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Molecular Characterization of the *vanD* Gene Cluster and a Novel Insertion Element in a Vancomycin-Resistant Enterococcus Isolated in Canada

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A single *vanD*-containing *Enterococcus faecium* strain (N97-330) was isolated in Canada. The *vanD*-containing region was cloned and sequenced. Although the proteins have more than 96% identity to a previously described *vanD* region in BM4339, the $vanS_D$ gene contains a frameshift mutation that leads to a predicted truncated protein. Furthermore, sequence analysis of the *ddl* gene revealed the presence of an IS*982*-like element (IS*Efm1*) which interrupted the D-Ala–D-Ala ligase. This suggested the constitutive expression of the *vanD* operon, which was confirmed. Pulsed-field gel electrophoresis fingerprinting demonstrated that BM4339 was not related to N97-330 (>15 band differences). Both strains contained multiple copies of the IS*982*-like element.

Vancomycin-resistant enterococci produce modified precursors that terminate in either D-alanyl-D-lactate (D-Ala-D-Lac) or D-alanyl-D-serine (D-Ala-D-Ser), which have a much lower affinity for glycopeptides than do unmodified precursors (2, 5). The genetic basis for resistance lies in genes whose products have homology to the bacterial D-Ala-D-Ala ligases, encoded by *ddl* genes, which produce the dipeptide target for glycopeptide antibiotics. High-level vancomycin resistance is conferred either by the transferable, inducible VanA or VanB D-Ala-D-Lac ligases (4, 11, 18) or by the nontransferable, constitutive VanD D-Ala-D-Lac ligase, which thus far has been described only for *Enterococcus faecium* BM4339 (7, 17) and *E. faecium* A902 (15). This report describes the genetic characterization of vancomycin resistance in a strain of *E. faecium* which is the first VanD-type strain isolated in Canada.

A vancomycin-resistant enterococcus was isolated from a stool specimen from a 59-year-old Ontario man who had had an orthoptic liver transplant 46 days before and had received multiple courses of antibiotics. Multiple attempts to amplify *vanA*, *vanB*, and *vanC1* using PCR with the previously reported primer sets (8) were negative. Initial confirmation of the isolate as a possible VanD-type strain was done courtesy of P. Courvalin (Institut Pasteur) using primers which amplify a 0.46-kb *vanD* fragment (17). The strain (N97-330) was identified as an *E. faecium* strain by using standard biochemical tests for strain identification (9). The MICs (micrograms per milliliter) of a number of antibiotics as determined by agar dilution (14) are shown in Table 1 and compared to those for the *vanD* strains BM4339 and A902 (15, 17). Expression of the vancomycin-resistance phenotype was not inducible in N97-330 (data not shown), suggesting constitutive expression of resistance genes similar to that in BM4339 (17) but unlike that in A902 (15). Macrorestriction analysis was carried out by separating *ApaI*- or *Sma*I-digested genomic DNA by pulsed-field gel electrophoresis using 1.1% agarose gels and a CHEF-DRIII (Bio-Rad, Hercules, Calif.) with the following pulse times: 1 to 10 s

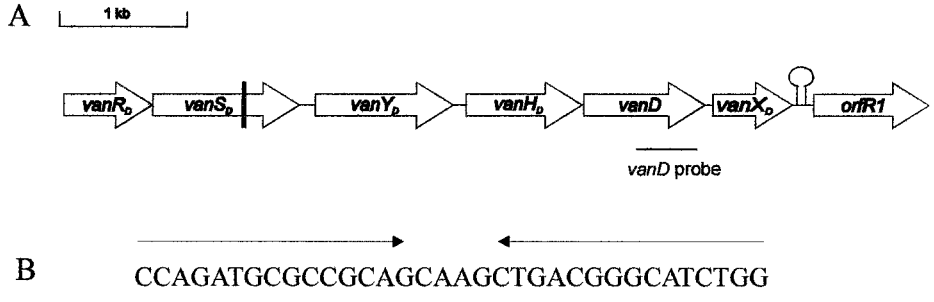

FIG. 1. Schematic representation of the *vanD* gene cluster and sequence of a putative stem-loop structure. (A) Predicted ORFs from a 6,793-bp region sequenced from two λ clones and a PCR product. Open arrows represent the coding sequences for the $vanR_D$, $vanS_D$, $vanY_D$, $vanH_D$, *vanD*, $vanX_D$, and *orfR1* genes. The vertical line in the $vanS_D$ ORF represents the position of the frameshift mutation leading to a predicted truncated protein. The *vanD* probe used in hybridization experiments to isolate the λ clones is shown below the corresponding region. (B) Sequence of the putative stem-loop structure downstream of $vanX_D$.

