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VanD-Type Glycopeptide-Resistant *Enterococcus faecium* BM4339

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Enterococcus faecium **BM4339 was constitutively resistant to vancomycin (MIC, 64 μg/ml) and to low levels of teicoplanin (MIC, 4 μg/ml). A 605-bp product obtained with the V1 and V2 primers for amplification of genes encoding D-Ala:D-Ala ligases and related glycopeptide resistance proteins was sequenced after cloning. The deduced amino acid sequence had 69% identity with VanA and VanB and 43% identity with VanC, consistent with the finding that BM4339 synthesized peptidoglycan precursors terminating in D-lactate. This new type of glycopeptide resistance phenotype was designated VanD.**

Vancomycin and teicoplanin interact with the C-terminal D-alanyl–D-alanine (D-Ala–D-Ala) residue of peptidoglycan precursors and block cell-wall synthesis by inhibition of the transglycosylation and transpeptidation reactions (15). Glycopeptide resistance in enterococci is phenotypically and genotypically heterogeneous (for a review, see reference 4). Two acquired resistance phenotypes, transferable by conjugation, have been characterized: the VanA type confers high-level inducible resistance to both vancomycin and teicoplanin, whereas the VanB type displays variable levels of inducible resistance to vancomycin only (1). The VanA phenotype is mediated by Tn*1546* or related elements (1), which carry a gene cluster that includes *vanR* and *vanS*, encoding a two-component regulatory system that activates transcription of *vanH*, *vanA*, and *vanX* in response to the presence of glycopeptides; and *vanY*, encoding an accessory resistance protein. The VanH dehydrogenase reduces pyruvate to D-lactate, one of the substrates for VanA (5). The VanA ligase is related to D-Ala:D-Ala ligases but synthesizes the depsipeptide D-alanyl–D-lactate (D-Ala–D-Lac) instead of the dipeptide D-Ala–D-Ala (5). The *vanX* gene encodes a D,D-dipeptidase which hydrolyzes the dipeptide D-Ala–D-Ala produced by the chromosomal D-Ala:D-Ala ligase, thus preventing pentapeptide synthesis (3, 16, 18). The *vanY* gene encodes a D,D-carboxypeptidase, insensitive to the activity of β-lactams such as penicillin G, which hydrolyzes the C-terminal residue of any remaining UDP-MurNAc-pentapeptide, thereby preventing the translocation of D-Ala–D-Ala-containing precursors to the cell surface (2, 10, 17). VanB-type resistance is mediated by a similar gene cluster (9). Intrinsically resistant *Enterococcus gallinarum*, *Enterococcus casseliflavus*, and *Enterococcus flavescens* produce a D-Ala:D-serine VanC ligase and a D-Ala–D-Ala ligase (13, 14).

Enterococcus faecium BM4339, isolated from urine, was resistant to penicillin G (MIC, 256 μg/ml), gentamicin (MIC, >2,000 μg/ml), streptomycin (MIC, >2,000 μg/ml), tetracycline (MIC, 16 μg/ml), and macrolide-lincosamide-streptogramin B-type antibiotics. It was also resistant to vancomycin (MIC, 64 μg/ml) and to low levels of teicoplanin (MIC, 4 μg/ml). Levels of growth of BM4339 in the absence of and following the addition of vancomycin (8 μg/ml) were identical (data not shown), indicating that resistance was expressed constitutively.

To determine the genotype responsible for the unusual resistance of BM4339, we used an assay, based on the specific amplification of fragments internal to genes encoding D-Ala:D-Ala ligases and related glycopeptide resistance proteins, which allows simultaneous identification of the glycopeptide resistance genotypes reported so far in enterococci and of the host at the species level (8). Despite several attempts, no PCR product was obtained with primers specific for glycopeptide resistance genes *vanA*, *vanB*, and *vanC*, whereas an amplification product was obtained with primers specific for the *E. faecium ddl* gene. Using degenerate V1 and V2 primers that allow PCR amplification of fragments internal to genes encoding related ligases (7), we obtained a 605-bp fragment which was cloned into *Escherichia coli* and sequenced on both strands (Fig. 1). The deduced amino acid sequence was aligned with those of VanA, VanB, and VanC (Fig. 2), and the percentage of identity was derived from this alignment (Table 1). This sequence shares 69% identity with the corresponding portions of VanA and VanB and 43% identity with VanC. In Southern blots under stringent conditions (6), the 605-bp PCR fragment hybridized with total DNA from BM4339 but not with that of VanA-, VanB-, or VanC-type strains (data not shown). These results suggest that we had sequenced an internal portion of a putative glycopeptide resistance gene of a new type that was designated *vanD*. Based on this sequence, primers specific for the *vanD* gene (5′ TAAGGCGCTTGCATATACCG 3′ and 5′ TGCAGCCAAGTATCCGGTAA 3′) were designed and used to amplify fragments internal to genes encoding related ligases from *Enterococcus* strains BM4147 (*vanA*), V583 (*vanB*), BM4174 (*vanC*), and BM4339 (*vanD*) under the following conditions: 30 s at 94°C, 15 s at 54°C, and 15 s at 72°C (30 cycles). A 461-bp product which was sequenced directly was obtained only with BM4339 DNA, confirming that the oligonucleotides were specific for *vanD*.

