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Glycopeptide Antibiotic Resistance Genes in Glycopeptide-Producing Organisms

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The mechanism of high-level resistance to vancomycin in enterococci consists of the synthesis of peptidoglycan terminating in D-alanyl-D-lactate instead of the usual D-alanyl-D-alanine. This alternate cell wall biosynthesis pathway is ensured by the collective actions of three enzymes: VanH, VanA, and VanX. The origin of this resistance mechanism is unknown. We have cloned three genes encoding homologs of VanH, VanA, and VanX from two organisms which produce glycopeptide antibiotics: the A47934 producer *Streptomyces toyocaensis* NRRL 15009 and the vancomycin producer *Amycolatopsis orientalis* C329.2. The predicted amino acid sequences are highly similar to those found in VRE: 54 to 61% identity for VanH, 59 to 63% identity for VanA, and 61 to 64% identity for VanX. Furthermore, the orientations of the genes, *vanH*, *vanA*, and *vanX*, are identical to the orientations found in vancomycin-resistant enterococci. Southern analysis of total DNA from other glycopeptide-producing organisms, *A. orientalis* 18098 (chloro-eremomycin producer), *A. orientalis* subsp. *lurida* (ristocetin producer), and *Amycolatopsis coloradensis* subsp. *labeda* (teicoplanin and avoparcin producer), with a probe derived from the *vanH*, *vanA*, and *vanX* cluster from *A. orientalis* C329.2 revealed cross-hybridizing DNA in all strains. In addition, the *vanH*, *vanA*, *vanX* cluster was amplified from all glycopeptide-producing organisms by PCR with degenerate primers complementary to conserved regions in VanH and VanX. Thus, this gene sequence is common to all glycopeptide producers tested. These results suggest that glycopeptide-producing organisms may have been the source of resistance genes in vancomycin-resistant enterococci.

The glycopeptide antibiotic vancomycin and the structurally related antibiotic teicoplanin are considered to be the last lines of defense against a variety of serious infections caused by gram-positive organisms, such as enterococci, methicillin-resistant *Staphylococcus aureus*, and *Clostridium difficile* (11). The recent rapid emergence and spread of vancomycin-resistant enterococci (VRE) has therefore become a grave concern of both medical practitioners and their patients (22, 27).

The molecular target of glycopeptide antibiotics is the D-alanyl-D-alanine (D-Ala-D-Ala) terminus of growing peptidoglycan, the rigid polymer which protects bacterial cells from osmotic lysis. By binding to this terminal dipeptide, glycopeptide antibiotics interfere with proper cell wall formation, which results in eventual cell death (6). Glycopeptide-resistant organisms avoid such a fate by modifying the drug's peptide target, specifically, by modifying it to the depsipeptide D-alanyl-D-lactate (D-Ala-D-Lac) (40). The biosynthetic machinery required to effect this transformation in vancomycin-resistant *Enterococcus faecium* is found on a transposable element, Tn*1546*, which incorporates five genes necessary and sufficient to confer high-level inducible glycopeptide resistance (2). Two of these gene products, VanR and VanS, are required for the vancomycin-induced resistance response and are members of a two-component regulatory system directing the transcription of *vanH*, *vanA*, and *vanX*. These genes encode the three proteins which are necessary for D-Ala-D-Lac synthesis and are thus essential for glycopeptide resistance (1). VanA is the pivotal enzyme, producing the ester D-Ala-D-Lac instead of the usual D-Ala-D-Ala dipeptide for incorporation into peptidoglycan precursors (9). VanH is an α-keto acid reductase (D-lactate dehydrogenase [D-LDH]) that supplies VanA with substrate by converting pyruvate into D-lactate (9), while VanX is a highly specific D,D-dipeptidase which hydrolyzes the D-Ala-D-Ala generated by the endogenous cell wall biosynthetic pathway but does not recognize D-Ala-D-Lac (34, 43). This elegant system is the means by which VRE can overcome current antibiotic regimens. A second variant of this mechanism and gene structure is encountered and distributed on plasmids (42), conjugative chromosomal elements (32), or transposons (e.g., Tn*1547* [31]) with the D-Ala-D-Lac ligase designated VanB (18, 33).