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TABLE 1. Antibiograms of *vanD*-containing *E. faecium* strains N97-330, BM4339 (17), and A902 (15)

Antibiotic	MIC (μg/ml)[a]		
	N97-330	BM4339	A902
Ampicillin	128	NA	64–128
Penicillin G	256	256	256
Chloramphenicol	8	NA	S
Doxycycline	0.12	NA	NA
Gentamicin	<500	>2,000	R
Streptomycin	>2,000	>2,000	R
Tetracycline	0.25	16	S
Vancomycin	>256	64	128
Teicoplanin	64	4	4[b]

[a] MICs for N97-330 and A902 were determined by agar dilution; the method of determining the MICs for BM4339 was not given. NA, MIC not available in the publication; S, susceptible to the antibiotic; R, resistant to high levels of the antibiotic.

[b] 16 to 32 μg/ml by broth microdilution.

for 12 h followed by 1 to 35 s for 31 h at 200 V in 0.5× Tris-borate-EDTA at 14°C. A comparison of the DNA fingerprints of N97-330 and BM4339 (courtesy of F. Tenover, Centers for Disease Control and Prevention) did not reveal any genetic relationship between the two strains (13, 23). *E. faecium* A902, which differed from BM4339 (≥6 band differences) (15), also appears not to be related to N97-330 (data not shown).

By using standard methods (20) and the 0.46-kb *vanD* PCR product as a probe, two overlapping clones were isolated from an N97-330 genomic library constructed in λEMBL3 (Promega, Madison, Wis.). These clones were subjected to partial sequence analysis (Fig. 1). The sequence of the 5′ end of the $vanR_D$ gene was obtained by sequencing a PCR product amplified from N97-330 genomic DNA using a primer designed based on a sequence upstream of BM4339 $vanR_D$ (7) and a primer from a previously sequenced region of the N97-330 $vanR_D$ gene. The complete nucleotide sequence of the 6,793-bp *vanD* region is shown in Fig. 1A. The BLAST programs (1) (http://www.ncbi.nlm.nih/BLAST) were used to identify putative products of detected open reading frames (ORFs). Six genes were found to have the same genetic organization as that of the *vanD* operon of *E. faecium* BM4339 (Fig. 1) (7). Comparisons of the amino acid products from the two strains revealed that pairs of homologous genes had at least 96% identity (data not shown). Further comparisons of the *vanD* sequence from N97-330 revealed 2.1 and 3.1% base pair differences with the genes from BM4339 and *E. faecium* A902 (15), respectively. These differences are less than those exhibited by the three designated *vanB* subtypes (3.6 to 5%) (10, 16) but the genes may still be considered *vanD* variants.

Beginning 161 bp downstream of the $vanX_D$ stop codon is an ORF of 909 bp (*orfR1*) coding for a putative product of 302 amino acids that shows low but significant homology to several regulatory proteins. The highest homology was with the YobV protein (313 residues) from *Bacillus subtilis* (accession no. AF027868), with which it has 30% identity. We have detected a region of dyad symmetry beginning 10 bp downstream of the $vanX_D$ stop codon that could form a putative hairpin structure with a $-\Delta G$ of 96 kJ/mol that may play a regulatory role (Fig. 1B). Transcription studies are needed to determine if *orfR1* is part of the *vanD* operon. Fifty base pairs of sequence downstream of the BM4339 $vanX_D$ is available (7), and an alignment with the N97-330 sequence (data not shown) reveals significant divergence, with the regions sharing only 40% identity. This may reflect a different genetic location of the *vanD* region in

