Genetic Characterization of *vanG*, a Novel Vancomycin Resistance Locus of *Enterococcus faecalis*

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***Enterococcus faecalis* strain WCH9 displays a moderate level of resistance to vancomycin (MIC = 16 μg/ml) and full susceptibility to teicoplanin but is negative by PCR analysis using primers specific for all known enterococcal vancomycin resistance genotypes (*vanA, vanB, vanC, vanD,* and *vanE*). We have isolated and sequenced a novel putative vancomycin resistance locus (designated *vanG*), which contains seven open reading frames, from this strain. These are organized differently from those of all the other enterococcal *van* loci, and, furthermore, the individual *vanG* gene products exhibit less than 50% amino acid sequence identity to other *van* gene products.**

Glycopeptides inhibit cell wall synthesis through the interaction of their N termini with the D-alanyl-D-alanine termini of peptidoglycan precursors (25). The resulting complexes prevent the transglycosylases from incorporating disaccharide pentapeptide subunits into the growing peptidoglycan chain, thereby inhibiting cell wall synthesis (4). In enterococci, both intrinsic and acquired resistance to glycopeptides occurs via a common mechanism involving modification of the peptidoglycan biosynthetic pathway (7). Six enterococcal vancomycin resistance genotypes have been described and characterized. Four of these (*vanA, vanB, vanD,* and *vanE*) are acquired mechanisms and the other two (*vanC1* and *vanC2/C3* specific to *Enterococcus gallinarum* and *Enterococcus casseliflavus/Enterococcus flavescens*, respectively [the *vanC2* and *vanC3* genotypes are closely related, with the loci containing minor sequence differences, and are therefore jointly referred to herein as *vanC2/C3*; likewise, there is debate over whether *E. casseliflavus* and *E. flavescens* are actually separate species, and they are jointly referred to herein as *E. casseliflavus/E. flavescens*]) are intrinsic properties (15, 18, 21).

The genes for the VanA vancomycin resistance phenotype are located on the 10.8-kb transposable element Tn*1546*, which encodes 7 polypeptides involved in resistance. These polypeptides function together to confer high-level resistance to vancomycin and teicoplanin (6, 7), as well as variable levels of resistance to all other glycopeptides (18). The VanB resistance phenotype confers variable levels of resistance to vancomycin but leaves the organism susceptible to teicoplanin (8, 13). VanD is characterized by resistance to moderate levels of vancomycin and to low levels of teicoplanin (21), while the VanE resistance phenotype exhibits low-level resistance to vancomycin but full susceptibility to teicoplanin (15).

Enterococci of the VanA, VanB, and VanD phenotypes produce a suite of functionally homologous proteins, including ligases (VanA, VanB, and VanD) resulting in the formation of peptidoglycan precursors terminating in D-Ala-D-Lac (18, 21), dehydrogenases (VanH, VanH$_B$, and VanH$_D$) which reduce pyruvate to D-lactate (7, 10, 11), dipeptidases (VanX, VanX$_B$, and VanX$_D$) which hydrolyze the glycopeptide-susceptible cell wall precursors ending in D-Ala-D-Ala (7, 11, 24), and carboxypeptidases (VanY, VanY$_B$, and VanY$_D$) (5, 11, 14) which cleave the C-terminal D-Ala residue of late peptidoglycan precursors (22). VanE ligase synthesizes the formation of peptides terminating in D-Ala-D-Ser, and serine racemase activity has also been detected in VanE strains (15).

VanC1 and VanC2/C3 are intrinsic enterococcal resistance phenotypes characterized by low-level resistance to vancomycin but susceptibility to teicoplanin (7). Three protein products of the *vanC1* operon have been characterized: VanC1, VanXY$_c$, and VanT. VanC1 is a ligase that catalyzes the synthesis of cell wall precursors terminating in D-Ala-D-Ser (19). VanXY$_c$ has both D,D-dipeptidase and D,D-carboxypeptidase activities (23), while VanT is a membrane-associated serine racemase which converts L-Ser to D-Ser (3). Genes encoding a putative two-component regulator (VanR$_c$-VanS$_c$) are located downstream of the *vanC1* locus (2).

