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Molecular Characterization of *Enterococcus faecalis* N06-0364 with Low-Level Vancomycin Resistance Harboring a Novel D-Ala-D-Ser Gene Cluster, *vanL*▽

David A. Boyd,[1] Barbara M. Willey,[2] Darlene Fawcett,[3] Nazira Gillani,[3] and Michael R. Mulvey[1]*

National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, Manitoba,[1] Mount Sinai Hospital and University Health Network, Toronto, Ontario,[2] and Rouge Valley Health Centre Centenary Site, Scarborough, Ontario,[3] Canada

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***Enterococcus faecalis* N06-0364, exhibiting a vancomycin MIC of 8 μg/ml, was found to harbor a novel D-Ala-D-Ser gene cluster, designated *vanL*. The *vanL* gene cluster was similar in organization to the *vanC* operon, but the VanT serine racemase was encoded by two separate genes, $vanTm_L$ (membrane binding) and $vanTr_L$ (racemase).**

Among enterococci, there are currently three characterized D-Ala-D-Ser operons that confer low-level vancomycin resistance, *vanC*, *vanE*, and *vanG* (1, 5, 6, 10, 14). The *vanC* operon is intrinsic to *Enterococcus gallinarum* (*vanC*) and *Enterococcus casseliflavus/flavescens* (*vanC2/3*), while *vanE* and *vanG* have been acquired by a small number of *Enterococcus faecalis* strains (21). Resistance is achieved by essentially the same mechanism in the three types: the ligases, encoded by *vanC*, *vanE*, or *vanG*, catalyze the formation of D-Ala-D-Ser, which is incorporated into peptidoglycan precursors, which subsequently have a low binding affinity for vancomycin. The *vanC* and *vanE* operons are organized similarly, *vanC* or *vanE*, *vanXY* (D,D-dipeptidase-D,D-carboxypeptidase), *vanT* (serine racemase), *vanR* (response regulator), and *vanS* (sensor histidine kinase) (1, 5, 14). The *vanG* operon begins with a three-component regulatory system, *vanU* (transcriptional regulator), *vanR*, *vanS*, *vanY* (D,D-carboxypeptidase) in the *vanG* operon which is absent in the *vanG2* operon, *vanW* (unknown function), and then *vanG*, *vanXY*, and *vanT* (6, 10). In this report we describe a novel D-Ala-D-Ser gene cluster, designated *vanL*.

The primers used in this study are listed in Table 1. PCR and thermal asymmetric interlaced PCR (TAIL-PCR) were carried out with AmpliTaq Gold (Applied Biosystems, Foster City, CA) or the FailSafe PCR system (Epicenter Biotechnologies, Madison, WI) as previously described (6, 7, 12, 22). DNA sequencing was carried out by the National Microbiology Laboratory's Genomics Core Facility. Homology comparisons were made using the BLAST suite of programs at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov/BLAST/). Vancomycin induction was studied as previously described (19). *E. faecalis* JH2-2 was used as a recipient in filter mating experiments with selection for transconjugants on 50 μg/ml rifampin, 100 μg/ml fusidic acid, and 2 μg/ml vancomycin. Antimicrobial susceptibilities were determined using broth microdilution according to CLSI guidelines (9) and by Etest (AB Biodisk, Solna, Sweden).

E. faecalis N06-0364 (vancomycin MIC, 8 μg/ml) was isolated from a single patient after a screen for vancomycin-resistant enterococci was carried out 2 days after hospitalization for a total hip resection. No vancomycin-resistant enterococci were isolated from this patient or any other in the hospital on follow-up screening 7 days later. *E. faecalis* N06-0364 was susceptible to teicoplanin, ampicillin, fluoroquinolones, erythromycin, gentamicin, streptomycin, linezolid, and tetracyclines. PCR analysis was positive for $ddl_{E.\ faecalis}$ but negative for $ddl_{E.\ faecium}$, *vanA*, and *vanB* in a multiplex PCR (12). PCRs for *vanD*, *vanC*, *vanE*, and *vanG*-type ligases were negative. A ~600-bp product amplified with the V3/V4 degenerate primers for D-Ala-D-Xxx ligases (15) was sequenced, and its putative translation product exhibited 57% identity to the VanC ligase. Using DNA sequence alignments of homologous genes from existing D-Ala-D-Ser operons, a number of degenerate primers from conserved regions were designed (Table 1). These primers and gene-specific primers were used in PCRs to obtain a total region of ~4 kb downstream of the V3/V4 PCR product (Fig. 1). An additional ~1.2-kb region upstream and ~2.5-kb region downstream were obtained by TAIL-PCR (Fig. 1). All products were sequenced to generate 8,295 bp of contiguous DNA sequence from *E. faecalis* N06-0364 (Fig. 1).

