ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 2007, p. 4111–4117 Vol. 51, No. 11
0066-4804/07/$08.00+0 doi:10.1128/AAC.00584-07
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vanD and *vanG*-Like Gene Clusters in a *Ruminococcus* Species Isolated from Human Bowel Flora[▽]

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Received 3 May 2007/Returned for modification 14 June 2007/Accepted 20 August 2007

A vancomycin-resistant, anaerobic, gram-positive coccus containing the *vanD* and *vanG*-like genes (strain CCRI-16110) was isolated from a human fecal specimen during a hospital surveillance program to detect carriers of vancomycin-resistant enterococci. Comparison of the 16S rRNA gene sequence of strain CCRI-16110 with databases revealed a potentially novel *Ruminococcus* species that was most similar (<94% identity) to *Clostridium* and *Ruminococcus* species. Strain CCRI-16110 was highly resistant to vancomycin and teicoplanin (MICs of >256 μg/ml). The complete DNA sequence of the *vanD* cluster was most similar (98.2% identity) to that of *Enterococcus faecium* BM4339, containing the *vanD1* allele. An *intD* gene with 99% identity with that of this *E. faecium* strain was found to be associated with the *vanD* gene cluster of this novel anaerobic bacterium. Strain CCRI-16110 also harbors genes encoding putative $VanS_G$, VanG, and $VanT_G$ proteins displaying 56, 73.6, and 55% amino acid sequence identity, respectively, compared to the corresponding proteins encoded by the *vanG1* and *vanG2* operons of *Enterococcus faecalis* BM4518 and N03-0233. This study reports for the first time an anaerobic bacterium containing the *vanD* gene cluster. This strain also harbors a partial *vanG*-like gene cluster. The presence of *vanD*- and *vanG*-containing anaerobic bacteria in the human bowel flora suggests that these bacteria may serve as a reservoir for the *vanD* and *vanG* vancomycin resistance genes.

The glycopeptide antibiotics vancomycin and teicoplanin interfere with cell wall synthesis in gram-positive bacteria by binding to the terminal dipeptide D-alanyl-D-alanine regions of the pentapeptide precursors of peptidoglycan side chains. Resistance to glycopeptide antibiotics was first described for enterococci (23, 35) and has now spread worldwide. Six different genes (*vanA*, *vanB*, *vanC*, *vanD*, *vanE*, and *vanG*) have been shown to confer glycopeptide resistance in enterococci (7).

The VanD-type resistance is constitutively expressed in enterococci (8, 11) and is characterized by moderate MICs of vancomycin (64 to 128 μg/ml) and teicoplanin (4 to 64 μg/ml). This VanD-type resistance results from the acquisition of the $vanR_D$, $vanS_D$, $vanY_D$, $vanH_D$, *vanD*, and $vanX_D$ cluster of genes, which directs synthesis of peptidoglycan precursors terminating in D-alanyl-D-lactate (11). The *vanD* gene cluster is located on the chromosome and is not transferable to other enterococci by conjugation in vitro (11). The VanD-type resistance in enterococci is also characterized by the presence of an impaired D-alanyl-D-alanine (Ddl) ligase due to mutations in the chromosomal ligase-encoding gene *ddl* (10). Although the Ddl ligase is inactive, the VanD-type enterococci are able to grow even in the absence of glycopeptide because the *vanD* cluster is expressed constitutively as a result of mutations in the $VanS_D$ sensor or in the $VanR_D$ regulator (10).

The VanG-type resistance in enterococci is characterized by low-level resistance to vancomycin (MIC, 16 μg/ml) and susceptibility to teicoplanin (9). This VanG-type resistance results from the acquisition of the $vanU_G$, $vanR_G$, $vanS_G$, $vanY_G$, $vanW_G$, *vanG*, $vanXY_G$, and $vanT_G$ cluster of genes, which directs synthesis of peptidoglycan precursors terminating in D-alanyl-D-serine (D-Ala-D-Ser) (9).

To date, the *vanD* and *vanG* gene clusters have been described only for *Enterococcus*. However, the complete genome sequence of the *Clostridium difficile* strain 630 recently revealed the presence of a cluster of genes with high similarity to the *vanG* cluster in enterococci (32). Moreover, our group has recently described a high prevalence of *vanB* (4.8%), *vanD* (37.9%), and *vanG* (9.3%) genes in fecal specimens which are not associated with vancomycin-resistant enterococci, during a vancomycin-resistant enterococcus surveillance program implemented in two North American hospitals (13).

In this study, we describe for the first time an anaerobic bacterium of the human bowel which is resistant to both vancomycin and teicoplanin and which contains *vanD* and *vanG*-like gene clusters.

(This study was presented in part at the 46th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, CA, 27 to 30 September 2006.)

MATERIALS AND METHODS

Isolation procedure and identification. A *vanD*-positive rectal specimen, ERV-110, collected from a patient at the Montreal General Hospital (Québec, Canada) in 2001 (13) was subcultured several times in brain heart infusion broth (Difco, Detroit, MI) supplemented with vitamin K (0.001 mg/ml), hemin (0.005 mg/ml), L-cystine (0.5 mg/ml), sodium lactate (10 mM), sodium pyruvate (10 mM), vancomycin (10 μg/ml), and aztreonam (100 μg/ml) under anaerobic conditions at 35 to 37°C. A subculture was then plated on agar medium containing the same supplement and incubated under the same anaerobic conditions for 7 days. Colonies were then screened for the presence of the *vanD* gene by PCR.

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▽ Published ahead of print on 27 August 2007.

