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Characterization of a Divergent *vanD*-Type Resistance Element from the First Glycopeptide-Resistant Strain of *Enterococcus faecium* Isolated in Brazil

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***Enterococcus faecium* 10/96A from Brazil was resistant to vancomycin (MIC, 256 μg/ml) but gave no amplification products with primers specific for known *van* genotypes. A 2,368-bp fragment of a *van* cluster contained one open reading frame encoding a peptide with 83% amino acid identity to $VanH_D$, and a second encoding a D-alanine-D-lactate ligase with 83 to 85% identity to VanD. The divergent glycopeptide resistance phenotype was designated VanD4.**

Four phenotypes of acquired glycopeptide resistance have been identified in enterococci. VanA and VanB are the most common types (18), whereas VanE is known from a single strain of *Enterococcus faecalis* (7) and VanD is known from three strains of *Enterococcus faecium*, one from New York (4, 14), one from Boston, Mass. (11), and one from Toronto, Ontario, Canada (3, 13). We report the characterization of a divergent *vanD*-type resistance element in the first glycopeptide-resistant enterococcus strain to be isolated in Brazil.

E. faecium 10/96A was isolated in August 1996 from the blood of a 9-year-old girl with aplastic anemia (6). It was the first glycopeptide-resistant enterococcus isolated in Brazil and probably the first isolated in South America, predating by 1 month a VanA strain reported from Argentina (10). Susceptibility to glycopeptides was determined with E-tests (Cambridge Diagnostics Ltd., Cambridge, United Kingdom) on Diagnostic Sensitivity Test agar (Oxoid, Basingstoke, United Kingdom) containing 5% lysed horse blood. All the PCRs used published primers and amplification conditions (see below). Selected amplicons were cloned into vector pCR2.1-TOPO (Invitrogen, Groningen, The Netherlands) and transformed into *Escherichia coli* strain TOP10 (Invitrogen). Sequencing was performed with a Dye-Labeled ddNTP Terminator Cycle Sequencing Kit (Beckman Coulter UK Ltd., High Wycombe, United Kingdom), and samples were analyzed on a CEQ 2000 automated sequencer (Beckman). Consensus sequences were assembled with Contig Express (Informax Inc., Oxford, United Kingdom); other manipulations of DNA and peptide sequences were performed as described previously (20). The composition of cytoplasmic peptidoglycan precursors was analyzed after growth of the *E. faecium* strain in the presence and absence of 4 μg of vancomycin/ml as described previously (2). Assays for D,D-dipeptidase and D,D-carboxypeptidase activities were performed on cell extracts also prepared from vancomycin-exposed and -unexposed cells, as described previously (1).

Strain 10/96A was highly resistant to vancomycin (MIC, 256 μg/ml) but was susceptible to teicoplanin (MIC, 4 μg/ml). It yielded no amplification products with primers specific for *vanA*, *vanB*, or *vanD* (18), which encode D-alanine-D-lactate (D-Ala-D-Lac) ligases, or with those for *vanC-1*, *vanC-2*, or *vanE* (7, 18), which encode D-Ala-D-Ser ligases. Despite this, the only confirmed mechanism of glycopeptide resistance in *Enterococcus* spp. is mediated by the production of D-Ala-D-X ligases; therefore, a novel ligase was sought in the strain. Degenerate primers van-V3 (5′-GAR GAT GGI TSC ATM CAR GGW-3′) and van-V4 (5′-MGT RAA ICC IGG CAK RGT RTT-3′) were used, with published cycling conditions (8). A 630-bp fragment was amplified, cloned into pCR2.1-TOPO to yield plasmid pARL00.17, and sequenced. The deduced 210-amino-acid partial peptide showed approximately 84% identity with the three VanD ligases listed in the GenBank database. These three sequences, all from strains of *E. faecium*, are not identical, and we propose numbering the alleles in accordance with their dates of deposition in GenBank. On that basis, *vanD1* is the allele of strain BM4339 from New York (GenBank accession no. AF130997) (4), *vanD2* is the allele of strain A902 from Boston (GenBank accession no. AF153050) (11), and *vanD3* is the allele of strain N97-330 from Toronto (GenBank accession no. AF175293) (3). Since the partial sequence from *E. faecium* 10/96A showed less than 20% amino acid divergence from these sequences, the allele was designated *vanD4*, in accordance with recommendations for standardizing gene nomenclature (15). Attempts to transfer the VanD4 phenotype to enterococcus recipient strains *E. faecalis* JH2-2 and *E. faecium* GE-1 (19) by conjugation were unsuccessful. Moreover, a digoxigenin-labeled probe (Roche, Lewes, United Kingdom) prepared from the 630-bp insert of pARL00.17 and used under stringent conditions hybridized only with the residual chromosomal DNA present in plasmid preparations of *E. faecium* 10/96A.

