BM4339 cloned into pUC18	This work
pAT656	0.6-kb PCR fragment (*vanD′*) of BM4339 cloned into pCR2.1	22
pAT657	1.0-kb PCR fragment ($vanR_D'S_D'$) of BM4339 cloned into pCR2.1	This work
pAT658	1.8-kb *Hin*dIII-*Cla*I fragment ($vanR_DS_D'$) of BM4339 cloned into pUC18	This work
pAT661	7.0-kb *Hin*dIII fragment (*ddl*) of BM4339 cloned into pGB2	This work
pAT662	1.2-kb *Sac*I-*Xba*I fragment (*ddl*) of BM4147 cloned into pAT79	This work

[a] Ap^r, ampicillin resistance; Km^r, kanamycin resistance; Sm^r, streptomycin resistance; Sp^r, spectinomycin resistance; Te^r, teicoplanin resistance; Vm^r, vancomycin resistance; P_2, promoter of the *aphA-3* gene from enterococcal plasmid pJH1.

termini of the effector domains of VanR, OmpR, and PhoB response regulators (5). The PCR product obtained with BM4339 total DNA as a template and *Taq* DNA polymerase (U.S. Biochemical-Amersham) was cloned into pCR2.1 (Invitrogen, Carlsbad, Calif.) and introduced into *E. coli* INVαF′ (Invitrogen) by transformation.

(iii) Plasmid pAT658. To complete the sequence of the $vanR_D$ gene, total DNA from *E. faecium* BM4339 was digested with *Eco*RI and *Cla*I, *Hin*dIII and *Cla*I, *Eco*RI and *Ssp*I, and *Hin*dIII and *Ssp*I, and the sizes of the fragments hybridizing with a 271-bp probe corresponding to the 5′ end of the pAT657 insert (Fig. 1) were estimated (26). Cloning was performed with restriction endonucleases generating fragments of more than 1 kb in length. The recombinant plasmids were screened by hybridization with the same probe, and plasmid pAT658, selected for further studies, contained a 1.8-kb *Hin*dIII-*Cla*I insert.

(iv) Plasmid pAT661. A strategy similar to that used with plasmid pAT658 was followed to clone the chromosomal *ddl* gene from BM4339. The 600-bp fragment internal to the *E. faecium* BM4147 *ddl* gene (13) was used as a probe. Plasmid pAT661 consisted of a 7-kb *Hin*dIII chromosomal fragment of BM4339 cloned into the low-copy-number vector pGB2.

(v) Plasmid pAT662. The *ddl* gene from *E. faecium* BM4147 with its ribosome binding site (RBS) was amplified by PCR with total DNA as a template and oligodeoxynucleotides 4147-1 and 4147-2 as primers. Primer 4147-1 (5′ ccgctgc agagctcTTAGAATACA*GGAGGA*C) contained a *Sac*I site (underlined) and 17 bases complementary to the sequence upstream from the BM4147 *ddl* gene (in uppercase letters) including the RBS (italicized). Primer 4147-2 (5′atttgggatcta gaTACGCAATCACTCCAGC) contained an *Xba*I site (underlined) and 17 bases complementary to the sequence downstream from the BM4147 *ddl* gene (in uppercase letters). The PCR product was digested with *Sac*I and *Xba*I and placed under the control of the constitutive promoter P_2 of the expression vector pAT79 (chloramphenicol resistant [Cm^r]), leading to plasmid pAT662.

Strain construction. *E. faecium* BM4409 was obtained by introduction of plasmid pAT662 (Cm^r Ω*ddl* BM4147) into *E. faecium* BM4339 by electrotransformation and selection on SR medium (28) containing chloramphenicol (10