TABLE 1. PCR primers used in this study to amplify and sequence the *vanD* and *vanG*-like clusters from *Ruminococcus* sp. strain CCRI-16110

Cluster	Primer name	Oligonucleotide sequence (5′→3′)	Nucleotide position[a] (ORF[s])	Source or reference
vanD	RD-TSP1	AAAAAACCGTTCACCCCATACTG	899 ($vanR_D$)	This study
	RD-TSP2	ACGCTCGCACAGATACCAAAG	519 ($vanR_D$)	This study
	RD-TSP3	GGCTGCTCTTGCTGATTGTC	443 ($vanR_D$)	This study
	RD1	CCGTTTAACCCGCTGGAA	324 ($vanR_D$)	25
	SD1	CTATCATGATCGGGATG	885 ($vanS_D$)	25
	SD2	CGAATGGTGGTATTCTC	1576 ($vanS_D$)	25
	YD1	GATTCGTCAACCGCATG	2453 ($vanY_D$)	25
	YD3F	GGCTGCATGTGTCACAC	2059 ($vanY_D$)	This study
	YD2	CTCTGGAACTGAGGGTA	2214 ($vanY_D$)	25
	HD1	CGTAAGCCATAAAGCGGA	3224 ($vanH_D$)	25
	HD3a	AACGAAACATAGTCCGC	3615 ($vanH_D$)	This study
	D1	TAAGGCGCTTGCATATACCG	4425 (*vanD*)	26
	D2	TGCAGCCAAGTATCCGGTAA	4866 (*vanD*)	26
	XD2	TATGTATCCGGGTATGG	5637 ($vanX_D$)	25
	ID1	GTAAAGGCCCAGACAGT	5995 (*intD*)	5
	ID-TSP1	TCAACGCTTTTCTGGAGTTC	6032 (*intD*)	This study
	ID-TSP2	GCGGGAACTGTCTGAAGCGG	6117 (*intD*)	This study
	ID-TSP3	GGAAAAAACCGCACTGTTCTTCT	6292 (*intD*)	This study
	ID2	ATTCAAGATCCGCTCGTG	6613 (*intD*)	5
vanG like	V1	GGIGARGAYGGIWSIHTICARGG	1511 (*vanG* like)	14
	V2a[b]	GTRAAICCIGGIADIGTRTT	2120 (*vanG* like)	This study
	GG1-TSP1	CCTTCCCCGTATATCTTTGC	1760 (*vanG* like)	This study
	GG1-TSP2	CCACAGCCTTCGGGACAGAT	1639 (*vanG* like)	This study
	GG1-TSP3	GAGGACGCCGCAGCCGACAA	1560 (*vanG* like)	This study
	GP20 reverse	GGCAAGTTCAAGTAATCCCT	1530 (*vanG* like)	This study
	GD1-TSP1	TCCTCACTGTGGGGAGAGTC	1887 (*vanG* like)	This study
	GD1-TSP2	CTGACGGGTGGATTCTTTGA	1919 (*vanG* like)	This study
	GD1-TSP3	ACAGGAGACGGCAAAGAACA	2017 (*vanG* like)	This study
	GP788	GGCTGTCAGGCATGATATTA	788 ($vanZ_F$ like)	This study
	GN2720	AGCGGTATCTTTTCAGTC	2720 ($vanT_G$ like)	This study
	GP1421	CCTGCGGTGCATGGAATACT	1421 (*vanG* like)	This study
	GN2038	AACATCCCAGCACTTGATAA	2038 (*vanG* like)	This study
	GG2-TSP1	GCATCGTCCTGAAAAATCTC	941 ($vanZ_F$ like)	This study
	GG2-TSP2	CATTAGCGGCAACCTGTGTA	866 ($vanZ_F$ like)	This study
	GD2-TSP1	AATGCGGAAAAGCTGGTGAG	2421 ($vanT_G$ like)	This study
	GD2-TSP2	GTTCGGGGACAGTTATTATG	2696 ($vanT_G$ like)	This study
	GP107	CCGATAAAATGCAGCGTGTAT	107 ($vanS_G$ like)	This study
	GN3916	ATTATGGGGCACTCAAAACAC	3916 ($vanT_G$ like)	This study
	GN3098	TGCCGGACTGACTGGTTTACA	3098 ($vanT_G$ like)	This study

[a] Nucleotide positions refer to sequences of the *vanD* cluster (accession number EF508033) or *vanG*-like cluster (accession number EF508032) from *Ruminococcus* sp. strain CCRI-16110.

[b] V2a represents the universal primer derived from reference 14 in which nucleotides GT have replaced the TG at the 5′ end.

Identification of the *vanD*-positive colony (strain CCRI-16110) was performed by methods previously described (15) and with the Rapid ID 32A system (bioMérieux, Marcy l'Étoile, France). Partial sequencing of a 1,466-bp region of the gene encoding 16S rRNA was performed as previously described (12).

Resistance studies. MICs of vancomycin and metronidazole were determined by the agar dilution method for anaerobes according to the CLSI (formerly NCCLS) (24), whereas the MIC of teicoplanin was determined by the Etest method (AB Biodisk, Sweden). The medium used for antimicrobial susceptibility testing was brucella agar (Difco, Detroit, MI) supplemented with laked sheep blood, vitamin K (0.001 mg/ml), and hemin (0.005 mg/ml).

Bacterial strains and plasmids. *Enterococcus faecium* BM4339 (resistant to vancomycin and teicoplanin and containing the *vanD* gene cluster) (5) and *Enterococcus faecalis* JH2-2 (resistant to fusidic acid and rifampin) (18) were obtained from P. Courvalin (Unité des Agents Antibactériens, Institut Pasteur, Paris, France) and from N. Woodford (Health Protection Agency, Collindale, London, United Kingdom), respectively. *E. faecium* BM4339 was used as a control strain, and *E. faecalis* JH2-2 was used as recipient in the conjugation experiments. *Escherichia coli* TOP10 (Invitrogen, Burlington, Ontario, Canada) was used as a host for recombinant plasmids. PCR products were cloned in the vector pCR2.1 using the TOPO TA cloning kit (Invitrogen, Burlington, Ontario, Canada).

Sequencing of the *vanD* gene cluster. The primers used to characterize the *vanD* cluster are described in Table 1. PCRs were performed using standard conditions with purified genomic DNA and the *Taq* DNA polymerase (Promega, Mississauga, Ontario, Canada) as previously described (20). Purification of the amplification products and sequencing reactions were performed as previously described (20). Comparison with known sequences was carried out using the programs from the GCG package (Wisconsin package version 10.3; Accelrys Inc., San Diego, CA).