Transfer of vancomycin resistance from BM4339 to *E. faecium* BM4107 and *Enterococcus faecalis* JH2-2 (12) by filter matings was attempted (6). The putative transconjugants were selected on brain heart infusion agar containing 20 μg of rifampin, 10 μg of fusidic acid, and 10 μg of erythromycin per ml or 1,000 μg of streptomycin or 8 μg of vancomycin per ml. In both recipients, only transfer of erythromycin and streptomycin resistance not associated with vancomycin resistance was observed.

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CTT GAA TTG TCA GGC ATT CCG TAT GTG GGA TGC GAT ATT CAA AGC TCC GTG ATC TGC ATG
L   E   L   S   G   I   P   Y   V   G   C   D   I   Q   S   S   V   I   C   M

GAT AAG GCG CTT GCA TAT ACC GTT GTG AAA AAT GCG GGT ATC ACT GTG CCT GGG TTC CGG
D   K   A   L   A   Y   T   V   V   K   N   A   G   I   T   V   P   G   F   R

ATC CTT CAG GAG GGT GAT CGC CTG GAA ACG GAG GAT TTC GTA TAT CCC GTT TTT GTA AAG
I   L   Q   E   G   D   R   L   E   T   E   D   F   V   Y   P   V   F   V   K

CCT GCC CGT TCC GGC TCA TCC TTT GGC GTA AAC AAG GTA TGC AAG GCA GAA GAA CTG CAG
P   A   R   S   G   S   S   F   G   V   N   K   V   C   K   A   E   E   L   Q

GCA GCA ATC GAA GAA GCA AGA AAA TAT GAC AGC AAG ATT TTG ATT GAA GAG GCC GTT ACC
A   A   I   E   E   A   R   K   Y   D   S   K   I   L   I   E   E   A   V   T

GGG AGT GAG GTA GGC TGC GCC ATA CTG GGA AAC GGA AAT GAT CTC ATG GCT GGC GAG GTG
G   S   E   V   G   C   A   I   L   G   N   G   N   D   L   M   A   G   E   V

GAT CAG ATT GAG CTG AGA CAC GGC TTT TTT AAG ATT CAT CAG GAA GCA CAG CCG GAG AAG
D   Q   I   E   L   R   H   G   F   F   K   I   H   Q   E   A   Q   P   E   K

GGA TCT GAA AAT GCA GTC ATC CGA GTT CCA GCC GCC TTA CCG GAT GAG GTA AGA GAA CAG
G   S   E   N   A   V   I   R   V   P   A   A   L   P   D   E   V   R   E   Q

ATT CAG GAA ACG GCA ATG AAG ATT TAC CGG ATA CTT GGC TGC AGA GGA TTG GCC CGC ATT
I   Q   E   T   A   M   K   I   Y   R   I   L   G   C   R   G   L   A   R   I

GAC CTG TTT TTG CGG GAG GAC GGT TGC ATT GTG CTG AAT GAA GTG AAC ACC TTC CCC GGC
D   L   F   L   R   E   D   G   C   I   V   L   N   E   V   N   T   F   P   G

TTC AC
F
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FIG. 1. Nucleotide and deduced amino acid sequences of the 605-bp PCR product obtained with degenerate V1 and V2 primers.