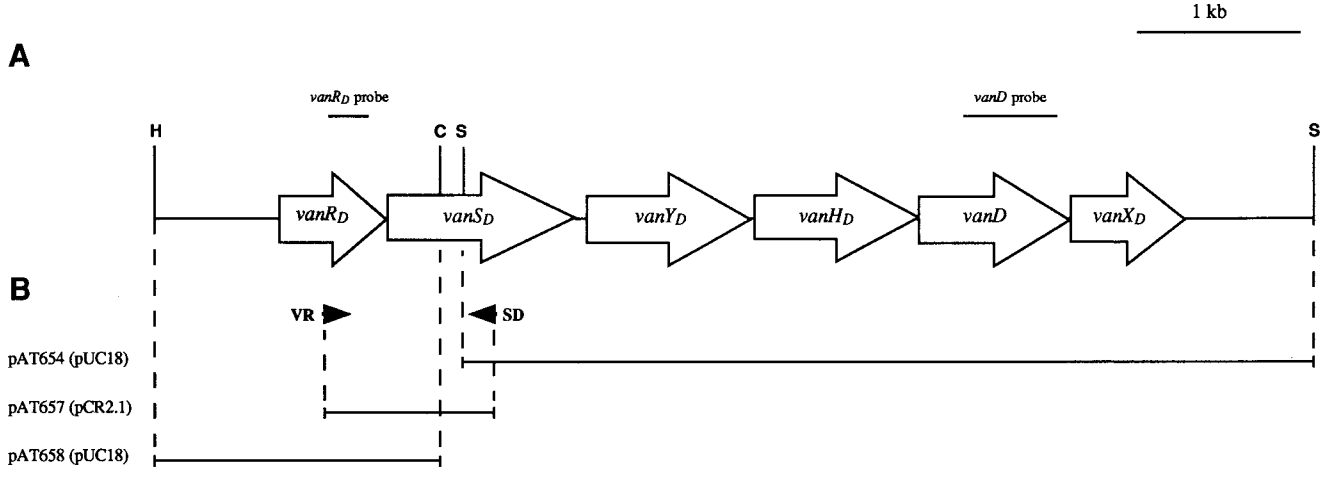

FIG. 1. Schematic representation of the *vanD* gene cluster and of recombinant plasmids. (A) Map of the 7.2-kb *Hin*dIII-*Sau*3AI fragment containing the $vanR_D$, $vanS_D$, $vanY_D$, $vanH_D$, *vanD*, and $vanX_D$ genes. Open arrows represent coding sequences. The PCR fragments internal to the $vanR_D$ and *vanD* genes used as probes in hybridization experiments are indicated above the corresponding regions. Abbreviations for restriction sites used for cloning are as follows: C, *Cla*I; H, *Hin*dIII; and S, *Sau*3AI. (B) Inserts in recombinant plasmids. The inserts are represented by solid lines, and the vectors are indicated in parentheses. Arrowheads indicate binding sites and orientations of the VR and SD oligodeoxynucleotides used for amplification.

TABLE 2. Levels of identity between the deduced sequences of the proteins encoded by the *vanA*, *vanB*, and *vanD* gene clusters

Sequence compared	% Identity											
	VanR	VanS	VanH	VanA	VanX	VanY	$VanR_B$	$VanS_B$	$VanH_B$	VanB	$VanX_B$	$VanY_B$
$VanR_D$	58						34					
$VanS_D$		42						19				
$VanH_D$			59						63			
VanD				69						69		
$VanX_D$					68						70	
$VanY_D$						13						15
VanR							35					
VanS								17				
VanH									68			
VanA										76		
VanX											75	
VanY												30

μg/ml). The presence of pAT662 in BM4409 was confirmed by plasmid DNA extraction (26).

Nucleotide sequencing. DNA sequencing was performed by the dideoxynucleotide chain termination method (27) with [α-^{35}S]dATP (Amersham) and the T7 Sequenase version 2.0 DNA sequencing kit (Amersham). The plasmid DNA used as the template was extracted with the commercial Wizard Plus Minipreps DNA Purification System (Promega, Madison, Wis.).

Computer analysis of sequence data. Sequence data were analyzed with the Sequence Analysis Software Package (version 7; Genetics Computer Group, Madison, Wis.).

Nucleotide sequence accession numbers. The 5,781-bp fragment containing the *vanD* gene cluster was submitted to GenBank and assigned accession no. AF130997. The nucleotide sequence of the 1,240-bp chromosomal region containing the BM4339 *ddl* gene was allotted accession no. AF130998.