Sequencing of the *vanG*-like gene cluster. PCR amplification using universal primers V1 and V2a (Table 1), which were designed to amplify an internal region of the genes encoding Ddl ligases and related vancomycin-resistant proteins (14), was performed using genomic DNA purified from strain CCRI-16110. PCR products of the expected size (630 bp) were purified and cloned in the vector pCR2.1. The inserts from these recombinant plasmids were sequenced using the universal M13 forward and M13 reverse primers (Invitrogen). Nucleotide and deduced amino acid sequences from the cloned 630-bp PCR product obtained were analyzed by using the BLASTN, TBLASTN, and BLASTP softwares. Subsequently, the DNA sequence flanking the 630-bp fragment containing the *vanG*-like gene was obtained by using a combination of PCR amplifications with the DNA Walking SpeedUp kit (Bio/Can Scientific, Mississauga, Ontario, Canada) and cloning techniques as previously described (12). Sequences obtained by these

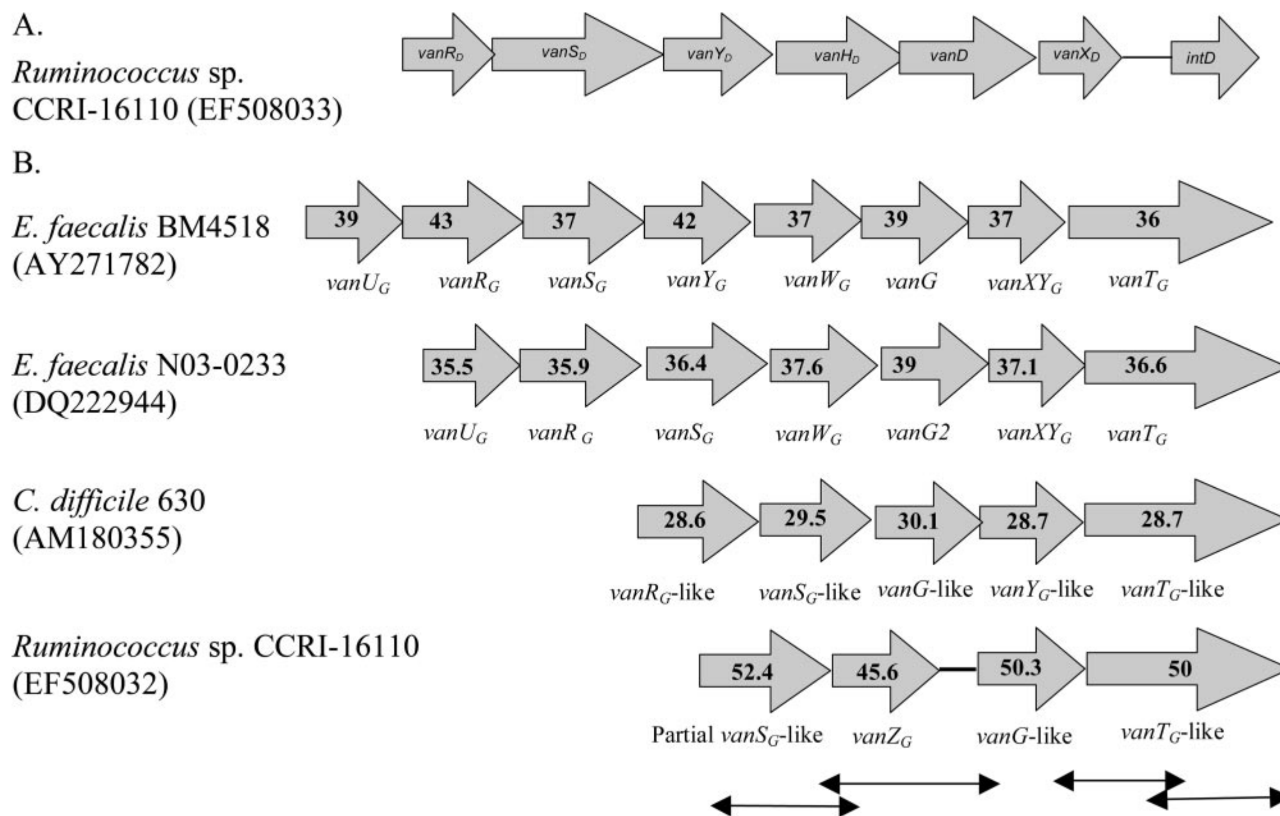

FIG. 1. Organization of the *vanD* and *vanG*-like operons from *Ruminococcus* sp. strain CCRI-16110. (A) Arrows represent the ORFs of the *vanD* operon. (B) Schematic organization of the *vanG* operon from *E. faecalis* BM4518 (accession number AY271782), the *vanG2* operon from *E. faecalis* N03-0233 (DQ222944), and the *vanG*-like operons from *C. difficile* 630 (AM180355) and *Ruminococcus* sp. strain CCRI-16110 (EF508032). Arrows represent the ORFs. The regions characterized by using the DNA walking method are indicated by two-headed arrows. The percent G+C is indicated within each arrow.

methods were confirmed by sequencing PCR products generated after amplification of total genomic DNA from strain CCRI-16110, using specific primers overlapping the cloned fragments (Table 1). The Artemis software (27) was used to analyze and collate data from the complete sequence.

Phylogenetic analysis of the *vanG*-like gene from strain CCRI-16110. To ascertain the phylogenetic position of the deduced protein sequence of the *vanG*-like ligase gene from strain CCRI-16110, phylogenetic analysis was performed using the deduced amino acid sequences from the Ddl ligases of gram-positive and gram-negative organisms as well as the D-Ala-D-Lac and D-Ala-D-Ser ligases of vancomycin-resistant organisms. Amino acid sequence alignment was performed with CLUSTALW (34). Phylogenetic analysis was carried out by the neighbor-joining method (28) using MEGA version 3.1 (21). The evolutionary distance was generated according to the Jones-Taylor-Thornton matrix for amino acid sequences (19). The tree topology was compared to that obtained by the maximum-parsimony method. Bootstrap values were calculated from 1,000 resamplings to test the robustness of the data and were displayed as percentages.