The origin of the *vanH*, *vanA*/*B*, and *vanX* (*vanHAX*) resistance cassette has remained obscure. Both the intrinsically vancomycin-resistant lactic acid bacteria, such as organisms from the genera *Leuconostoc*, *Pediococcus*, and *Lactobacillus*, and the glycopeptide-producing organisms are potential reservoirs. Alternatively, the genes may have arisen through mutations in homologous genes within enterococci or other organisms. The vancomycin-resistant lactic acid bacteria such as *Leuconostoc mesenteroides* have both the requisite D-Ala-D-Lac ligase (15, 29) and D-LDH (12) enzymes, although the D-Ala-D-Lac ligases differ in their primary sequence from VanA/B, notably in the ω-loop region which is critical to catalysis (16). We have recently cloned a gene encoding a VanA/B homolog, designated DdlM, from the glycopeptide antibiotic-producing organism *Streptomyces toyocaensis* NRRL 15009 (23). The striking homology between VanA/B and DdlM (62% identity, >77% similarity) suggested that these enzymes evolved from a common ancestor. Similarly, we reported a segment of the ligase gene (*ddlN*) cloned from the vancomycin-producing organism *Amycolatopsis orientalis* C329.2 which also encoded a clear VanA/B homolog (23). Importantly, the ω-loop segment is conserved between VanA, VanB, DdlM, and DdlN. Thus, these results suggested that the VanA/B enzymes originated in

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TABLE 1. Sequences of PCR primers used in this study

Primer[a]	DNA sequence	Associated protein	Target amino acid consensus sequence
p1 (fwd)	5′-CA(T/C) TC(G/C) CGI GGI (T/A)C(C/G) (A/G)C(C/G) ATC GAC-3′	VanX	HSRG(S/T)(A/T)ID
p2 (rev)	5′-G(T/A)A GTG CCA CCA (T/C)T(T/C)-3′	VanX	EWWHY
p3 (fwd)	5′-GGI GAG GAC GGI (T/A)(C/G)I (C/A)TI CAG GG-3′	VanA	GEDGAMQG
p4 (rev)	5′-GTG AAI CC(G/C) GGI A(T/G)I GTG TT-3′	VanA	NT(L/I)PGFT
p5 (fwd)	5′-GGI TGC/T GGI T/A/CC/GG/C GAC GAG/A GC-3′	VanH	GCG(S/P)DEA

[a] fwd, forward; rev, reverse.

glycopeptide-producing organisms but that the origins of the VanH and VanX accessory proteins were unknown. We now report on the cloning and sequencing of genes encoding VanH and VanX homologs from the same glycopeptide producers as well as the presence of *vanHAX* gene clusters in several other glycopeptide-producing organisms. The strong homology of the gene products and the arrangement of the cloned genes indicate a common origin for the vancomycin resistance genes in VRE and antibiotic-producing organisms.