Recently, we have investigated the genotype of Australian isolates of vancomycin-resistant enterococci (VRE) (9). Among the strains examined were four *Enterococcus faecalis* isolates which exhibited moderate resistance to vancomycin (MIC range, 12 to 16 μg/ml), but full susceptibility to teicoplanin (MIC = 0.5 μg/ml), a phenotype similar to that of VanB and VanE strains. The four isolates were indistinguishable by pulsed-field gel electrophoresis and were isolated from inpatients at the same hospital in Brisbane, Queensland, Australia, in 1997. However, these strains were negative by PCR using primers specific for all enterococcal vancomycin resistance genotypes known at the time (*vanA, vanB, vanC1, vanC2/C3,* and *vanD*) (9) and have since tested negative by PCR using primers specific for the recently described *vanE* genotype (15). In the present study, we isolated and characterized the putative vancomycin resistance locus from one of these isolates (WCH9).

Isolation of putative vancomycin resistance locus from *E. faecalis* WCH9. In order to isolate a fragment of the vancomycin resistance locus, *E. faecalis* WCH9 DNA was extracted using the Wizard genomic DNA purification kit (Promega, Madison, Wis.), and subjected to PCR amplification using the degenerate primers V1 and V2 previously described by Perichon et al. (21). These primers were designed to direct amplification of an internal fragment from genes encoding glycopeptide resistance-associated ligases from a number of gram-positive species. The resultant 1.63-kb PCR product was much larger than expected and was purified using the

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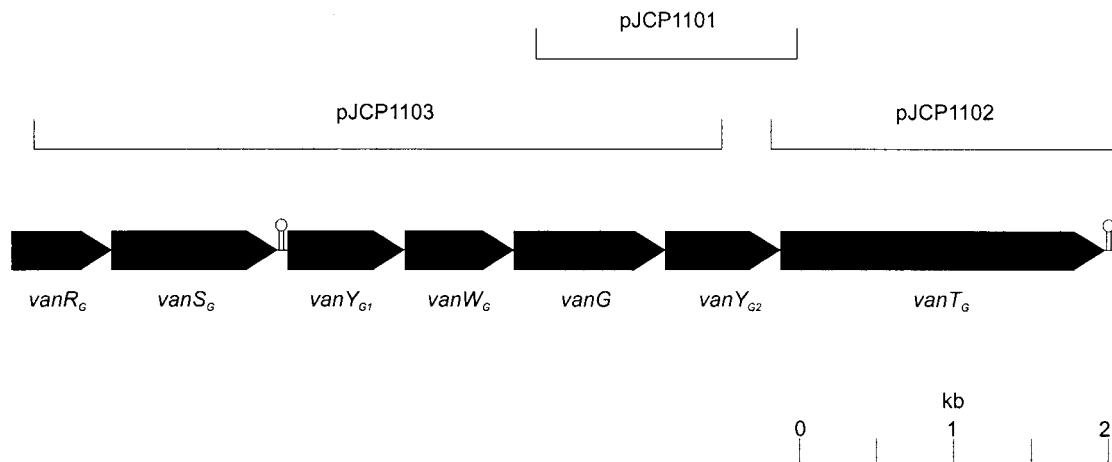
FIG. 1. Map of region of *E. faecalis* WCH9 chromosome containing the *vanG* locus, showing the location of the various subclones used to determine the DNA sequence. The locations and direction of transcription of the various ORFs are shown as solid pointed boxes. The locations of two putative transcription terminators are also indicated.

BRESAspin PCR purification kit (Bresatec Ltd., Adelaide, Australia) and cloned into pGEMT-easy (Promega), generating plasmid pJCP1101. The enterococcal DNA insert of this plasmid was then sequenced using dye terminator chemistry on an ABI model 373A automated DNA sequencer. Interestingly, analysis of the sequence revealed that amplification of the 1.63-kb fragment was a consequence of priming with only one of the degenerate primers (V1), presumably due to the presence of an inverted repeat of the target sequence. Nevertheless, BLASTX analysis (1) indicated that the fragment encoded a putative protein with similarities to the enterococcal VanB and VanA ligases (approximately 50 and 48% amino acid sequence identity, respectively), and another with approximately 50% identity to the VanY D,D-carboxypeptidase of the *vanA* locus.