Putative D,D-dipeptidase and D,D-carboxypeptidase activities in *E. faecium* BM4339 were assayed by determining the amount of D-Ala released from hydrolysis of the dipeptide D-Ala–D-Ala and of the pentapeptide L-Ala–γ-D-Glu–L-Lys–D-Ala–D-Ala, respectively (3). The latter activity was also investigated by high-performance liquid chromatography (HPLC) analysis of the substrate UDP-MurNAc-pentapeptide and product UDP-MurNAc-tetrapeptide of assays carried out in the presence and absence of penicillin. Experiments were performed with the 100,000 × *g* supernatant of lysed bacteria that had been grown in the presence of various concentrations of vancomycin as an inducer (0, 8, and 16 μg/ml). The dipeptide D-Ala–D-Ala was not hydrolyzed by cytoplasmic extracts from induced or noninduced *E. faecium* BM4339 (Table 2). The lack of D,D-peptidase activity was surprising. The extracts were prepared by two methods (lysozyme plus sonication and lysozyme-muramidase osmotic lysis) on several occasions in two laboratories, but no activity was detected even by sensitive assays. It

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VanD   L ELSGIPYVGC DIQSSVICMD KALAYTVVKN AGITVPGFRI LQEGDRLET. .....E...D  52
VanA   F ELSGIPFVGC DIQSSAICMD KSLTYIVAKN AGIATPAFWV INKDDRPVA. .....A...T  52
VanB   F ELSGIPYVGC DIQSSAACMD KSLAYILTKN AGIAVPEFQM IEKGDKPEA. .....R...T  52
VanC   L ELMNLPYVGC HVAASALCMN KWLLHQLADT MGIASAPTLL LSRYENDPAT IDRFIQ...D  58
 Efe   L ETLNMPYVGA GVLTSACAMD KIMTKYILQA AGVPQVPYVP VLKNQWKENP KKVFDQCEGS  61

VanD   FVYPVFVKPA RSGSSFGVNK VCKAEELQAA IEEARKYDSK ILIEEAVTGS EVGCAILGNG  112
VanA   FTYPVFVKPA RSGSSFGVKK VNSADELDYA IESARQYDSK ILIEQAVSGC EVGCAVLGNS  112
VanB   LTYPVFVKPA RSGSSFGVTK VNSTEELNAA IEAAGQYDGK ILIEQAISGC EVGCAVMGNE  112
VanC   HGFPIFIKPN EAGSSKGITK VTDKTALQSA LTTAFAYGST VLIQKAIAGI EIGCGILGNE  118
 Efe   LLYPMFVKPA NMGSSVGITK AENREELQNA LATAYQYDSR AIVEQGIEAR EIEVAVLGNE  121

VanD   NDLMAGEVDQ IELRHGFFKI HQEAQPEKGS ENAVIRVPAA LPDEVREQIQ ETAMKIYRIL  172
VanA   AALVVGEVDQ IRLQYGIFRI HQEVEPEKGS ENAVITVPAD LSAEERGRIQ ETAKKIYKAL  172
VanB   DDLIVGEVDQ IRLSHGIFRI HQENEPEKGS ENAMIIVPAD IPVEERNRVQ ETAKKVYRVL  172
VanC   .QLTIGACDA ISLVDGFFDF EEKYQL.... ISATITVPAP LPLALESQIK EQAQLLYRNL  173
 Efe   .DVRTTLPGE VVKDVAFYDY EAKYINNK.I E...MQIPAE VPEEVYQKAQ EYAKLAYTML  176

VanD   GCRGLARIDL FLREDGCIVL NEVNTFPGF.                                  201
VanA   GCRGLARVDM FLQDNGRIVL NEVNTLPGF                                   201
VanB   GCRGLARVDL FLQEDGGIVL NEVNTLPGF                                   201
VanC   GLTGLARIDF FVTNQGAIYL NEINTMPGF                                   202
 Efe   GGSGLSRCDF FLTNKNELFL NEL......                                   199
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FIG. 2. Alignment of the deduced partial amino acid sequence of VanD and of the corresponding regions of VanA, VanB, and VanC. Identical amino acids are indicated in boldface. Efe, D-Ala:D-Ala ligase of *E. faecium*. The numbering of VanD and Efe is according to the VanA coordinates.

TABLE 1. Sequence identity between the deduced amino acid sequences of VanD and related ligases

Sequence compared	% Sequence identity			
	VanD	VanA	VanB	VanC
VanA	69			
VanB	69	76		
VanC	43	40	40	
Efe[a]	34	34	36	34

[a] Efe, D-Ala–D-Ala ligase of *E. faecium.*

can be inferred from the wall precursor assays (vide infra) that the presence of this enzyme is unnecessary in this strain. The lack of D,D-dipeptidase activity could be due to either the absence of the *vanX* gene or production of an inactive VanX protein. Low levels of D,D-carboxypeptidase were detected in membrane preparations from BM4339, and there was no difference in enzyme activity between uninduced and induced bacteria (Table 3). Penicillin G dramatically inhibited this activity, since after addition of this antibiotic (10 mM), no D,D-carboxypeptidase activity was detectable. Penicillin-binding proteins display a D,D-carboxypeptidase activity that is susceptible to penicillin G, whereas VanY D,D-carboxypeptidase activity is penicillin insensitive. The results obtained with *E. faecium* BM4339 suggest that VanY is either absent, inactive, or, less likely, susceptible to penicillin G.