MATERIALS AND METHODS

Cloning of *vanX* from *S. toyocaensis* NRRL 15009. Cloning of a *vanX* gene from *S. toyocaensis* NRRL 15009 ($vanX_{st}$) was achieved by the isolation of a specific gene probe by PCR amplification and degenerate deoxyoligonucleotide primers p1 and p2 designed to amplify an internal *vanX* fragment of approximately 360 bp (Table 1). PCR amplification reactions contained *S. toyocaensis* NRRL 15009 total DNA as template (500 ng), 1 U of *Taq* polymerase, each deoxynucleoside triphosphate (dNTP) at a concentration of 0.4 mM, 1 μM primers, and 2 mM $MgCl_2$. The 360-bp product was cloned into the pGEM-T plasmid vector (Promega), and both strands were sequenced. To isolate a clone encompassing the entire $vanX_{st}$ gene, the probe fragment was randomly labeled with ^{32}P by using the Klenow fragment (4). Labeling reactions contained 200 ng of template, 1 μl of each PCR primer, 1× Klenow buffer (10 mM Tris-Cl [pH 7.5], 5 mM $MgCl_2$, 7.5 mM dithiothreitol), and 10 U of the Klenow fragment in a final volume of 55 μl. This probe was used to screen Southern hybridization blots of total *S. toyocaensis* NRRL 15009 DNA which had been digested to completion with several restriction enzymes in separate experiments. Hybridizing fragments (5.2 kb) of DNA from digestion with *Pst*I were cloned into pBluescript II KS+ (Stratagene), transformed into *Escherichia coli* DH5α, and confirmed to contain the $vanX_{st}$ gene by colony hybridization. A 5.2-kb fragment was found to contain the $vanX_{st}$ gene and was sequenced in its entirety.

The *vanH* gene was located immediately upstream of the *ddlM* gene, whose sequence we have reported previously (23).

Cloning of *van* genes from *A. orientalis* C329.2. Cloning of the *A. orientalis* C329.2 *vanHAX* cluster was achieved essentially as described for the *vanX* gene of *S. toyocaensis* NRRL 15009. A *van* gene probe was generated from *A. orientalis* C329.2 genomic DNA by degenerate PCR with primers p2 and p3 complementary to conserved sequences in *vanA* and *vanX* (Table 1). Each amplification reaction mixture consisted of 500 ng of total DNA from *A. orientalis* C329.2, 1× *Taq* buffer, 5% dimethyl sulfoxide, 2 mM $MgCl_2$, each dNTP at a concentration of 0.4 mM, 1 U of *Taq* polymerase, and 1 μM primers. The 1.3-kb product was cloned into pGEM-T (Promega), and this clone was used as a template to generate a ^{32}P-radiolabeled oligonucleotide probe with the Klenow fragment. This probe was used to screen Southern blots of restriction endonuclease-digested *A. orientalis* C329.2 genomic DNA to identify a 3.5-kb *Bam*HI product containing *van* genes. This fragment was cloned into pGEM-7Z and was isolated by standard methods.

DNA sequencing. The nucleotide sequence of the *S. toyocaensis* NRRL 15009 gene cluster was determined with a series of nested deletions constructed by *Exo*III-S1 nuclease digestion and through the synthesis of specific oligonucleotide primers. The gene cluster for *A. orientalis* C329.2 was sequenced by primer walking. DNA cycle sequencing was performed by B. Allore on an Applied Biosystems automated DNA sequencer at the MOBIX central facility at McMaster University.

Analysis of gene clusters in glycopeptide producing organisms. Total DNA was isolated from glycopeptide-producing organisms, and 10 to 20 μg was separated on a 1% TAE (Tris-acetate-EDTA)–agarose gel after digestion to completion with *Bam*HI. The DNA was blotted onto nitrocellulose by standard methods. A *vanHAX* cluster oligonucleotide probe radiolabeled with ^{32}P was generated as described above by using the cloned 3.5-kb *Bam*HI fragment from *A. orientalis* C329.2 as template and primers p3 and p4 (Table 1). This probe was hybridized to genomic DNA restriction products, and the blot was washed under stringent conditions prior to exposure.

PCR amplification of *vanHAX* from glycopeptide-producing organisms. The presence of the *vanHAX* cluster in glycopeptide producers was also assessed by amplification of the cluster by PCR. Degenerate oligonucleotide primers complementary to conserved regions in VanH (primer p5) and VanX (primer p2) were designed to amplify nearly the entire cluster (Table 1). The PCR mixtures (100 μl) contained 500 ng of total DNA, 1 U of *Taq* polymerase, 1× Taq buffer, 5% dimethyl sulfoxide, 4 mM $MgCl_2$, each dNTP at a concentration of 0.4 mM, and 1 μM primers. Forty cycles of 94, 55, and 72°C (1, 1, and 2 min, respectively) were required to produce a visible band of approximately 2.6 kb. This product was isolated, applied to a 1% TAE agarose gel, and blotted onto nitrocellulose. A ^{32}P-labeled *vanHAX* probe was generated from *A. orientalis* C329.2 total DNA and primers p3 and p4 (Table 1).