RESULTS AND DISCUSSION

Identification of the *van* genes and protein sequence analysis. Partial digests of *E. faecium* BM4339 total DNA were cloned into *E. coli*, and transformants were screened by hybridization with a *vanD* internal probe (Fig. 1). Plasmid pAT654 (*vanS*$_D$′ *vanY*$_D$*H*$_D$*DX*$_D$) carried an insert of 5.3 kb that was sequenced with specific primers. Analysis of the sequence revealed five ORFs with the same orientation, the 5′ one being truncated (Fig. 1). The deduced amino acid sequences were compared to those of the proteins encoded by the *vanA* and *vanB* operons (Table 2). Based on homology, four ORFs could be assigned to the 3′ end of the *vanS*$_D$ gene, to *vanH*$_D$, to *vanD*, and to *vanX*$_D$. The identities between the $VanH_D$, VanH, and $VanH_B$ dehydrogenases; the VanD, VanA, and VanB ligases; and the $VanX_D$, VanX, and $VanX_B$ DD-dipeptidases were high (from 59 to 70%) (Table 2). The three conserved residues, Arg, Glu, and His, predicted to participate in substrate binding and catalysis of D-Lac dehydrogenases (31) were present in $VanH_D$ at positions 232, 260, and 292, respectively (data not shown). $VanH_D$ also contained the GXGXXG(17X)D sequence (positions 154 to 177) characteristic of nucleotide-binding domains in NAD^+ cofactor-dependent dehydrogenases (31). The PEKG motif specifically found in the ω loop of VanA and VanB D-Ala:D-Lac ligases (13) was also present in VanD between positions 249 and 252 (data not shown). The presence of $VanH_D$, which was homologous to dehydrogenases producing D-Lac, and of VanD, which was related to D-Ala:D-Lac ligases, is consistent with the fact that the *vanD* gene cluster (22), like the *vanA* and *vanB* operons (6, 8), confers glycopeptide resistance by production of D-Ala–D-Lac-ending peptidoglycan precursors. The deduced product of the fifth ORF was homologous to penicillin-binding proteins (PBPs) displaying DD-carboxypeptidase activity (Fig. 1), and the gene was designated *vanY*$_D$. $VanY_D$ displayed 26% identity with a PBP from *Streptomyces* sp. strain K15 (20), with the putative DacF DD-carboxypeptidase involved in the sporulation of *Bacillus subtilis* MB24 (34), and with PBP 6 from *E. coli* (9). $VanY_D$ contained in the right order the motifs predicted to define the active sites of these PBPs (20): SXXK, which includes the catalytic serine, the SG(C/N) triad, and the KTG motif (Fig. 2). Consistent with these observations, the DD-carboxypeptidase activity detected in BM4339 was sensitive to penicillin G (22), unlike the activities of VanY (4) and $VanY_B$ (14).

A PCR strategy was used to complete the sequence of *vanS*$_D$. Since the gene organization in BM4339 was similar to those of the *vanA* and *vanB* clusters (6), an ORF related to *vanR* and *vanR*$_B$ was expected to be located upstream from *vanS*$_D$. In two-component regulatory systems, response regulators display well-conserved N-terminal domains (15). Based on the alignment of VanR, OmpR, and PhoB (5), the degenerate oligodeoxynucleotide VR was designed and used in combination with primer SD, specific for *vanS*$_D$ (Fig. 1). A product with the 1-kb expected size was obtained and cloned. Recombinant plasmid pAT657 (*vanR*$_D$′*S*$_D$′) contained the 5′ missing portion of *vanS*$_D$ and the 3′ half of the *vanR*$_D$ gene (Fig. 1). To recover the 5′ extremity of *vanR*$_D$, total DNA from BM4339 was digested and cloned into *E. coli* and transformants were

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VanY_D          NH2---120---TAKIAPASTAKMIMALTA---39---LIALMLPSGNDAAYTLA---106---RPEVIGLKTGTSSLGGA---39---COOH
S. K15 PBP      NH2----56---DTRRSTGSTTKIMTAKVV---43---LYGLMLPSGCDAAYALA---100---YSGAIGVKTGSGPEAKY---40---COOH
B. subtilis PBP NH2----56---NERLAPASMTKIMTMLLI---42---LKGIAIASGNDASVAMA----89---YPGVDGVKTGYTGEAKY---74---COOH
E. coli PBP 6   NH2----58---DEKLDPASLTKIMTSYVV---48---NKGVIIQSGNDACIALA----86---NLNVDGMKTGTTAGAGY---74---COOH

Conserved motifs                 motif I                  motif II                 motif III
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FIG. 2. Partial alignment of the deduced amino acid sequences of $VanY_D$ from *E. faecium* BM4339, PBP from *Streptomyces* sp. strain K15 (20), the putative DacF DD-carboxypeptidase from *B. subtilis* MB24 (34), and PBP 6 from *E. coli* (9). Conserved motifs involved in the scaffolding of the active site are indicated in boldface. The numbers of amino acids between the NH_2 terminus and motif I, motifs I and II, motifs II and III, and motif III and the COOH terminus are indicated.