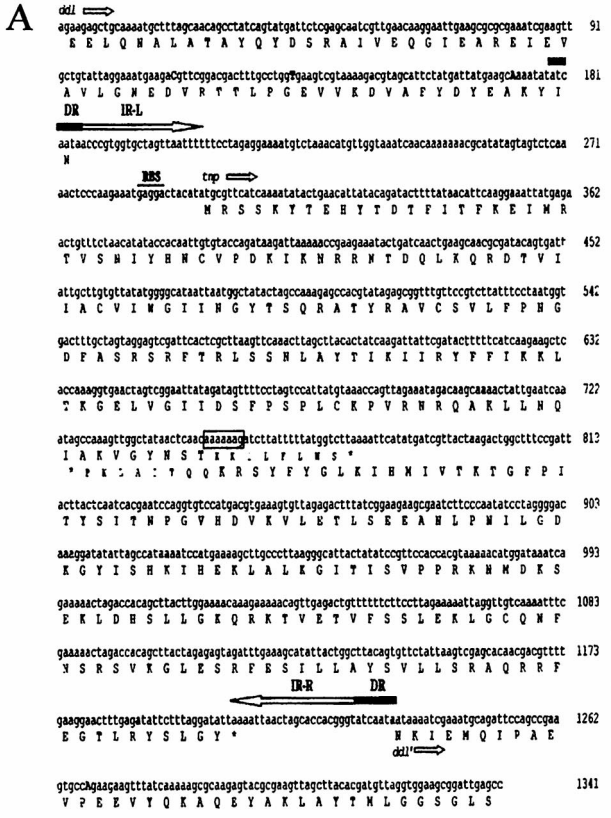

FIG. 2. Sequence of the IS*Efm1* element found in the *ddl* gene and prevalence of the insertion element in selected strains. (A) Complete nucleotide sequence and predicted amino acid sequence of the IS*Efm1* element and partial *ddl* gene. Capital letters indicate nucleotide differences in *ddl* compared to the published sequence of *E. faecium* BM4147-1. Open arrows indicate the coding sequence for the *ddl* and *tnp* genes. *ddl′* denotes the sequence of the *ddl* gene downstream of the IS*Efm1* element. DR, IR-L, and IR-R indicate direct repeats, the left inverted repeat, and the right inverted repeat, respectively. The open box indicates the translational frameshift region. (B) Southern blot of *Cla*I-digested DNA probed with the IS*Efm1* PCR product. Lane 1, 1-kb extension ladder (Life Technologies; sizes, in kilobases, of the fragments are indicated at the left); lane 2, *E. faecium* N97-330 (*vanD*); lane 3, *E. faecium* BM4339 (*vanD*); lane 4, *E. faecium* N98-638 (*vanA*); lane 5, *E. faecium* ATCC 19434; lane 6, *E. faecalis* ATCC 29212.

BM4339 compared to that in N97-330 or may be due to interstrain sequence divergence.

Comparison of the *vanS$_D$* genes from BM4339 and N97-330 revealed that the latter has a 1-bp deletion at nucleotide position 670 of the BM4339 gene sequence, which results in a frameshift leading to, presumably, a truncated nonfunctional protein of 233 amino acids. The *vanA* and *vanB* operons are activated in the presence of an inducer when the VanR protein becomes phosphorylated either by the cognate autophosphorylated VanS (VanS ~ P) or by an unknown kinase (3, 22). In N97-330, the absence of a functional $VanS_D$ protein may lead to a high steady-state level of VanR ~ P and thus to constitutive expression of the vancomycin resistance operon, as growth studies have shown. Vancomycin resistance is constitutive in BM4339, although it carries an intact *vanS$_D$* gene (7, 17).

During initial characterization of N97-330, PCR was carried out with primers specific for the *ddl* gene of *E. faecium* (8). A product approximately 1 kb larger than the expected size was obtained (data not shown). Sequence analysis of 1,341 bp from this region revealed that a 1,041-bp insertion sequence (IS), defined by 22-bp perfect terminal inverted repeats, had been inserted in the *ddl* gene (Fig. 2A). Two overlapping ORFs were identified in the IS, and putative translation products showed between 38 and 43% identity with parts of the putative transposase protein (Tnp) from the IS*982* family of insertion elements (12). Further analysis of the two ORFs revealed that a translational frameshift in the region from bases 748 to 754 (Fig. 2A) could lead to the translation of a fusion protein of 302 residues which can be aligned to the Tnps of IS*982* family members. Control of transposition by programmed translational frameshifting is common in a number of bacterial *tnp* genes, though it has not been found in the IS*982* family (12). Alternatively, a frameshift mutation may have occurred in the *tnp* gene after the IS was acquired by the genome, resulting in a truncated inactive protein. We propose naming this element IS*Efm1* (12). In order to determine the number of copies of IS*Efm1* in various strains of enterococci, we probed *Cla*I digests with a fragment of the *tnp* gene generated by PCR (Fig. 2B). Multiple copies exist in the *E. faecium vanA* and *vanD* strains, whereas a vancomycin-sensitive strain (ATCC 19434) appears to have a single copy. The probe did not hybridize to the type strain *E. faecalis* ATCC 29212. Several faint bands visible on the autoradiograph in the *E. faecium* lanes may be due to a low level of homology between the probe and the *tnp* genes of other IS elements found in *E. faecium*. It will be interesting to expand this work to include additional species of *Enterococcus* to determine if IS*Efm1* is specific to *E. faecium*.

Enterococci with impaired Ddl activity that require the presence of a glycopeptide for growth have been reported (6, 19, 21, 24). Although N97-330 appears to have a nonfunctional Ddl due to insertion of an IS element, a glycopeptide is not required for growth due to a frameshift mutation in *vanS$_D$*, which most likely leads to the constitutive expression of the *van* operon.

Nucleotide sequence accession numbers. The complete nucleotide sequence of the 6,793-bp *vanD* region shown in Fig. 1A has been deposited in the GenBank database under accession number AF175293. The sequence of the *ddl* region containing IS*Efm1* (Fig. 2A) has been deposited in the GenBank database under the accession number AF138282.

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