Filter mating. To study the transfer by conjugation of the glycopeptide resistance phenotype of strain CCRI-16110, mating on filters was performed as described previously (12). *E. faecalis* JH2-2 was used as the recipient, while strain CCRI-16110 was used as a donor.

Nucleotide sequence accession numbers. The GenBank accession numbers for the *vanD* gene cluster and partial *vanG*-like gene cluster sequences of strain CCRI-16110 are EF508033 and EF508032, respectively.

RESULTS

Isolation and identification of *Ruminococcus* sp. strain CCRI-16110 containing *vanD*. We isolated a strictly anaerobic, gram-positive coccus (strain CCRI-16110) from fecal specimen ERV-110. Biochemical tests performed in triplicate using the Rapid ID 32A system remained negative for all substrates tested with this system. Analysis of the partial sequence of its 16S rRNA gene revealed that strain CCRI-16110 was most similar to *Clostridium* and *Ruminococcus* species ($<$94% identity) belonging to the *Clostridium coccoides* cluster of organisms (rRNA cluster XIVa) (6). Based on these phenotypic and genotypic analyses, strain CCRI-16110 is a potentially novel *Ruminococcus* species. This strain was resistant to vancomycin and teicoplanin (MICs, $>$256 μg/ml) and susceptible to metronidazole (MIC, $<$0.125 μg/ml).

Characterization of the *vanD* gene cluster from *Ruminococcus* sp. strain CCRI-16110. The *vanD* gene cluster from *Ruminococcus* sp. strain CCRI-16110 was characterized to determine its genetic organization. Nucleotide sequence analysis of the *vanD* gene cluster of this strain showed that the gene order of this cluster was similar to that of known *vanD* clusters (Fig. 1A). The complete DNA sequence of the CCRI-16110 *vanD* operon (from *vanR*$_D$ to *vanX*$_D$, 5,655 bp) exhibited 98% identity with the *vanD1* operon (accession number AF130997), 97% identity with the *vanD3* operon (accession number AF175293), 95% identity with the *vanD2* operon (accession number AF153050), 93% identity with the *vanD5* operon (accession number AY489045), and 89% identity with the *vanD4* operon (accession number AY082011). The amino acid sequences of VanR$_D$, VanS$_D$, VanH$_D$, and VanX$_D$ of the *vanD*

TABLE 2. Comparison of nucleotide and amino acid sequences of the *vanD* gene cluster and *intD* gene from *Ruminococcus* sp. strain CCRI-16110 with those of the *vanD1*, *vanD2*, *vanD3*, *vanD4*, and *vanD5* gene clusters

Gene	Nucleotide/amino acid identity (%)[a] to:				
	vanD1	*vanD2*	*vanD3*	*vanD4*	*vanD5*
$vanR_D$	99.4/100	NA	97.3/98.7	9.3/98.7	97.0/100
$vanS_D$	98.4/98.2	NA	97.0/96.0	90.0/94.0	92.0/96.0
$vanY_D$	98.0/97.5	88.0/71.4	99.0/98.3	82.0/59.7	77.4/39.2
$vanH_D$	98.0/100	96.5/98.1	98.5/100	80.0/88.0	94.0/96.6
vanD	98.1/98.3	97.3/97.1	98.3/99.1	82.6/89.5	85.8/91.0
$vanX_D$	99.0/100	98.0/100	97.4/99.0	86.0/90.0	99.0/97.0
intD	99.3/99.6	NA	NA	NA	NA

[a] Sequence accession numbers for the *vanD* gene clusters are as follows: *vanD1*, *E. faecium* BM4339 (AF130997); *vanD2*, *E. faecium* A902 (AF153050); *vanD3*, *E. faecium* N97-330 (AF175293); *vanD4*, *E. faecium* 10/96A (AY082011); *vanD5*, *E. faecium* N03-0072 (AY489045). NA, data not available.

operon from this *Ruminococcus* strain were most similar to those of the *vanD1* operon from *E. faecium* BM4339, while the amino acid sequences of $VanY_D$ and VanD were most similar to those of the *vanD3* operon from *E. faecium* N97-330 (Table 2). The H, N, G1, F, and G2 conserved motifs found in histidine kinase proteins were present in the $VanS_D$ sensor of strain CCRI-16110 (10). Comparison of the sequence of the $VanS_D$ sensor of strain CCRI-16110 with that of BM4339 revealed point mutations in critical regions of these conserved motifs: (i) a $V_{165}A$ mutation within the H block beside the putative autophosphorylation site and (ii) a $Q_{308}K$ mutation beside the G1 ATP binding block. An *intD* gene, encoding a putative integrase-like protein showing 99.2% amino acid sequence identity with that of *E. faecium* BM4339, was found to be associated with the *vanD* gene cluster (Fig. 1A).