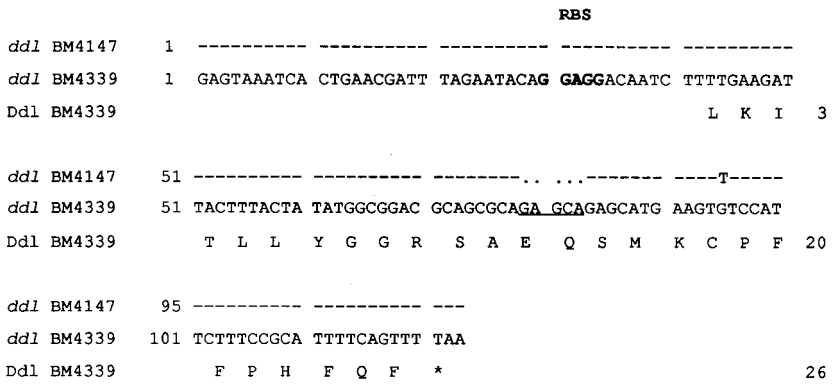

FIG. 3. Partial alignment of the nucleotide sequence of the *ddl* genes from *E. faecium* BM4339 and *E. faecium* BM4147 (14a). Numbers at the left refer to the position of the first nucleotide in the corresponding line. Identical bases are indicated by dashes in the BM4147 sequence. The putative RBS is indicated in boldface lettering. The 5-bp insertion in the BM4339 sequence is underlined and corresponds to a gap represented by dots in the BM4147 sequence. The deduced amino acid sequence of the *E. faecium* BM4339 *ddl* gene is indicated below the alignment. Numbers at the right refer to the position of the last amino acid of the corresponding line. The putative translation stop codon is indicated by an asterisk.

screened by hybridization with a probe internal to $vanR_D$ (Fig. 1). Recombinant plasmid pAT658 ($vanR_DS_D'$) carried a 1.8-kb insert which contained the entire $vanR_D$ gene (Fig. 1). A higher degree of identity was observed between $VanR_D$ and VanR and between $VanS_D$ and VanS than with the corresponding proteins encoded by the *vanB* gene cluster (Table 2). $VanR_D$ and $VanS_D$ were respectively as related to $VanR_B$ and $VanS_B$ as are VanR and VanS (Table 2) (14). No genes homologous to *vanZ* and *vanW* from the *vanA* and *vanB* operons, respectively, were found.

Cloning and sequence analysis of the *E. faecium* BM4339 *ddl* gene. The insert in recombinant plasmid pAT661 (*ddl* BM4339) (Table 1) was sequenced on 1,300 consecutive base pairs with divergent primers complementary to the termini of the 600-bp fragment internal to the BM4147 *ddl* gene (13) and specific oligodeoxynucleotides. In turn, the sequence of the BM4339 *ddl* region allowed the cloning by PCR of the entire BM4147 *ddl* gene (14a). Comparison of the two *ddl* sequences revealed the presence of a 5-bp insertion near the 5′ end in the BM4339 gene (Fig. 3). The insertion was responsible for a frameshift leading to the synthesis of a 26-amino-acid peptide instead of the putative 358-amino-acid Ddl. Production of a truncated protein accounts for the lack of D-Ala–D-Ala-containing peptidoglycan precursors in BM4339 (22). VanA-type (25, 29) and VanB-type (7, 32) mutants of *Enterococcus* impaired in Ddl activity grow only in the presence of glycopeptides. These antibiotics are required to induce production of the resistance ligase and dehydrogenase and, therefore, to synthesize peptidoglycan from D-Ala–D-Lac- instead of D-Ala–D-Ala-containing precursors. In *E. faecium* BM4339, constitutive expression of glycopeptide resistance (22) accounts for the fact that this strain is not glycopeptide dependent.