In order to confirm that the isolated sequences were specific for non-*vanABCDE* strains such as WCH9, a variety of VRE and sensitive enterococci were subjected to PCR using primers VANG1 (5′-CGGTTGTGCCGTACTTGGC-3′) and VANG2 (5′-GGGTAAAGCCATAGTCTGGGGC-3′). These primers direct amplification of an 810-bp fragment from the portion of the pJCP1101 insert encoding the ligase homologue. The PCR amplification conditions used were the same as those previously described by Bell et al. (9) for detection of the *vanA*, *vanB*, *vanC1*, and *vanC2/C3* genotypes. The only strains that yielded 810-bp PCR products were WCH9 and the three other related non-*vanABCDE* VRE isolates. No PCR products were obtained when DNA extracts from vancomycin-sensitive *Enterococcus faecium*, *E. faecalis vanA*, *E. faecalis vanB*, *E. gallinarum vanC1*, or *vanC2/C3 E. casseliflavus/E. flavescens* strains were tested (result not shown).

In order to isolate the remainder of the apparently novel *van* locus, DNA from WCH9 was subjected to PCR using primers on the basis of preliminary sequence analysis of inverse PCR fragments. Primers SM5 (5′-GTTGACGAATTCATATTCCC AGATAAA-3′) and SM6 (5′-CGAAAGGAATTCTGGCAT TGATTATTTTAG-3′) directed amplification of a 1,999-bp PCR product, which was cloned into the *Eco*RI site of pBluescript KS (Stratagene, La Jolla, Calif.) to generate plasmid pJCP1102. Plasmid pJCP1103 was constructed by cloning the 5,004-bp PCR product obtained using primers VANG2 and SM7 (5′-CGGGACAAATAATTGGATGTC-3′) into pGEMT-easy (see Fig. 1).

DNA sequence analysis. The sequence of the complete putative vancomycin resistance locus was then determined by analysis of the recombinant plasmids, and nested deletion derivatives thereof, constructed according to the method of Henikoff (16). Sequence data in the 5′ direction of the region cloned in pJCP1103 were also obtained by direct sequence analysis of an additional inverse PCR product using primers SM30 (5′-CGATTGGAAAATCTGATCAATG-3′) and SM31 (5′-CGTAACCTCCCGATATGAGCC-3′) on *Nde*I-digested, recircularized DNA. Although existing enterococcal vancomycin resistance loci have been named *vanA* through *vanE*, we designated this novel locus *vanG*, because the name *vanF* was recently used for a *vanA*-like locus in *Paenibacillus popillae* (20). Examination of the compiled sequence indicated that the *vanG* locus was 7,652 nucleotides long and contained seven complete open reading frames (ORFs), arranged as shown in Fig. 1. BLASTX analyses were performed to identify the putative functions of the proteins, and the results are summarized in Table 1. The 5′ end of the locus contains genes designated $vanR_G$ and $vanS_G$, encoding a putative two-component regulatory system. The $vanS_G$ gene was immediately followed by a stem-loop structure ($\Delta G = -28.5$ kcal/mol), suggesting that these two genes may be transcribed separately from the remainder of the locus, which encodes two putative D,D-peptidases ($VanY_{G1}$ and $VanY_{G2}$), the ligase (VanG), a racemase ($VanT_G$), and a protein with unknown function ($VanW_G$). Interestingly, no obvious promoter region could be found upstream of $vanY_{G1}$, and the nearest site with similarity to consensus *Escherichia coli* promoter sequences is located near the 3′ end of $vanW_G$. Thus, the ORFs designated $vanY_{G1}$ and $vanW_G$ may or may not be transcribed. Each of the genes in the *vanG* cluster is preceded by a potential ribosome binding site, although that for $vanY_{G2}$ is very close to the initiation codon, which may result in a low translation efficiency. An additional stem-loop structure ($\Delta G = -23.0$ kcal/mol) is located approximately 20 nucleotides downstream of the $vanT_G$ termination codon. BLASTX analysis of sequences downstream of this indicated a low degree of similarity to a protein of unknown function (ORF9) from the 3′ end of the *E. faecalis* conjugative transposon Tn*916* (GenBank accession number U09422).