Characterization of the *vanG*-like operon from *Ruminococcus* sp. strain CCRI-16110. Since the *ddl* gene, encoding the Ddl ligase, is mutated in enterococci harboring the *vanD* gene (4, 5, 10), we searched for a *ddl* gene in strain CCRI-16110 by using the universal primers V1 and V2a (Table 1) (14). A similarity search using TBLASTN revealed that the sequence of the 630-bp PCR amplification product generated using these primers encoded a putative protein that was most similar (66% amino acid sequence identity) to the known VanG of enterococci, and hence this gene encoding the VanG variant was designated *vanG* like. From this partial *vanG*-like gene sequence, the successive combination of PCR amplification, cloning, and sequencing allowed us to characterize a 3,950-bp genomic DNA fragment. Sequence analysis of this genomic DNA fragment revealed the presence of three complete open reading frames (ORFs) and one partial ORF encompassing the *vanG*-like gene of strain CCRI-16110. Two complete ORFs displayed the highest amino acid sequence identities with the VanG D-Ala-D-Ser ligases (63.6% to 65.3%) and the $VanT_G$ serine racemases (58.6 to 59.6%) of the *vanG* operons of enterococci (Table 3). The partial ORF (which resulted from the shortened PCR fragment used for sequencing) was most similar to the $VanS_G$ histidine kinase sensors (63.4 to 64.8% identities). The fourth complete ORF displayed significant amino acid sequence identity to the VanZ protein encoded in the *vanA* operon of *E. faecium* BM4147 (27.1%) and the *vanF* operon of *Paenibacillus popilliae* (27.5%) (Table 3), as well as in the *skin* (*sigK* intervening sequence) element of *C. difficile* 630 (28.2%). Therefore, the gene encoding the putative VanZ protein in the *vanG*-like cluster of strain CCRI-16110 was named $vanZ_G$.

Comparison of the organization of the partial *vanG*-like operon from strain CCRI-16110 (the region upstream of the $vanS_G$-like gene has not been characterized) with those of the *vanG* operons from *E. faecalis* BM4518 (*vanG1*), *E. faecalis* N03-0233 (*vanG2*), and *C. difficile* 630 (*vanG* like) showed that the genes $vanY_G$ (encoding the carboxypeptidase), $vanXY_G$ (encoding the bifunctional dipeptidase and carboxypeptidase enzyme), and $vanW_G$ (with unknown function) were absent in strain CCRI-16110 (Fig. 1B). The $vanZ_G$ gene, with unknown function, was present only in the *vanG*-like operon from CCRI-16110 (Fig. 1B). The percent G+C content of the genes forming the *vanG*-like operon of strain CCRI-16110 was higher than that of the *vanG* operons from enterococci and *C. difficile* (Fig. 1B).

TABLE 3. Percent identity of the amino acid sequences from the deduced proteins of the *vanG*-like gene cluster to those from other *van* gene clusters

VanG-like cluster from CCRI-16110	% Identity with Van type[a]:									
	VanA	VanB	VanD	VanC	VanE	VanF	VanG1	VanG2	VanG like (*C. difficile*)	Ddl (*C. innocuum*)
$VanS_G$[b]	44.4	24.8	56.6	41.7	34.5	38.6	**64.8**	63.4	60	NA
$VanZ_G$	27.1	NA	NA	NA	NA	**27.5**	NA	NA	NA	NA
VanG	44.7	45.1	43.5	45.1	41.5	44.5	65	63.6	**65.3**	46.1
$VanT_G$	NA	NA	NA	37.5	40.4	NA	58.6	**59.6**	57.8	36.1

[a] Sequence accession numbers for the Van-type proteins are as follows: VanA, *E. faecium* BM4147 (M97297); VanB, *E. faecalis* V583 (EFU35369); VanD, *E. faecium* BM4339 (AF130997); VanC, *E. gallinarum* BM4174 (AF162694); VanE, *E. faecalis* N00-410 (AF430807); VanF, *Paenibacillus popilliae* ATCC 14706 (AF155139); VanG1, *E. faecalis* BM4518 (AY272782); VanG2, *E. faecalis* N03-0233 (DQ222944); VanG like, *C. difficile* 630 (AM180355); Ddl, *C. innocuum* NCIB 10674 (AY479979). The numbers in boldface indicate the highest percent identity with VanG-type proteins. NA, not applicable.

[b] The partial deduced sequence of $VanS_G$ (190-amino-acid sequence) of CCRI-16110 was used for comparison.