A 2,368-bp fragment of the *vanD4* cluster from strain 10/96A was amplified with primers 3-forward (5′-TTT CAG AAA TTG TGG CAA GCA-3′) and 3-reverse (5′-ATG TGG CAT ATT TGG CAT CC-3′) (11), cloned into pCR2.1-TOPO to yield plasmid pARL00.30, and then sequenced. The fragment contained the complete *vanD4* gene, which was predicted to encode a D-Ala-D-Lac ligase of 343 amino acids. It is likely that the *vanD4* allele was not detected with published *vanD* primers

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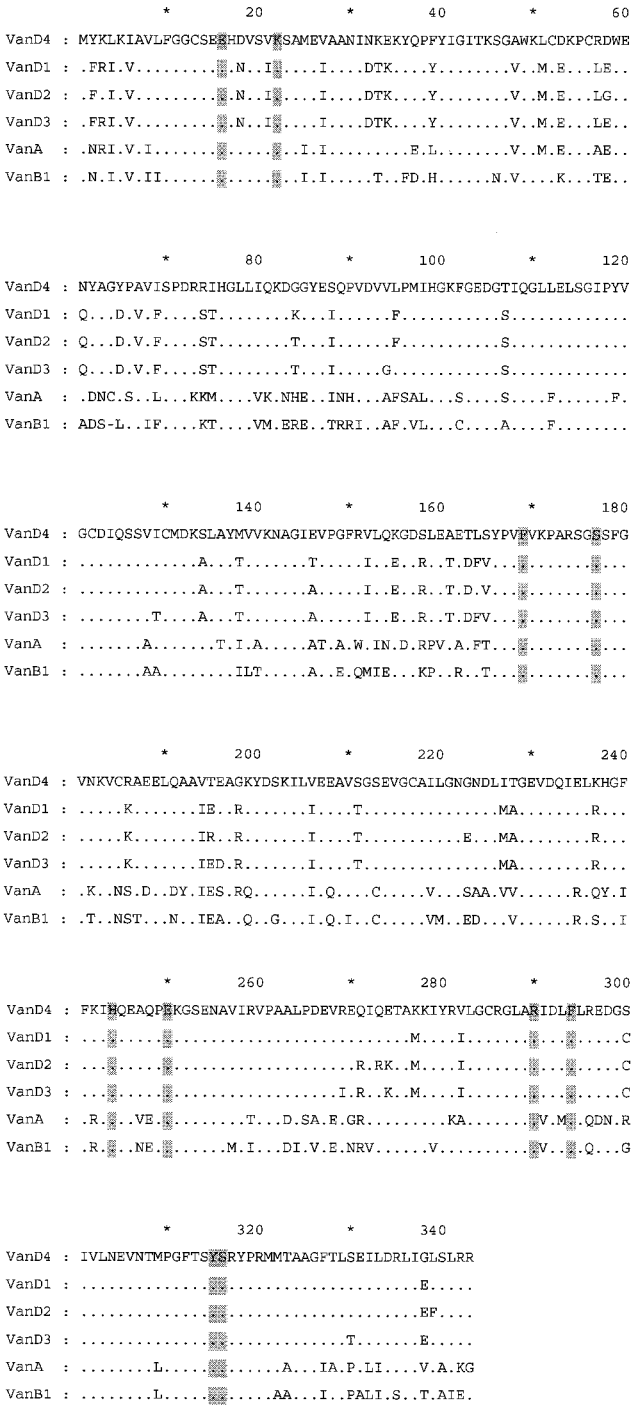

FIG. 1. Comparison of the amino acid sequences of VanD4 and other D-Ala-D-Lac ligases from glycopeptide-resistant enterococci. The conserved residues that form the active site, and those important for ligand binding in the VanA ligase, are shaded. These are Glu-16, Lys-22, Phe-169, Ser-177, His-244, Glu-250, Arg-290, Phe-294, Tyr-315, and Ser-316 (9, 16). Data for comparison are taken from Genbank accession no. AF130997 (VanD1), AF153050 (VanD2), AF175293 (VanD3), M97297 (VanA), and U35369 (VanB1).

(14, 18) because, although the reverse primer had only one mismatch with *vanD4* and would be expected to anneal, the forward primer had five mismatches, two of which were located at the 3′ end of the primer. The VanD4 peptide showed 83 to 85% amino acid identity to the VanD1, VanD2, and VanD3 ligases and 68% identity with the VanA and VanB ligases (Fig. 1; Table 1). The residues believed to comprise the active site of VanA are conserved in all enterococcal D-Ala-D-Lac ligases, including VanD4, as are those associated with ligand binding (9, 16) (Fig. 1).