Similarities to other enterococcal *van* loci and gene products. The comparative genetic organization for the five VRE

TABLE 1. Summary of ORFs in the *vanG* locus

Gene	Putative function	Location in sequence[a]	Predicted M_r	No. of amino acids	% G+C content	Similar protein(s) (GenBank accession no.; gene)	% Identity, % similarity (no. of aa)
$vanR_G$	Response regulator	1 to 706	26,858	235	42.3	$VanR_D$ of *E. faecium* (AF130997; *vanD*)	54, 71 (238)
						VanR of *Bacillus circulans* (Y15706)	46, 63 (239)
						VanR, *E. faecium* (M68910; *vanA*)	46, 61 (238)
$vanS_G$	Sensor protein kinase	722 to 1825	42,027	367	27.7	$VanS_D$ of *E. faecium* (AF130997; *vanD*)	57, 69 (330)
						VanS of *B. circulans* (Y15707)	39, 56 (364)
						VanS of *E. faecium* (M68910; *vanA*)	38, 57 (365)
$vanY_{G1}$	D,D-Peptidase	1962 to 2798	31,926	278	42.9	$VanY_B$ of *E. faecalis* (U35369; *vanB*)	48, 63 (211)
						Carboxypeptidase of *Bacillus subtilis* (AF015775)	31, 47 (246)
						YokZ of *B. subtilis* (AF006665)	31, 47 (246)
$vanW_G$	Unknown	2979 to 3713	28,301	244	35.8	VanW of *E. faecalis* (U35369; *vanB*)	45, 60 (244)
vanG	Ligase	3715 to 4764	38,727	349	39.0	VanB of *E. faecalis* (U00456)	46, 64 (215)
						VanA of *B. circulans* (Y15704)	49, 66 (202)
						VanB2 of *E. faecium* (U81452)	47, 64 (212)
$vanY_{G2}$	D,D-Peptidase	4761 to 5525	29,362	253	37.3	VayY of *B. circulans* (Y17303)	35, 54 (262)
						VanY of *E. faecium* (M90647; *vanA*)	34, 53 (256)
						YokZ of *B. subtilis* (AF006665)	34, 51 (191)
$vanT_G$	Racemase	5518 to 7653	80,159	711	36.1	VanT of *E. gallinarum* (AF077816; *vanC1*)	35, 55 (698)
						Alanine racemase of *Bacillus psychrosaccharolyticus* (AB021683)	36, 53 (383)
						Alanine racemase of *Synechocystis sp.* (D64003)	35, 53 (362)

[a] Nucleotide positions are given.

van loci that have been fully sequenced to date is shown in Fig. 2. Although except in the case of *vanC1* response regulator (*R*) and sensor protein kinase (*S*) genes are located at the 5′ end of the loci, the organization of subsequent genes differs widely between *van* types, suggesting diverse origins. Marked differences also occur at the deduced amino acid sequence level. The alignment of the putative ligase VanG with the respective ligases VanA, VanB, VanC1, and VanD is shown in Fig. 3, and the percent amino acid identity between each ligase is shown in Table 2. This shows that the degree of similarity between VanG and the other ligases (39 to 47%) is no greater than between any of the other proteins (values observed range from 38% identity between VanC1 and VanD to 76% identity between VanA and VanB). Thus, VanG is clearly not a minor variant of one of the other enterococcal proteins and represents a distinct class of ligases.

The putative regulatory proteins $VanR_G$ and $VanS_G$ had greatest identity with $VanR_D$ and $VanS_D$ (54 and 57%, respectively). In addition, $VanR_G$ also has 46% identity with VanR (over 236 amino acids [aa]) and 28% identity with $VanR_B$ (over 169 aa), while $VanS_G$ has 42% identity with VanS (over 365 aa) and 20% identity with $VanS_B$ (over 274 aa). $VanR_G$ has similar degrees of amino acid identity between both the N- and C-terminal portions, which are the phosphorylation site and DNA-binding regions, respectively. $VanS_G$ has a percent amino acid identity in the C-terminal two-thirds, which include the histidine kinase activity motif, greater than that in the N-terminal third, which is involved in ligand binding.