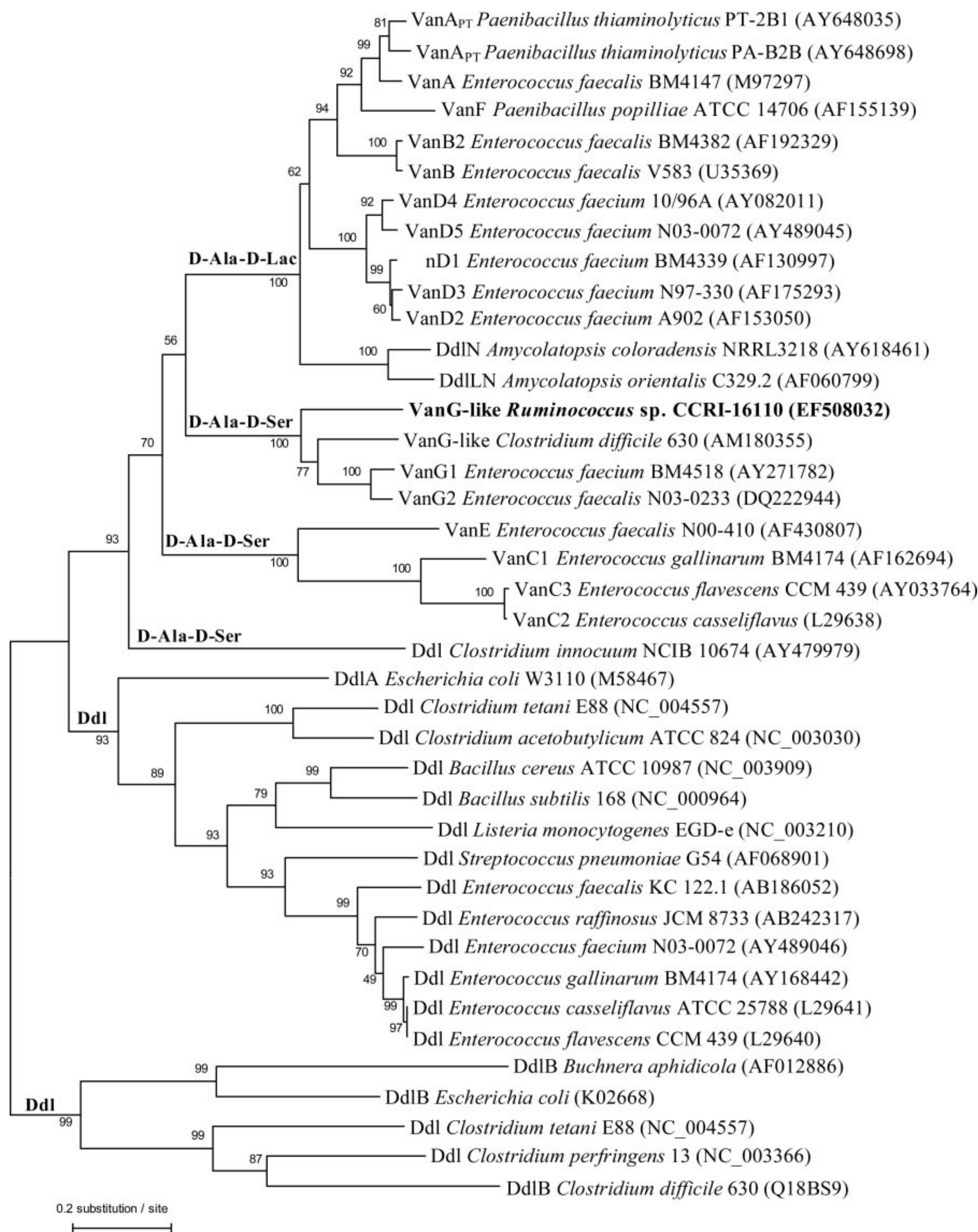

FIG. 2. Tree showing the phylogenetic relationships of the deduced amino acid sequences of the *vanG*-like genes from strain CCRI-16110 encoding the Ddl, D-Ala-D-Lac, and D-Ala-D-Ser ligases. The tree was constructed using the neighbor-joining method based on a comparison of 175 amino acid positions. Bootstrap values, expressed as a percentage of 1,000 replications, are given at each branching point. GenBank accession numbers are given in parentheses for each amino acid sequence.

The branching pattern of the phylogenetic tree constructed using sequences of Ddl, D-Ala-D-Ser, and D-Ala-D-Lac ligases showed that the family of D-Ala-D-Ser ligases is organized in three distinct phylogenetic clades that were supported by significant bootstrap resampling values (Fig. 2). Clearly, the VanG-like protein of strain CCRI-16110 was most closely related to the VanG-type D-Ala-D-Ser family of ligases. The two other clades were formed by (i) the VanE and VanC D-Ala-D-

Ser ligases and (ii) the D-Ala-D-Ser ligases of *Clostridium innocuum*.

Transfer of glycopeptide resistance from *Ruminococcus* sp. strain CCRI-16110 by conjugation. We performed mating experiments between *Ruminococcus* sp. strain CCRI-16110 and *E. faecalis* JH2-2 to determine whether the glycopeptide resistance of *Ruminococcus* sp. strain CCRI-16110 was transferable by conjugation. No transfer was observed in several mating experiments.

DISCUSSION

We isolated for the first time a potentially novel species of *Ruminococcus* (strain CCRI-16110) displaying a high level of resistance to vancomycin and teicoplanin. This strain was isolated from a human fecal specimen and harbors the *vanD* gene. Work is in progress to characterize and assign a taxonomic name to this novel species of *Ruminococcus*.

Genetic analysis revealed that *Ruminococcus* sp. strain CCRI-16110 carries a *vanD* gene cluster closely related to the *vanD1* gene cluster in enterococci. So far, the *vanD* gene cluster has been described only for enterococci (4, 10, 33), and the present report is the first descriptive study of the *vanD* gene cluster in a nonenterococcal species.

While searching for a *ddl* gene in *Ruminococcus* sp. strain CCRI-16110 and potential alterations in this gene, we identified a cluster of four ORFs, three of which displayed high identities to the $vanS_G$, *vanG*, and $vanT_G$ genes of *E. faecalis* BM4518 and N03-0233, whereas one ORF was more closely related to the *vanZ* gene. The *vanZ* gene has been described in various genetic elements, including the enterococcal *vanA* operon, the *vanF* operon of *Paenibacillus popilliae*, and the *skin* element of *C. difficile* 630 (7, 32). The *skin* element of *C. difficile* 630 is a 14.66-kb prophage-like element inserted in the gene *sigK*, encoding a sporulation-specific sigma factor (7, 17, 32). The function of the *vanZ* gene is unknown, but it was shown to be involved in low-level resistance to teicoplanin in *E. faecium* BM4147 containing the *vanA* operon (1, 2).

Phylogenetic analysis showed that the VanG-like ligase of strain CCRI-16110 is closely related to the family of VanG-type D-Ala-D-Ser ligases, suggesting that these proteins share a common ancestor. However, the organization of the *vanG* operon of this strain differs from that of the *vanG* operons of enterococci and *C. difficile*. In CCRI-16110, the $vanY_G$ and $vanW_G$ genes are absent, while a *vanZ*-like gene is described for the first time in a *vanG*-like operon. The presence of the $vanU_G$ and $vanR_G$ genes as well as the entire sequence of the $vanS_G$ gene remains to be characterized in strain CCRI-16110. The percent G+C of the genes forming the *vanG*-like operon of this strain is higher than those of the *vanG* operons of enterococci and *C. difficile*, suggesting different origins for these genes. Therefore, the origin of the *vanG* and *vanG*-like operons could be due to a step-by-step mechanism of gene acquisition from various *van* operons as well as a one-step transfer of the *vanG* cluster of genes due to mobile genetic elements. Such a one-step acquisition of a *vanG* cluster was described during the transfer of the *vanG* operon from *E. faecalis* BM4518 to *E. faecalis* JH2-2 and was associated with the movement, from chromosome to chromosome, of genetic elements of ca. 240 kb (9). The transfer of *vanG* genes could explain the presence of a *vanG*-like operon in *C. difficile* 630, considering that this strain harbors several conjugative transposons, including CTn*2*, CTn*4*, and CTn*5*, which have a conjugation module related to that of Tn*1549* (32), a conjugative transposon responsible for VanB-type vancomycin resistance (16). It has been shown that the *vanG*-like operon was not present in all *C. difficile* strains (32). This observation further supports the horizontal transfer of this operon in *C. difficile*. Despite the presence of a *vanG*-like operon in *C. difficile* 630, this strain is susceptible to glycopeptides (32). Based on these data, we can suggest that the presence of the *vanG* cluster of genes in some anaerobic bacteria is an evolutionary process towards the expression of glycopeptide resistance.