Analysis of sequence data. Contiguous sequences were constructed by using the MegAlign software from DNA Star (Madison, Wis.). Open reading frames (ORFs) were screened in all six frames by using the program FRAME (8) to identify probable streptomycete ORFs. Protein sequence alignments were performed by using CLUSTAL W software (39), version 1.7, with the Blosum matrix with a gap penalty of 10 and a gap extension of 0.05.

Nucleotide sequence accession numbers. The nucleotide sequences of $vanH_{st}$ and *ddlM* (accession no. U82965), $vanX_{st}$ (accession no. AF039028), and the gene cluster from *A. orientalis* C329.2 (accession no. AF060799) have been deposited in GenBank.

RESULTS AND DISCUSSION

Cloning of *van* genes from *S. toyocaensis* NRRL 15009 and *A. orientalis* C329.2. *S. toyocaensis* NRRL 15009 and *A. orientalis* C329.2 are gram-positive microbes which produce the "aglyco" glycopeptide antibiotic A47934, a structural homolog of teicoplanin (44), and vancomycin, respectively. We cloned and sequenced an expanse of *S. toyocaensis* NRRL 15009 DNA spanning 2,650 bp and a 3,470-bp fragment of DNA from *A. orientalis* C329.2. Each of these contains three ORFs whose products bear striking homology to VanH, VanA (23), and VanX of VRE (Fig. 1). These genes have been designated $vanH_{st}$, *ddlM* (23), and $vanX_{st}$, respectively, in *S. toyocaensis* and $vanH_{aov}$, *ddlN*, and $vanH_{aov}$, respectively, in *A. orientalis* C329.2. (This rather ponderous terminology is necessitated by the fact that various *A. orientalis* strains produce different glycopeptide antibiotics; thus, the subscript *ao* designates *A. orientalis* and the subscript *v* indicates that it is vancomycin producer.)

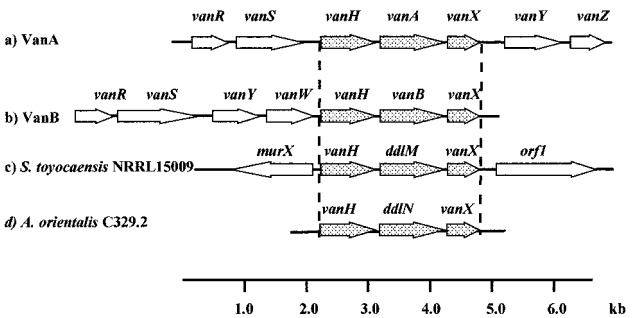

FIG. 1. Comparison of glycopeptide resistance gene clusters from VRE and glycopeptide antibiotic-producing organisms.

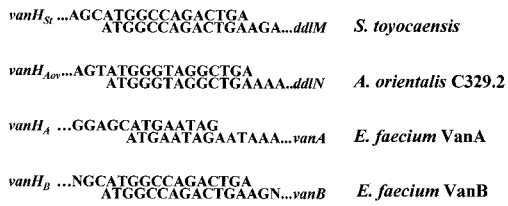

FIG. 2. Overlap of *vanH* and *vanA* homolog genes.