The putative peptidase $VanY_{G1}$, which may or may not be transcribed, has a deduced amino acid sequence with 46% identity with that of $VanY_B$ (over 214 aa) and 27% identity with the putative protein $VanY_{G2}$ (over 177 aa). In addition, the putative peptidase $VanY_{G2}$ has 32% identity (over 247 aa) and 33% identity (over 184 aa) with the VanY and $VanY_B$ proteins, respectively. $VanY_{G2}$ is approximately 49 and 14 amino acids shorter than its VanY and $VanY_B$ counterparts, respectively. The *vanG* locus lacks a putative VanX dipeptidase, indicating that the putative $VanY_G$ proteins may be similar to $VanXY_c$ of the *vanC1* locus and display both carboxypeptidase and dipeptidase activities.

Attempted transfer of vancomycin resistance. Transfer of vancomycin resistance from *E. faecalis* WCH9 to *E. faecium* ATCC 19434 was attempted by filter mating (26), with selection on phenol red agar containing phenol red broth base
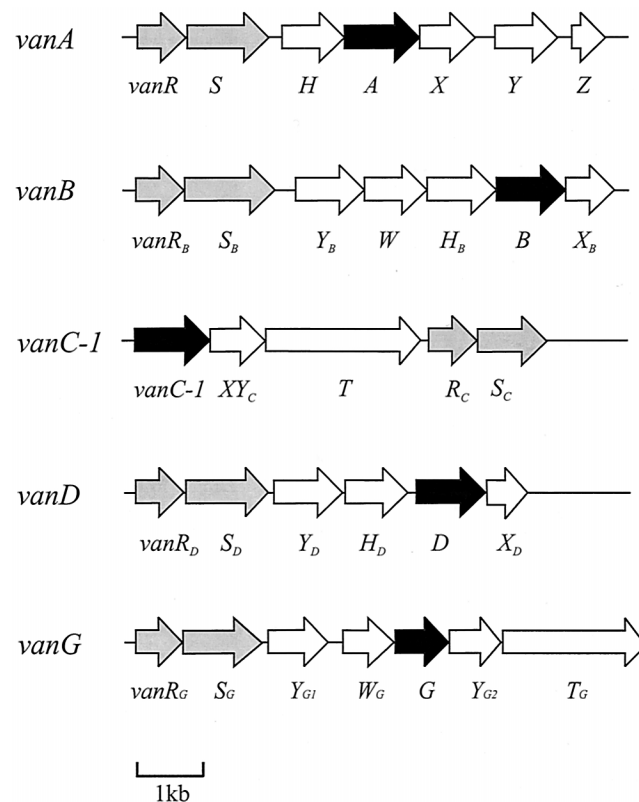

FIG. 2. Genetic organization of the *vanG* locus, compared with that of other enterococcal vancomycin resistance loci, based on published sequence data for the *vanA*, *vanB*, *vanC1*, and *vanD* loci (2, 6, 7, 11). ORFs encoding putative ligases and two-component regulatory systems are shown in black and grey, respectively.

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VanG     1  MNKKAIFGGNSTEYVSQSASAFENINTN--KDIPIGITSGE-WYHYTGEKE
VanA     1  MNRIKVAIFGGCSEEHDVSKSAIEIAANINKE--KYPYIGITKSGV-WKCEKP--
VanB     1  MNIKVAIIFGGCSEEHDVSKSAIEIAANINTE--KDPHYIGITKNGV-WKCKKP--
VanC1    1  --MKKAIIFGGNSPEYTVSASATSAIEA  SPYDYDLSLIGIAPDDWYYTGELE
VanD     1  MFRIKVAFGGCSEEHVSKSAEIAANITK--KYQPYIGITKSGV-WKCEKP--

VanG    58  KILNNTWFEDKNLCPVVSQRSVGFLEASDKYRIIVDFPVLHGKNGEGQQG
VanA    56  ---CAEWEDN--CYSAVLSPDKMHGLLVKKNEYEINVDVAFSALHGKSGEDGSIQG
VanB    56  ---CTEWEAD---SLPAFSPDRKTHGLLVKEEYETRDVAFPVLHGKCGEDGIQG
VanC1   59  NIRQDTWLLD--KHKQKIQFLFEGNGFWSEEQ--TLVPDVFPHGKGEDGSIQG
VanD    56  ---CLEWEYA--GDPVVFSPDRSTHGLLQKGYEIQPVDVFPHGKGEDGSIQG