Ruminococcus sp. strain CCRI-16110 displays a high level of vancomycin and teicoplanin resistance (MICs of both antibiotics, >256 μg/ml), which usually corresponds to the VanA phenotype, although no *vanA* gene was detected in this strain (data not shown). Therefore, the presence of both *vanD* and *vanG*-like operons in *Ruminococcus* sp. strain CCRI-16110 could be responsible for the high level of resistance to vancomycin and teicoplanin. Moreover, mutations in the $VanS_D$ sensor could be responsible for constitutive expression of vancomycin resistance in CCRI-16110, as described for *E. faecium* BM4339 (10). However, the function of each operon as well as the role of the mutations in $vanS_D$ in the vancomycin and teicoplanin resistance phenotype of this strain remains to be described. In addition, the presence of a *ddl* gene encoding a Ddl ligase in this strain remains to be demonstrated.

The anaerobic bacteria from the intestinal flora seem to represent a reservoir of *vanB*, *vanD*, and *vanG* gene clusters (13) and could be involved in the dissemination of vancomycin resistance genes in other important anaerobic or aerobic gram-positive pathogens. However, in vitro conjugative transfer of the glycopeptide resistance phenotype from *Ruminococcus* sp. strain CCRI-16110 to *E. faecalis* JH2-2 could not be demonstrated. While the transfer of the *vanG* gene cluster has already been described (9), no other *vanD* gene cluster has been successfully transferred by conjugation in vitro to date (3, 10). The absence of in vitro transfer of the *vanD* and *vanG*-like gene clusters in the present study does not rule out the possibility of an in vivo horizontal transfer mechanism that could occur in the intestinal environment. Indeed, the transfer of the *vanB* gene cluster from *Clostridium symbiosum* to enterococcal strains has been demonstrated in the gut of gnotobiotic mice (22). Infact, the human gut represents a natural ecosystem where nutrients, biofilm bacteria, antibiotic resistance genes, and genetic material exchange are abundant (29–31).

ACKNOWLEDGMENTS

This study was supported by grant PA-15586 from the Canadian Institutes of Health Research (CIHR) and by grant 2201-181 from the Valorisation Recherche Québec (VRQ). M.-C. Domingo is a research fellow from Bayer Healthcare (Bayer Healthcare/CIHR/AMMI Canada/FCMI).

REFERENCES

1. **Arthur, M., F. Depardieu, C. Molinas, P. Reynolds, and P. Courvalin.** 1995. The *vanZ* gene of Tn*1546* from *Enterococcus faecium* BM4147 confers resistance to teicoplanin. Gene **154:**87–92.
2. **Arthur, M., F. Depardieu, P. Reynolds, and P. Courvalin.** 1996. Quantitative analysis of the metabolism of soluble cytoplasmic peptidoglycan precursors of glycopeptide-resistant enterococci. Mol. Microbiol. **21:**33–44.