Analysis of the genes and their putative proteins indicated the presence of a novel D-Ala-D-Ser gene cluster, designated *vanL*. The percent identities of the *vanL* gene cluster proteins with the corresponding proteins from other D-Ala-D-Ser operons are shown in Table 2. The *vanL* gene cluster was organized essentially the same as the *vanC* and *vanE* operons; however, serine racemase activity appears to be encoded by two genes, $vanTm_L$ and $vanTr_L$ (discussed below). The putative VanL exhibited 51 and 49% identity to the VanE and VanC ligases, respectively, and the EKYQ motif involved in substrate recognition was conserved at residues 252 to 255 of VanL (Fig. 2A) (13).

The putative $VanXY_L$ exhibited 46% identity to $VanXY_C$

* Corresponding author. Mailing address: Nosocomial Infections, Public Health Agency of Canada, 1015 Arlington St., Winnipeg, Manitoba, Canada R3E 3R2. Phone: (204) 789-2133. Fax: (204) 789-5020. E-mail: Michael_mulvey @phac-aspc.gc.ca.

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TABLE 1. Primers used in this study

Name	Sequence (5′ to 3′)[a]	Coordinates (bp)[b]	PCR no. (strand)[c]	Reference
vanA1	GGGAAAACGACAATTGC	NA[d]	NA	11
vanA2	GTACAATGCGGCCGTTA	NA	NA	11
vanB-F	AAGCTATGCAAGAAGCCATG	NA	NA	12
vanB-R	CCGACAATCAAATCATCCTC	NA	NA	12
ddlFm-1	ATTACAAAGGCAGAAAACCG	NA	NA	This study
ddlFm-2	TGTCAAAAAGAAATCGCACC	NA	NA	This study
ddlFs-1	TTATTTTGTTGTATGGCGGC	NA	NA	This study
ddlFs-2	AAAGTCAGTAAAACCAGGCA	NA	NA	This study
vanC1-93	GAAAGACAACAGGAAGACCGC	NA	NA	8
vanC2-93	ATCGCATCACAAGCACCAATC	NA	NA	8
vanD-U1	TATTGGAATCACAAATCCGG	NA	NA	7
vanD-U2	CGGCTGTGCTTCCTGATG	NA	NA	7
VANE1	TGTGGTATCGGAGCTGCAG	NA	NA	14
VANE2	GTCGATTCTCGCTAATCC	NA	NA	14
vanG5	TTCGATTTCATCAACTCTGC	NA	NA	This study
vanG6	CAGGAATACCTGTTGTTGG	NA	NA	This study
AD5	NTCGASTWTSGWGTT	NA	1 (+), 9 (−)	22
364U4	TTTCCGCCTAAATCAATTCC	1081–1100	1 (−)	This study
364U5	GAGTTTCTAGTACACTTACTG	1028–1048	1 (−)	This study
364U6	ATATTCCGAAGATTGACCTCC	988–1008	1 (−)	This study
AD3	AGWGNAGWANCAWAGG	NA	2 (+)	22
364U1	AGCATCCTCTAGCTTATCTGG	1537–1557	2 (−)	This study
364U2	TAGTGCGAATTATGAGCGTAG	1409–1429	2 (−)	This study
364U3	CACTCTGAGCAAACTCATGC	1371–1390	2 (−)	This study
V3	GARGATGGITSCATMCARGGW	1274–1293	3 (+)	15
V4	MGTRAAICCIGGCAKRGTRTT	1882–1902	3 (−)	15
364D4	AGCAAAGGTAAATATACCTGC	1728–1748	4 (+)	This study
vanXY-U	CCNACRTANCKRAARTGCCA	2468–2487	4 (−)	This study
vanXYL-D3	GCACATGATATCATAGCACC	2336–2355	5 (+)	This study
vanT-U	TDATNVDNGCNGGNARRTACCA	2941–2963	5 (−)	This study
vanTL-D1	TCTATCACTTTGGATATGTC	2869–2888	6 (+)	This study
vanT-U2	CCNARNCKRTGCAMNCCNGTRTC	3939–3961	6 (−)	This study
vanTL-D2	CTTATCCATTACGAACTTACC	3843–3873	7 (+)	This study
RserU1	AYRTCNGGNARCATNACRTC	4887–4906	7 (−)	This study
364RD1	AAGGGTTTCAAGTAACAACC	4804–4823	8 (+)	This study
SerU1	GGNGTNYKNARRTCRTGNGC	5863–5882	8 (−)	This study
364SD4	GAAGGGATATTGATTATCGGTGTG	5605–5628	9 (+)	This study
364SD5	CCAGAACAGCGTGTTAAGCTATC	5725–5747	9 (+)	This study
364SD1	TAATGCTGACAATCGCGCAG	5799–5818	9 (+)	This study
ISvL-D1	ATGGACTATTACGAGAGTACC	7481–7502	10 (+)	This study
ISvL-D2	TGATTCACAGGCTCCTTAGC	7524–7543	10 (+)	This study
ISvL-D3	TCAGAAGTTCAGTGAAGCTAC	7568–7588	10 (+)	This study
AD1	NGTCGASWGANAWGAA	NA	10 (−)	22