VanG   118  FELGIPVVGCDTLSSACMDKDRAHKVSLAGIVPKSVTFRFNEEAA---EIEA
VanA   111  LFELSGIPVGCDIQSSACMDKSLTYIAKNAGIATP---AFWINKDD-----PVAA
VanB   110  LFELSGIPYVGCDIQSSAACMDKSLAYITKNAGIAVP---EFQIEKGD-----PEAR
VanC1  115  LFELMKPYVGCGAGSACMKWLLHQAAAAIGQSAP--IILTNQQQEQEAFIQ
VanD   111  LLELSGIPYVGCDIQSSVCMDKLAYTVKNAGITVP---GFEGD-----LETE

VanG   175  NLTYPFKPVRGSSFGTKVIEKELDAAIELAFHDEEETINGFEVGCAVLG-
VanA   163  TFTYPVFVKPARSGSSFGVKKVNSAELDYAIESARQYDSKILIEQASGCEVGCAVLGN
VanB   162  TLTYPVFVKPARSGSSFGVTKVNSTEELAAIEAAGQYDGKILIEQAISGCEVGCAVGN
VanC1  173  THGPVFFKPNEGSSKGTKVTCVEEAAKEAFTYCSALKNIGVEGCLG-
VanD   163  DFVYPVFVKPARSGSSFGVNKVCKAEELQAAIEEARKYDSKILIEEAGSEVGCALGN

VanG   234  IDLIVGRVDIELSSGFFDYT-EKYTLKSSK---IYPARDAEAEKRIQEAAVTIYA
VanA   223  SAALVGEVDQIRLQYGIFIHQEVEPEKGSENAVITVPADISAEERGRIQETAKKIYA
VanB   222  EDDLIVGEVDQIRLSHGIFIHQENEPEKGSENAIIVPADPVEERNRQETAKKYRV
VanC1  232  NDSLTVCACDAISLVDGFFDFE-EKYQLISK---ITVPAPLPETIETKEQAQLYR
VanD   223  GDLAGEVDQIELRHGFFIHQEAQPEKGSENAVIRVPAALPDEVREQIQETAMKIYRI

VanG   290  LGCSGFRVDFYTPSGEIVFNEVNTPGFTSHSRYPNMMKIGFSLDLIGLYE
VanA   283  LGCRGLARVDFLQGRIVLNEVNTPGFTSYSRYPRMMAAAGALPELDLIVLALK
VanB   282  LGCRGLARVDFLQEDGGIVLNEVNTPGFTSYSRYPRMAAAAGLPALDSLITLAE
VanC1  288  LGLGLARDFFTERGEYLNENTPGFTSHSRYPAMMAAVGYQBLLQLVLAKE
VanD   283  LGCRGLARDFLREDGCIVLNEVNTPGFTSYSRYPRMMAAGFLSELDLIELLR