Analysis of the cytoplasmic peptidoglycan precursors that accumulated in cultures incubated in the presence of ramoplanin to inhibit wall synthesis after formation of the precursors indicated that UDP-MurNAc-pentadepsipeptide, UDP-MurNAc-tetrapeptide, and UDP-MurNAc-tripeptide were the main compounds present and that there was only a small quantity (2%) of UDP-MurNAc-pentapeptide (Table 3). The HPLC profiles were identical whether the bacteria had been grown in the presence (8 μg/ml) or absence of vancomycin, a finding consistent with the observation that the growth curves of BM4339 in the absence or presence of the same concentration of vancomycin were indistinguishable. The large proportion of UDP-MurNAc-pentadepsipeptide indicates that the mechanism of vancomycin resistance in BM4339 is similar to that in VanA and VanB strains. UDP-MurNAc-tripeptide is an "early" precursor in the pathway of peptidoglycan synthesis and is not normally present in substantial amounts in glycopeptide-susceptible or -resistant enterococci. Its presence in BM4339 implies that the rate of synthesis of D-Ala–D-Ala or D-Ala–D-Lac substrates is limiting. It is possible that the host ligase might be inactive; this would be consistent with the

TABLE 2. D,D-Dipeptidase and D,D-carboxypeptidase activities in extracts from *E. faecium* BM4339

Concn of vancomycin for induction (μg/ml)	Sp act (nmol min^{-1} mg^{-1})[a]		
	D,D-Dipeptidase	D,D-Carboxypeptidase[b]	
		No penicillin G	Penicillin G (10 mM)
0	2.84 ± 0.5	10.38 ± 2.88	$<10^{-1}$
8	2.52 ± 0.4	11.77 ± 3.12	0.65 ± 0.28
16	2.77 ± 0.6	11.91 ± 2.75	$<10^{-1}$

[a] Activity in the 100,000 × *g* supernatant of lysed bacteria was measured. Values are means ± standard deviations of a minimum of three independent experiments.

[b] Activity in the resuspended pellet fraction was measured after centrifugation of lysed bacteria at 100,000 × *g* for 45 min.

TABLE 3. Peptidoglycan precursors synthesized by *E. faecium* BM4339[a]

Culture	% of precursor synthesized			
	UDP-MurNAc-tripeptide	UDP-MurNAc-tetrapeptide	UDP-MurNAc-pentapeptide	UDP-MurNAc-pentadepsipeptide
Uninduced	19	21	2	58
Induced (vancomycin, 8 μg/ml)	17	17	2	64

[a] Cultures were incubated with ramoplanin to inhibit peptidoglycan synthesis for the equivalent of 0.5 mean time generation. Cell extracts were prepared and precursors were analyzed by HPLC as described previously (3). The individual precursors were characterized by amino acid analysis and mass spectroscopy.

virtual absence of UDP-MurNAc-pentapeptide and of VanX-type activity from the cytoplasm. The activities of purified D-Ala:D-Lac ligases are low in comparison with those of D-Ala:D-Ala ligases (4, 5). A relationship between these values and the in vivo activities could account for the accumulation of UDP-MurNAc-tripeptide in the cytoplasm of BM4339 when wall synthesis is inhibited. The absence of a penicillin-insensitive D,D-carboxypeptidase (VanY-type activity) is surprising in view of the presence of UDP-MurNAc-tetrapeptide in the cytoplasm. This compound is only likely to have been derived from the hydrolysis of UDP-MurNAc-pentapeptide or UDP-MurNAc-pentadepsipeptide. The results suggest the compound may have originated from the latter precursor, though the enzyme activity involved has not yet been identified. Finally, resistance only to low levels of glycopeptides in BM4339 remains to be explained, since pentapeptide represents a very small amount of the peptidoglycan precursors in this strain. A decrease in the proportion of UDP-MurNAc-pentapeptide has been correlated with an increase in the MIC of vancomycin (3), although the genetic background of enterococcal strains may influence the MIC of vancomycin or teicoplanin (11).

Taken together, these data suggest that *E. faecium* BM4339 expressed a new type of resistance to glycopeptides. The *vanD* gene encoded a D-Ala:D-Lac ligase related to VanA and VanB and was not transferable by conjugation. Studies are in progress to elucidate the organization of the *vanD* gene cluster in BM4339 and thus the mechanism of resistance to glycopeptides in this strain.

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