[a] K is G or T, M is A or C, R is A or G, S is G or C, W is A or T, Y is C or T, D is A, G, or T, V is A, C, or G, I is inosine, and N is any base.
[b] Coordinates are from accession no. EU250284.
[c] PCR number as indicated in Fig. 1.
[d] NA, not applicable.

and contained all the conserved active site residues of other VanXY proteins and also those found in VanX and VanY proteins (Fig. 2B) (20).

The third and fourth open reading frames, *vanTm*$_L$ and *vanTr*$_L$, respectively, appear to encode serine racemase activity (Fig. 1). *vanTm*$_L$ and *vanTr*$_L$ are in different reading frames, and a single-base insertion between bases 3497 and 3564 (coordinates are from accession no. EU250284) would lead to a

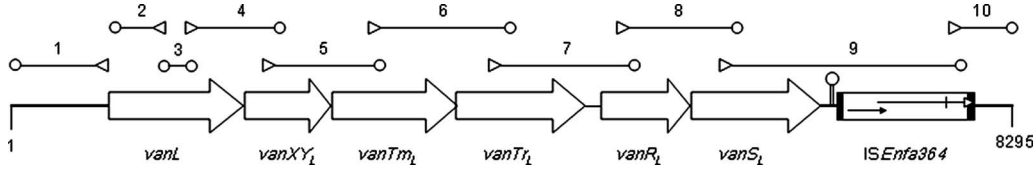

FIG. 1. Schematic diagram of the VanL operon and flanking regions. Regions amplified and sequenced are indicated at the top and are numbered (see Table 1), with a triangle indicating a sequence-specific primer and an oval indicating a degenerate primer. The putative stem-loop region between *vanS*$_L$ and IS*Enfa364* is shown. The inverted repeats of IS*Enfa364* are indicated by black bars, the two overlapping reading frames coding for *tnpA* are shown as arrows, and the in-frame stop codon is indicated by a vertical line. Coordinates are from GenBank accession number EU250284.

TABLE 2. Extents of identity of proteins from the VanL operon with the corresponding proteins from other D-Ala-D-Ser operons

Protein	% Identity with protein from other indicated operon					
	VanL	$VanXY_L$	$VanTm_L$[a]	$VanTr_L$[a]	$VanR_L$	$VanS_L$
VanC	49	46	45	51	72	52
VanE	51	41	44	53	59	39
VanG1	42	38	35	41	60	40
VanG2	42	38	34	40	62	40

[a] As aligned with the corresponding domain of VanT proteins.

single 2,085-bp putative $vanT_L$ gene. Whether extant *vanT* genes arose in this way or whether $vanTm_L$ and $vanTr_L$ evolved by a deletion in a $vanT_C$-like gene is purely speculative. An alignment of $VanTm_L$ and $VanTr_L$ with the $VanT_C$ protein is shown in Fig. 3. $VanTm_L$ exhibits 45% identity with the $VanT_C$ N-terminal region, which is postulated to function in the transport of L-serine (2, 3). The $VanTr_L$ protein exhibits 51% identity to the $VanT_C$ C-terminal region and shares all the residues important in structure and function of pyridoxal 5′-phosphate-dependent alanine racemases (2) (Fig. 3). Since L-serine transport and racemase activities are not codependent for function (3), having these two domains separately encoded would not necessarily compromise VanT activity in *E. faecalis* N06-0364. The $vanR_L$ and $vanS_L$ genes follow $vanTr_L$ (Fig. 1). Alignments of $VanR_L$ and $VanR_C$ and of $VanS_L$ and $VanS_C$ are shown in Fig. 4. The deduced $VanR_L$ exhibited 72% identity to $VanR_C$ and contained the conserved residues (D10, D53, and K102) found in response regulators from gram-positive two-component systems (Fig. 4A) (18). The deduced $VanS_L$ exhibited 52% identity to $VanS_C$ and contained the six conserved amino acid motifs (H, X, N, G1, F, and G2 boxes) of the C-terminal transmitter module of VanS proteins (16) (Fig. 4B). In addition, the $VanS_L$ N-terminal region contained two transmembrane regions characteristic of the sensor proteins in two-component systems (Fig. 4B) (4). $VanS_L$ has none of the substitutions (R200, D312, and G320) shown in $VanS_C$ to be associated with constitutive vancomycin resistance (19). This is consistent with growth experiments which revealed that vancomycin resistance was inducible in *E. faecalis* N06-0364 (data