***trans* complementation of the insertional mutation in the BM4339 *ddl* gene.** The *ddl* gene from *E. faecium* BM4147 was cloned under the control of the heterologous enterococcal P_2 promoter in the gram-positive expression vector pAT79 (5), leading to pAT662 (*ddl* BM4147) (Table 1). The recombinant plasmid was introduced by electrotransformation into *E. faecium* BM4339, and transformants, such as BM4409 (Table 1), were susceptible to vancomycin and teicoplanin (MICs = 0.5 μg/ml). The decrease in glycopeptide resistance was most likely due to expression of the heterologous Ddl since no VanX DD-dipeptidase activity is present in cytoplasmic extracts from *E. faecium* BM4339 and only low levels of VanY DD-carboxypeptidase activity are found in membrane preparations (22).

Peculiarities of VanD-type glycopeptide resistance in *E. faecium* BM4339. Inducible expression of the resistance genes in VanA- and VanB-type strains is regulated by the two-component systems VanRS and $VanR_BS_B$, respectively. VanB-type constitutive variants harbor mutations in the $vanS_B$ sensor gene (7) that are thought to impair dephosphorylation of the $VanR_B$ regulator (2, 7). The sequences of $VanR_D$ and $VanS_D$ were analyzed for the presence of the amino acids involved in protein phosphorylation and of the motifs conserved in response regulators and protein kinases (21). The three amino acids Asp10, Asp53 (which corresponds to the putative site of phosphorylation), and Lys101, highly conserved in the effector domains of response regulators (21), were present in $VanR_D$ (data not shown). The five motifs characteristic of protein kinases (21), namely, H (positions 164 to 172), N (273 to 284), G1 (309 to 317), F (324 to 328), and G2 (340 to 346), including the histidine at position 166, which is the putative site of autophosphorylation (data not shown), were found in $VanS_D$. The constitutive phenotype of BM4339 may be due to mutations located near the putative autophosphorylation site and known to alter the phosphatase activity of $VanS_B$ (7). Alternatively, the signal recognition properties of $VanS_D$ may be impaired, leading to phosphorylation of $VanR_D$ even in the absence of glycopeptides. Another possibility is that alternate phosphorylation of $VanR_D$ by acetyl phosphate or by a heterologous protein kinase (2) maintains high concentrations of $VanR_D$-phosphate in spite of $VanS_D$ phosphatase activity.

As already mentioned, insertional inactivation of the BM4339 chromosomal *ddl* gene accounts for the absence of D-Ala–D-Ala-containing peptidoglycan precursors in this strain (22). Lack of a substrate for DD-dipeptidase hydrolysis makes $VanX_D$ superfluous in achieving glycopeptide resistance in BM4339. As a matter of fact, although $VanX_D$ does not exhibit mutations in the conserved residues involved in zinc binding and catalysis (17) (data not shown), BM4339 does not produce DD-dipeptidase activity (22). It has been shown that a mutation in the host *ddl* gene can compensate for inactivation of *vanX* in VanA-type strains (1a). Conversely, loss of production of $VanX_D$ DD-dipeptidase activity in BM4339 may be secondary to the impairment of the host ligase.

The $VanY_D$ DD-carboxypeptidase exhibited the same hydro-

phobicity profile as VanY (4) and $VanY_B$ (14), with a cluster of hydrophobic residues near the N terminus of the protein (data not shown). $VanY_D$ may thus be a membrane-anchored protein that acts like VanY and $VanY_B$. The DD-carboxypeptidase contributes to resistance by hydrolyzing precursors containing the D-Ala–D-Ala target of glycopeptides (1a, 3). This activity in BM4339 may explain the presence of tetrapeptide peptidoglycan precursors (17% of the precursors synthesized [22]). In this strain, the substrates for the DD-carboxypeptidase may be the pentapeptides (which represent only 2% of all precursors [22]) and, to a minor extent, the pentadepsipeptides. The pentapeptide precursors may conceivably originate from a very low rate of production of D-Ala–D-Ala by VanD. Like VanA (10) and VanB (18), the related VanD ligase may display broad substrate specificity, leading to synthesis of D-Ala–D-Ala at a level lower than that of D-Ala–D-Lac but in sufficient amount to require a weak contribution of $VanY_D$ DD-carboxypeptidase activity.

In conclusion, *E. faecium* BM4339 harbors the *vanD* gene cluster responsible for glycopeptide resistance. The D-Ala:D-Ala ligase in this strain is not functional following a mutation in the chromosomal *ddl* gene. However, replacement of the host metabolic pathway for synthesis of D-Ala-ending peptidoglycan precursors by the constitutively expressed resistance pathway leading to production of D-Lac-terminating precursors allows glycopeptide-independent growth of BM4339.

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