TABLE 1. Percent amino acid identities between VanD4 and selected D-Ala-D-Lac ligases

Ligase	% Amino acid identity					
	VanD4	VanD1	VanD2	VanD3	VanA	VanB1
VanD4	100	85	84	83	68	68
VanD1		100	96	97	68	67
VanD2			100	96	67	67
VanD3				100	68	67
VanA					100	75
VanB1						100

A second complete open reading frame (ORF) was located upstream of *vanD4*. This encoded a putative keto acid dehydrogenase with 83% amino acid identity to the three published $VanH_D$ peptides (Table 2). A partial ORF of 91 amino acids, located upstream of $vanH_{D4}$, had 93 to 97% identity with the $VanY_{D1}$ through $VanY_{D3}$ peptides, and another partial ORF of 23 amino acids, located downstream of *vanD4*, had homology with the $VanX_{D1}$ through $VanX_{D3}$ peptides. Hence the genetic organization of the *vanD4* cluster—$vanY_{D4}vanH_{D4}vanD4$ $vanX_{D4}$—resembles those reported in other VanD strains.

Pools of cytoplasmic peptidoglycan precursors were analyzed from cells of strain 10/96A grown in the presence or absence of 4 μg of vancomycin/ml. In both cases, the pools contained 95% UDP-MurNAc-pentadepsipeptide, 3% UDP-MurNAc-pentapeptide, and 2% UDP-MurNAc-tetrapeptide. This supported the role of VanD4 as a D-Ala-D-Lac ligase and indicated that glycopeptide resistance was expressed constitutively. D,D-carboxypeptidase ($VanY_{D4}$) activity was detected in membrane fractions of strain 10/96A (Table 3) and was not inhibited significantly by penicillin, even at 100 μg/ml, which contrasts with the $VanY_D$ activities of other VanD enterococci studied (13, 14). Negligible D,D-dipeptidase ($VanX_{D4}$) activity was detected in the cytoplasmic fractions of strain 10/96A (Table 3). Two other VanD strains, BM4339 (14) and BM4416 (13) (also published as N97-330 [3]), also had undetectable or very weak D,D-dipeptidase activity. Despite the lack of $VanX_D$ activity, both strains expressed vancomycin resistance because of impaired D-Ala-D-Ala ligase (Ddl) activity; glycopeptide dependence was obviated by constitutive expression of the *van* clusters. It is possible that strain 10/96A also has impaired Ddl activity, as it also expressed constitutive vancomycin resistance and had negligible $VanX_{D4}$ activity.

In summary, we have reported a strain of vancomycin-resis-

TABLE 2. Percent amino acid identities between $VanH_{D4}$ and selected α-keto acid dehydrogenases from glycopeptide-resistant enterococci

Dehydrogenase	% Amino acid identity					
	$VanH_{D4}$	$VanH_{D1}$	$VanH_{D2}$	$VanH_{D3}$	VanH	$VanH_{B1}$
$VanH_{D4}$	100	83	83	83	59	61
$VanH_{D1}$		100	97	99	58	63
$VanH_{D2}$			100	98	59	63
$VanH_{D3}$				100	59	63
VanH					100	67
$VanH_{B1}$						100

TABLE 3. D,D-Dipeptidase ($VanX_{D4}$) and D,D-carboxypeptidase ($VanY_{D4}$) activity in extracts of *E. faecium* 10/96A

Concn of vancomycin (μg/ml)	D,D-Dipeptidase activity[a] (nmol min^{-1} mg^{-1})	D,D-Carboxypeptidase activity[b] (nmol min^{-1} mg^{-1})	
		Without penicillin G	With 100 μg of penicillin G/ml
0	1.0	60	52
4	1.3	62	54

[a] Hydrolysis of 10 mM D-Ala-D-Ala measured in the supernatant of osmotically lysed bacteria after centrifugation at 40,000 × *g* for 20 min.

[b] Hydrolysis of 10 mM UDP-MurNAc-pentapeptide measured in the resuspended pellet fraction after centrifugation at 40,000 × *g* for 20 min.

tant *E. faecium* from Brazil that contained a novel *vanD* allele. No other similar strains were isolated at the hospital, and the source of this strain is unknown; the patient had no known links with the United States or Canada. The three other published VanD ligases share >96% amino acid identity, but VanD4 showed greater divergence. The geographical scatter of the strains and the divergence in the genes suggest multiple escapes of *vanD* clusters into *E. faecium* from as yet unrecognized donor species. The allelic nature of VanD resistance is similar to that seen with VanB resistance (5, 8); by contrast, the most globally widespread and prevalent form of glycopeptide resistance in enterococci, VanA, shows remarkable sequence homogeneity, with only a few point mutations identified. VanA resistance elements typically vary by deletions and the presence of insertion sequences in nonessential regions, not by variation in the sequences of the resistance genes themselves (12, 17).

Nucleotide sequence accession number. The complete nucleotide sequence of the 2,368-bp insert of plasmid pARL00.30 has been deposited under accession no. AF277571.

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