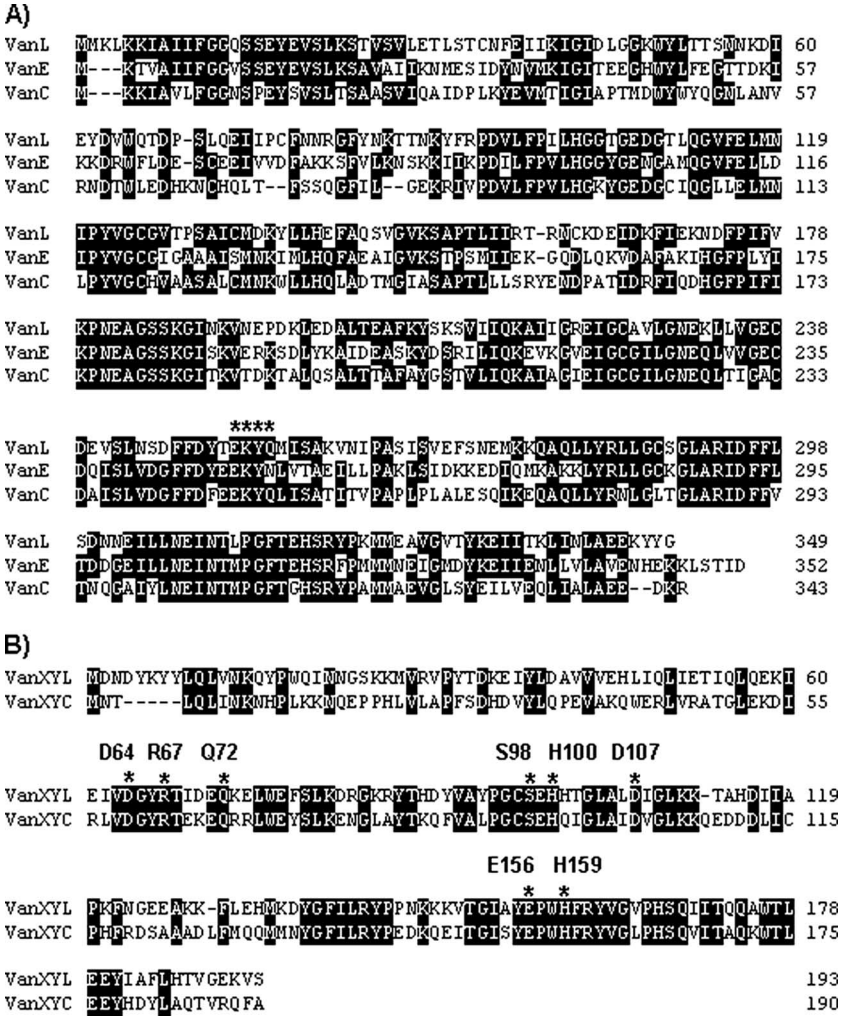

FIG. 2. Alignment of the VanL, VanE, and VanC proteins (A) and the $VanXY_L$ and $VanXY_C$ proteins (B). Identical residues are boxed in black. The EKYQ motif putatively involved in substrate binding is overlined by asterisks in panel A. The residues conserved in VanX, VanY, and VanXY proteins are indicated in panel B.