3. **Boyd, D. A., P. Kibsey, D. Roscoe, and M. R. Mulvey.** 2004. *Enterococcus faecium* N03-0072 carries a new VanD-type vancomycin resistance determinant: characterization of the VanD5 operon. J. Antimicrob. Chemother. **54:**680–683.
4. **Boyd, D. A., M. A. Miller, and M. R. Mulvey.** 2006. *Enterococcus gallinarum* N04-0414 harbors a VanD-type vancomycin resistance operon and does not contain a D-alanine:D-alanine 2 (*ddl2*) gene. Antimicrob. Agents Chemother. **50:**1067–1070.
5. **Casadewall, B., P. E. Reynolds, and P. Courvalin.** 2001. Regulation of expression of the *vanD* glycopeptide resistance gene cluster from *Enterococcus faecium* BM4339. J. Bacteriol. **183:**3436–3446.
6. **Collins, M. D., P. A. Lawson, A. Willems, J. J. Cordoba, J. Fernandez-Garayzabal, P. Garcia, J. Cai, H. Hippe, and J. A. Farrow.** 1994. The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. Int. J. Syst. Bacteriol. **44:**812–826.
7. **Courvalin, P.** 2006. Vancomycin resistance in gram-positive cocci. Clin. Infect. Dis. **42**(Suppl. 1)**:**S25–S34.
8. **Dalla Costa, L. M., P. E. Reynolds, H. A. Souza, D. C. Souza, M. F. Palepou, and N. Woodford.** 2000. Characterization of a divergent *vanD*-type resistance element from the first glycopeptide-resistant strain of *Enterococcus faecium* isolated in Brazil. Antimicrob. Agents Chemother. **44:**3444–3446.
9. **Depardieu, F., M. G. Bonora, P. E. Reynolds, and P. Courvalin.** 2003. The *vanG* glycopeptide resistance operon from *Enterococcus faecalis* revisited. Mol. Microbiol. **50:**931–948.
10. **Depardieu, F., M. Kolbert, H. Pruul, J. Bell, and P. Courvalin.** 2004. VanD-type vancomycin-resistant *Enterococcus faecium* and *Enterococcus faecalis*. Antimicrob. Agents Chemother. **48:**3892–3904.
11. **Depardieu, F., P. E. Reynolds, and P. Courvalin.** 2003. VanD-type vancomycin-resistant *Enterococcus faecium* 10/96A. Antimicrob. Agents Chemother. **47:**7–18.
12. **Domingo, M. C., A. Huletsky, A. Bernal, R. Giroux, D. K. Boudreau, F. J. Picard, and M. G. Bergeron.** 2005. Characterization of a Tn*5382*-like transposon containing the *vanB2* gene cluster in a *Clostridium* strain isolated from human faeces. J. Antimicrob. Chemother. **55:**466–474.
13. **Domingo, M. C., A. Huletsky, R. Giroux, K. Boissinot, F. J. Picard, P. Lebel, M. J. Ferraro, and M. G. Bergeron.** 2005. High prevalence of glycopeptide resistance genes *vanB*, *vanD*, and *vanG* not associated with enterococci in human fecal flora. Antimicrob. Agents Chemother. **49:**4784–4786.
14. **Dutka-Malen, S., C. Molinas, M. Arthur, and P. Courvalin.** 1992. Sequence of the *vanC* gene of *Enterococcus gallinarum* BM4174 encoding a D-alanine: D-alanine ligase-related protein necessary for vancomycin resistance. Gene **112:**53–58.
15. **Engelkirk, P. G., J. Duben-Engelkirk, and J. V. R. Dowell.** 1992. Laboratory procedures, p. 331–357. *In* S. Hoffman (ed.), Principles and practice of clinical anaerobic bacteriology. Star Publishing Company, Belmont, CA.
16. **Garnier, F., S. Taourit, P. Glaser, P. Courvalin, and M. Galimand.** 2000. Characterization of transposon Tn*1549*, conferring VanB-type resistance in *Enterococcus* spp. Microbiology **146:**1481–1489.
17. **Haraldsen, J. D., and A. L. Sonenshein.** 2003. Efficient sporulation in *Clostridium difficile* requires disruption of the σ^K gene. Mol. Microbiol. **48:**811–821.
18. **Jacob, A. E., and S. J. Hobbs.** 1974. Conjugal transfer of plasmid-borne multiple antibiotic resistance in *Streptococcus faecalis* var. *zymogenes.* J. Bacteriol. **117:**360–372.
19. **Jones, D. T., W. R. Taylor, and J. M. Thornton.** 1992. The rapid generation of mutation data matrices from protein sequences. Comput. Appl. Biosci. **8:**275–282.
20. **Ke, D., F. J. Picard, F. Martineau, C. Ménard, P. H. Roy, M. Ouellette, and M. G. Bergeron.** 1999. Development of a PCR assay for rapid detection of enterococci. J. Clin. Microbiol. **37:**3497–3503.
21. **Kumar, S., K. Tamura, and M. Nei.** 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief. Bioinform. **5:**150–163.
22. **Launay, A., S. A. Ballard, P. D. Johnson, M. L. Grayson, and T. Lambert.** 2006. Transfer of vancomycin resistance transposon Tn*1549* from *Clostridium symbiosum* to *Enterococcus* spp. in the gut of gnotobiotic mice. Antimicrob. Agents Chemother. **50:**1054–1062.
23. **Leclercq, R., E. Derlot, J. Duval, and P. Courvalin.** 1988. Plasmid-mediated resistance to vancomycin and teicoplanin in *Enterococcus faecium*. N. Engl. J. Med. **319:**157–161.
24. **NCCLS.** 2004. Methods for antimicrobial susceptibility testing of anaerobic bacteria; approved standard, 6th ed. M11-A6. NCCLS, Wayne, PA.
25. **Périchon, B., B. Casadewall, P. Reynolds, and P. Courvalin.** 2000. Glycopeptide-resistant *Enterococcus faecium* BM4416 is a VanD-type strain with an impaired D-alanine:D-alanine ligase. Antimicrob. Agents Chemother. **44:** 1346–1348.
26. **Périchon, B., P. Reynolds, and P. Courvalin.** 1997. VanD-type glycopeptide-resistant *Enterococcus faecium* BM4339. Antimicrob. Agents Chemother. **41:**2016–2018.
27. **Rutherford, K., J. Parkhill, J. Crook, T. Horsnell, P. Rice, M. A. Rajandream, and B. Barrell.** 2000. Artemis: sequence visualization and annotation. Bioinformatics **16:**944–945.
28. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:**406–425.
29. **Salyers, A. A., A. Gupta, and Y. Wang.** 2004. Human intestinal bacteria as reservoirs for antibiotic resistance genes. Trends Microbiol. **12:**412–416.
30. **Salyers, A. A., K. Moon, and M. S. Schlessinger.** 2007. The human intestinal tract—a hotbed of resistance gene transfer? Part I. Clin. Microbiol. Newsl. **29:**17–21.
31. **Salyers, A. A., K. Moon, and M. S. Schlessinger.** 2007. The human intestinal tract—a hotbed of resistance gene transfer? Part II. Clin. Microbiol. Newsl. **29:**25–30.
32. **Sebaihia, M., B. W. Wren, P. Mullany, N. F. Fairweather, N. Minton, R. Stabler, N. R. Thomson, A. P. Roberts, A. M. Cerdeno-Tarraga, H. Wang, M. T. Holden, A. Wright, C. Churcher, M. A. Quail, S. Baker, N. Bason, K. Brooks, T. Chillingworth, A. Cronin, P. Davis, L. Dowd, A. Fraser, T. Feltwell, Z. Hance, S. Holroyd, K. Jagels, S. Moule, K. Mungall, C. Price, E. Rabbinowitsch, S. Sharp, M. Simmonds, K. Stevens, L. Unwin, S. Whithead, B. Dupuy, G. Dougan, B. Barrell, and J. Parkhill.** 2006. The multidrug-resistant human pathogen *Clostridium difficile* has a highly mobile, mosaic genome. Nat. Genet. **38:**779–786.
33. **Tanimoto, K., T. Nomura, H. Maruyama, H. Tomita, N. Shibata, Y. Arakawa, and Y. Ike.** 2006. First VanD-type vancomycin-resistant *Enterococcus raffinosus* isolate. Antimicrob. Agents Chemother. **50:**3966–3967.
34. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
35. **Uttley, A. H., C. H. Collins, J. Naidoo, and R. C. George.** 1988. Vancomycin-resistant enterococci. Lancet **i:**57–58.