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

LIBERA M. DALLA COSTA,¹,² PETER E. REYNOLDS,³ HELENA A. P. H. M. SOUZA,² DILAIR C. SOUZA,² MARIE-FRANCE I. PALEPOU,¹ AND NEIL WOODFORD¹*

Antibiotic Resistance Monitoring and Reference Laboratory, Central Public Health Laboratory, London NW9 5HT;¹ and Department of Biochemistry, University of Cambridge CB2 1QW,³ United Kingdom, and Hospital de Clinicas—Universidade Federal do Paraná, Curitiba, Paraná, Brazil²

Received 26 June 2000/Returned for modification 22 August 2000/Accepted 12 September 2000

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, 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. Enterococcus 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 Diagnostico 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 Enterococcus 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 GTT TSC ATM CAR GGW-3') and van-V4 (5'-MGT RAA ICC IGG CKG 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 Enterococcus 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. AF135050) (11), and vanD3 is the allele of strain N97-330 from Toronto (GenBank accession no. AF175293) (3). Since the partial sequence from Enterococcus 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 Enterococcus 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 TTG 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.
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).

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 VanHD peptides (Table 2). A partial ORF of 91 amino acids, located upstream of vanHD4, had 93 to 97% identity with the VanYD1 through VanYD3 peptides, and another partial ORF of 23 amino acids, located downstream of vanD4, had homology with the VanXD1 through VanXD3 peptides. Hence the genetic organization of the vanD4 cluster—vanYD4vanHD4vanD4vanXD4—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 (VanYD4) 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 VanYD activities of other VanD enterococci studied (13, 14). Negligible D,D-dipeptidase (VanXD4) 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 VanXD 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 VanXD4 activity.

In summary, we have reported a strain of vancomycin-resistant enterococci and its genetic and enzymatic characterization.
TABLE 3. D,D-Dipeptidase (VanXD4) and D,D-carboxypeptidase (VanYD4) activity in extracts of E. faecium 10/96A

<table>
<thead>
<tr>
<th>Conc of vancomycin (µg/ml)</th>
<th>D,D-Dipeptidase activity (nmol min⁻¹ mg⁻¹)</th>
<th>D,D-Carboxypeptidase activity&lt;sup&gt;a&lt;/sup&gt; (nmol min⁻¹ mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without penicillin G</td>
<td>With 100 µg of penicillin G/ml</td>
</tr>
<tr>
<td>0</td>
<td>1.0</td>
<td>60</td>
</tr>
<tr>
<td>4</td>
<td>1.3</td>
<td>62</td>
</tr>
</tbody>
</table>

<sup>a</sup> Hydrolysis of 10 mM D-Ala-D-Ala measured in the supernatant of osmotically lysed bacteria after centrifugation at 40,000 x g for 20 min.

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

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.

L.M.D.C. was supported by a grant from the Conselho Nacional de Desenvolvimento Científico e Tecnológico—CNPq, process number 200520/99-7.

We are grateful to Dave Roper (York Structural Biology Laboratory, York, United Kingdom) for helpful discussions during the preparation of this report.

REFERENCES

Downloaded from http://aac.asm.org/ on August 27, 2017 by guest