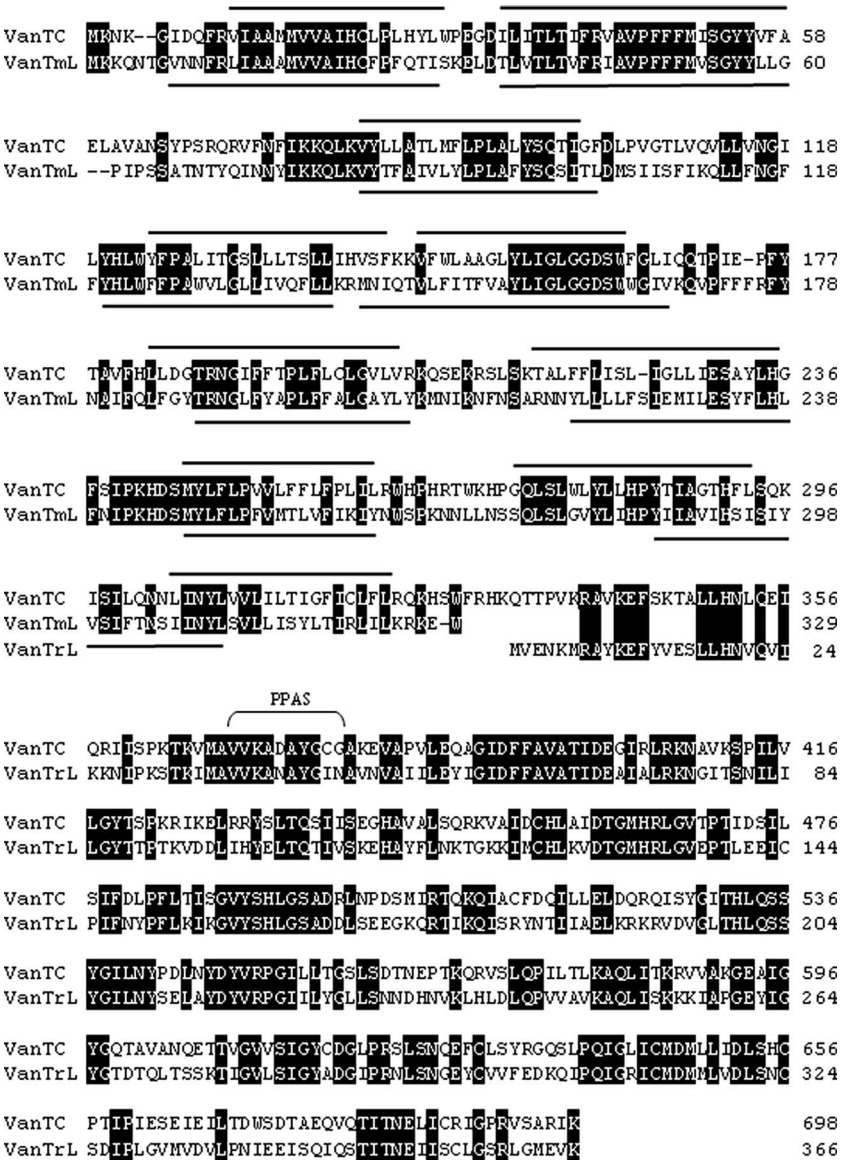

FIG. 3. Alignment of $VanT_C$ with the $VanTm_L$ and $VanTr_L$ proteins. Identical residues are boxed in black, hydropobic transmembrane domains are indicated by solid lines, and the pyridoxal phosphate attachment site (PPAS) is labeled.

not shown). Blast searches of the GenBank database with the ~950 bp upstream of *vanL* failed to return any matches. Beginning 110 bp downstream of $vanS_L$ is a 61-bp region with the potential to form a large stem-loop structure with a ΔG of −15.64 kcal/mol which may act as a transcriptional terminator (Fig. 1). Inserted 5 bp downstream of the putative stem-loop region is a 1,055-bp insertion sequence, designated IS*Enfa364*, with ends that are defined by 25-bp inverted repeats (IR) and that is most closely related to elements in the IS*30* family (17). The IS*Enfa364* transposase appears to be coded for by two overlapping reading frames whose products would presumably become linked through translational frameshifting (17). However, an in-frame stop codon would presumably lead to an inactive truncated protein. Typically, IS*30* family members are flanked by short 2- to 4-bp direct repeats created upon insertion (17). IS*Enfa364* is flanked by a single adenine residue. However, it was noted that the 4 bp flanking IR-L, ATGA, are repeated 2 bp downstream of IR-R.

The *vanL* region has a G+C content of 32%, while the *E. faecalis* genome has a G+C content of 38%, indicating acquisition from an organism with a more-AT-rich genome.