No genes encoding the homologs of the two-component regulatory elements (VanR and VanS) of VRE, the D,D-carboxypeptidase (VanY), or the vancomycin-resistant enterococcus-associated proteins of unknown function (VanW or VanZ) were found in the DNA flanking the *vanHAX* cluster in either organism, although we did not explicitly screen for these and they may be located elsewhere in the chromosome. The 5′ region immediately upstream of $vanH_{st}$ contains an ORF which encodes a putative D-Ala-D-Ala-adding enzyme (MurF) homolog, which we have designated MurX (23), and part of a gene homologous to *femA* which is required for the synthesis of the penta-Gly bridge that joins the peptidoglycan peptide strands and is associated with high-level methicillin resistance in staphylococci (13).

A 1.6-kb ORF, designated ORF1, is located 195 bp downstream from $vanX_{st}$. This ORF has associated promoter −30 and −10 sequences and thus does not appear to be part of a $vanH_{st}$-*ddM*-$vanX_{st}$ polycistronic message. The ORF1 amino acid sequence shows a high degree of homology [P(N)10^{-170} to 10^{-42} by BLAST search] to other bacterial proteins of unknown function. Analysis against the PROSITE database did not reveal any distinguishing or revealing features. Further sequencing of 2 kb of DNA downstream of ORF1 did not reveal any genes encoding proteins which could obviously be ascribed to peptidoglycan or glycopeptide antibiotic biosynthesis.

No complete ORFs were detected in the regions flanking the *vanHAX* cluster from *A. orientalis*, although only approximately 800 bp of flanking DNA was sequenced.

Putative streptomycete −20 (CGGGC) and −10 (CACA TA) promoter sequences (36) are located upstream of $vanH_{st}$. No additional promoter sequences were found upstream of *ddlM* or $vanX_{st}$; thus, these three genes appear to be driven by a single promoter, as is the case with the sequences of VRE. Putative −20 (CGGGG) and −10 (CCCATA) promoter sequences were also identified in the *A. orientalis* C329.2 cluster. Additionally, a possible terminator sequence located between bases 3228 and 3273 was identified.

The $vanH_{st}$ gene terminates 10 bases within the predicted 5′ end of the *ddlM* gene, and similarly, $vanH_{aov}$ also terminates 10 bases within *ddlN* (Fig. 2). These observations directly parallel the arrangement of $vanH_A$ and *vanA* in Tn*1546*, where the $vanH_A$ termination codon begins 5 bases into the *vanA* gene (3) and in the VanB gene cluster where the $vanH_B$ termination codon similarly begins 5 bases into the *vanB* gene (Fig. 2) (17).

Sequence analysis of *vanH* and *vanX* from glycopeptide producing organisms. The predicted amino acid sequence of $VanH_{st}$ and $VanH_{aov}$ clearly identify them as D-LDHs. Amino acid residues which are thought to participate in catalysis based on site-directed mutagenesis studies (21, 37, 38) of D-LDHs, as well as the three-dimensional structure of the *Lactobacillus pentosus* D-LDH (35), are conserved in $VanH_{st}$ and $VanH_{aov}$. For example, a conserved Arg (Arg237 in $VanH_{st}$ and Arg255 in $VanH_{aov}$) is predicted to bind to the carboxylate and polarize the ketone carbonyl for reduction, and an invariant His

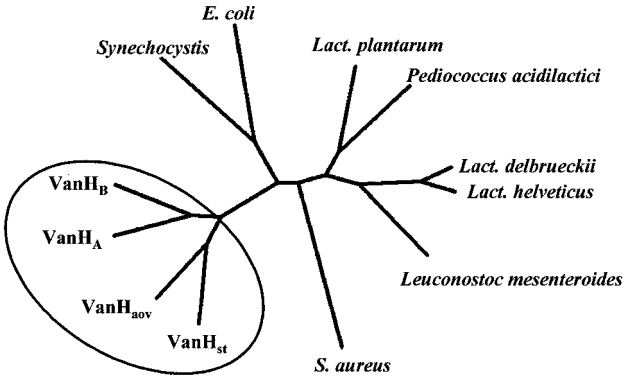

FIG. 3. Phylogenetic relationship among VanH homologs as calculated by the CLUSTAL W method (39). The tree was generated with the PHYLIP program Drawtree (19). *Lact.*, *Lactobacillus.*