VanG   350  ---
VanA   343  G--
VanB   342  R--
VanC1  348  EVK
VanD   343  R--
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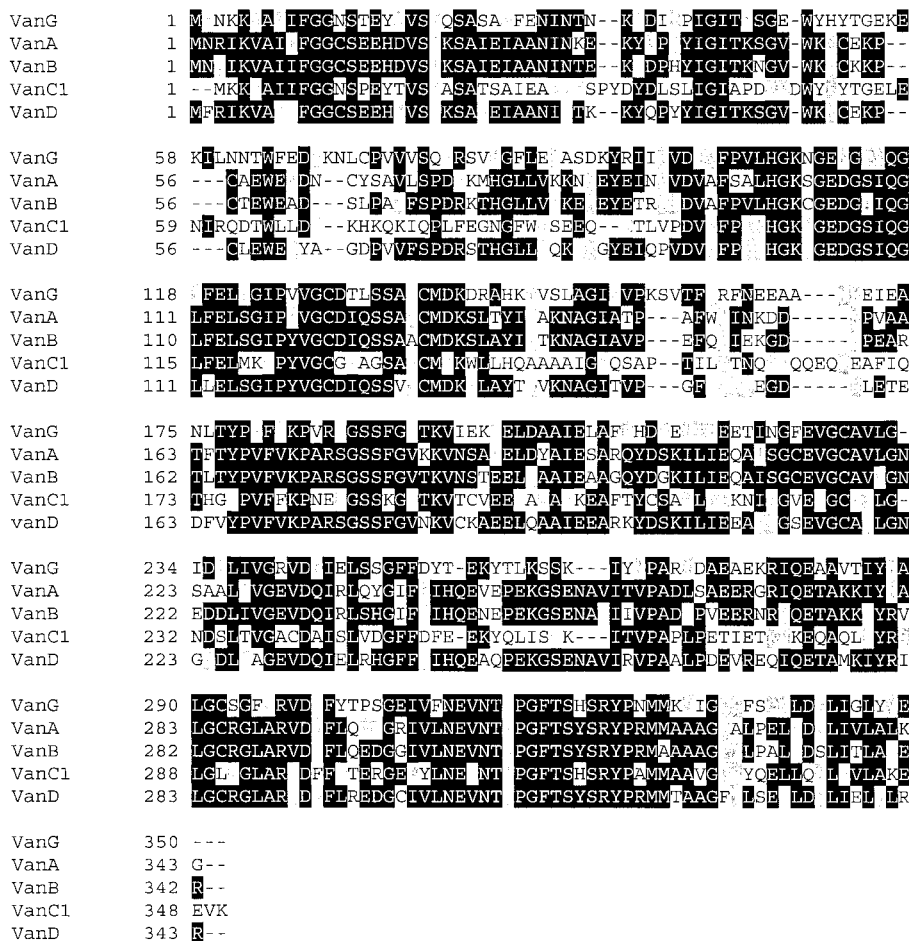
FIG. 3. Comparison of VanG with other enterococcal ligases. The deduced amino acid sequence of VanG was aligned with those for VanA (6), VanB (14), VanC1 (12), and VanD (11) using CLUSTAL (17). Amino acid position numbers are shown at the left of the sequences. Identical residues are shaded black; residues with similar properties are shaded grey. Gaps (dashes) have been introduced by the program to optimize the alignment.

(1.6% [wt/vol]), Bacto agar (1% [wt/vol]), L-arabinose (1% [wt/vol]), and vancomycin (5 μg/ml). However, although transmission of vancomycin resistance occurred when vanB E. faecalis ATCC 51299 was mated with the sensitive Enterococcus faecium recipient, transmission was not demonstrated when the vanG strain was used as donor. Fines et al. (15) were similarly unable to demonstrate transmission of the VanE phenotype. Transfer of vancomycin resistance from E. faecalis WCH9 to another E. faecalis strain was not attempted due to the lack of a selective marker capable of distinguishing donor and recipient strains.

Conclusions. In this study, we have isolated and characterized a novel vancomycin resistance locus, designated vanG, from E. faecalis strain WCH9, which locus displays a moderate level of resistance to vancomycin and full susceptibility to teicoplanin. The vanG locus contains seven ORFs, but these are organized differently from all the other enterococcal van loci characterized to date. Moreover, the individual vanG gene products exhibit less than 50% amino acid sequence identity to their respective homologues from the other enterococcal van loci, suggesting diverse evolutionary origins of vancomycin resistance in this genus. VanG VRE appear to be rare, at least in Australia. The four original strains were isolated from patients in a single institution over a relatively short period of time, consistent with nosocomial spread. With the exception of reisolation from one of the four original VanG VRE-carrying patients 12 months later, no further strains belonging to this genotype have been detected. However, its prevalence in other parts of the world remains to be investigated.

Nucleotide sequence accession number. The DNA sequence of the vanG locus described here has been deposited with GenBank under accession number AF253562.

TABLE 2. Sequence identity between the deduced amino acid sequence of VanG and those of other enterococcal ligases

Sequence compared[a]	% Sequence identity (no. of overlapping amino acids)				
	VanG	VanA	VanB	VanC	VanD
VanA	44 (351)				
VanB	47 (354)	76 (341)			
VanC	42 (347)	38 (349)	39 (350)		
VanD	46 (347)	69 (342)	67 (343)	38 (350)	
VanE	39 (201)	44 (206)	42 (206)	54 (202)	44 (206)

[a] Based on complete sequence data published for VanA (6), VanB (14), VanC1 (12), and VanD (11), and the partial sequence published for VanE (15).

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