Despite several attempts, transfer of the *vanL* gene cluster could not be demonstrated in mating experiments with *E. faecalis* JH2-2 as a recipient. Plasmids were not visualized on gels from multiple plasmid preparations from *E. faecalis* N06-0364 (data not shown). It appears likely that the *vanL* gene cluster is located in the chromosome. All acquired D-Ala-D-Ser gene clusters identified to date, *vanE*, *vanG*, and *vanG2*, are chromosomally located (5, 6, 10, 14). Definitive proof of *vanL* functionality awaits cloning and transfer experiments and biochemical analysis of cell wall precursors.

vanL expands the so-called *van* alphabet and highlights the

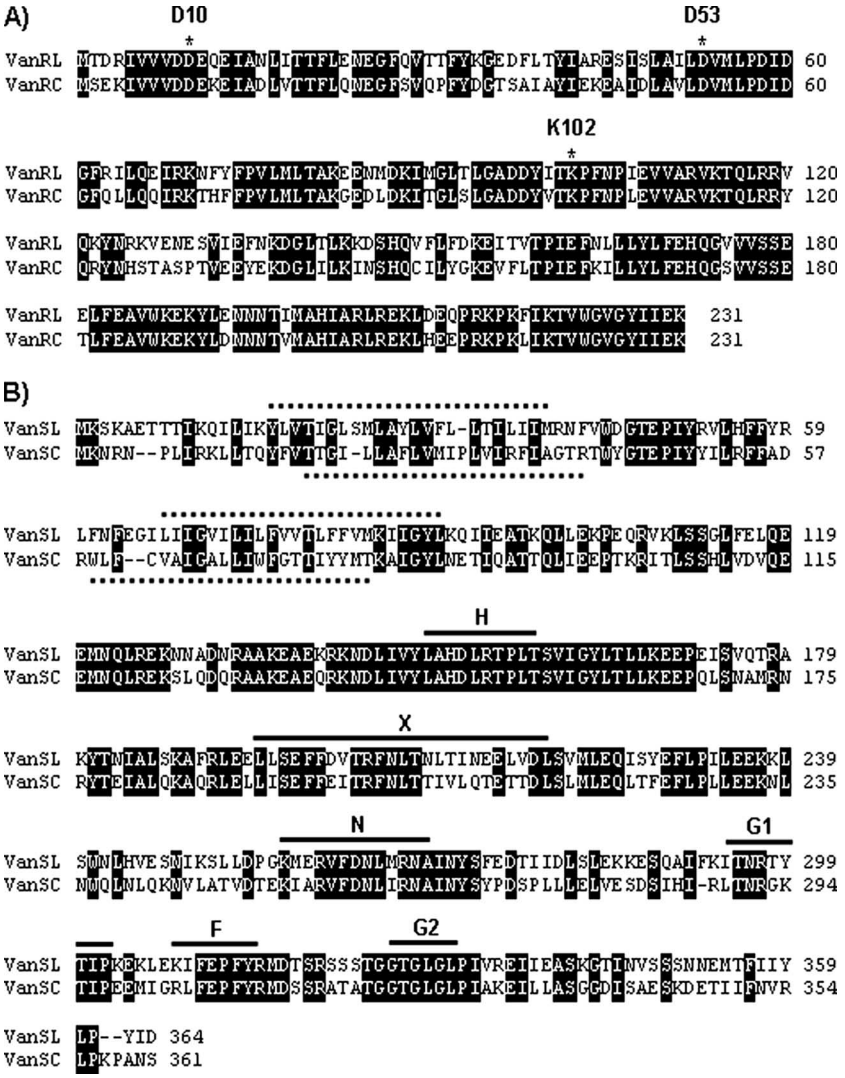

FIG. 4. Alignments of the $VanR_L$ and $VanR_C$ proteins (A) and the $VanS_L$ and $VanS_C$ proteins (B). Identical residues are boxed in black. The conserved aspartate and lysine residues of gram-positive response regulators are labeled in panel A. The transmembrane regions are indicated by hatched lines, and the conserved amino acid motifs H, X, N, G1, F, and G2 of VanS proteins are labeled and indicated by solid lines in panel B.

importance of characterization of *E. faecalis* and *Enterococcus faecium* isolates exhibiting low-level vancomycin resistance. As both the origin of the *vanL* gene cluster by this strain and the way this strain was acquired by this patient are unknown, the clinical significance of this finding remains to be established.

Nucleotide sequence accession number. The *vanL* gene cluster characterized in this study has been assigned accession number EU250284 in the GenBank Database.

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