(His298 in $VanH_{st}$ and His316 in $VanH_{aov}$) is predicted to act as an active-site general acid donating a proton during pyruvate reduction. Protein sequence alignment of $VanH_{st}$ and $VanH_{aov}$ with the VanH enzymes from VRE yielded overall amino acid identities of 51 to 55%, while alignment with other D-LDHs resulted in identities ranging from 26 to 31%. Phylogenetic analysis clearly places all the VanH enzymes in a distinct branch of the D-LDH family (Fig. 3), an observation which parallels the clustering of DdlM and DdlN with VanA and VanB within the D-Ala-D-Ala ligase family (Fig. 4) (23).

Phylogenetic analysis of the D-Ala-D-Ala ligases indicates four major groups (Fig. 4): the A and B groups, which are bona fide D-Ala-D-Ala ligases but which also contain the D-Ala-D-Lac ligases from lactic acid bacteria; the VanC group, which are D-Ala-D-Ser ligases and which confer constitutive low-level vancomycin resistance in several organisms such as *Enterococcus gallinarum* and *Enterococcus casseliflavus* (14, 28); and the VanA group, which consists of the D-Ala-D-Lac ligases from VRE and glycopeptide-producing organisms.

Amino acid sequence alignment of $VanX_{st}$ and $VanX_{aov}$ with the homologs from VRE confirms the presence of the Zn^{2+} binding ligands His116, Asp123, and His184 as well as the putative active site base Glu181 (25) (Fig. 5). The $VanX_{st}$

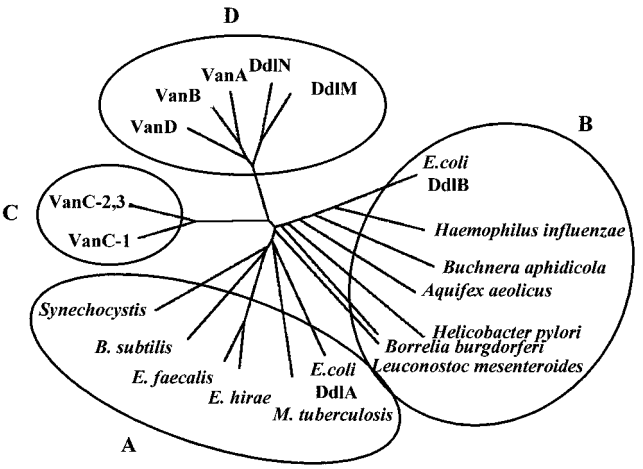

FIG. 4. Phylogenetic relationship among D-Ala-D-Ala ligase homologs as calculated by the CLUSTAL W method (39). The tree was generated with the PHYLIP program Drawtree (19). The four major branches of the tree are labeled A, B, C, and D.

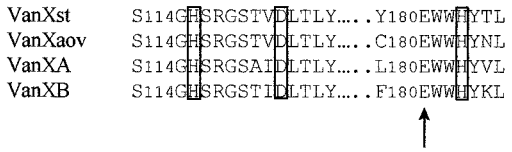

FIG. 5. Overlap of the Zn^{2+} binding regions of VanX proteins. The alignment was as calculated by the CLUSTAL W method (39). The Zn^{2+} ligands are boxed, and the putative active site base is indicated with an arrow.

and $VanX_{aov}$ proteins show 61 to 63% identity (>75% similarity) to the homologous proteins in VRE.

Identification of *vanHAX* gene clusters in other glycopeptide antibiotic-producing organisms. Several other glycopeptide-producing organisms were screened for the presence of the *vanHAX* genes by two methods. First, a part of the gene cluster from *A. orientalis* C329.2 was amplified and used to probe total DNA that had been isolated from several glycopeptide producers and digested to completion with *Bam*HI. A single hybridizing band was observed in producers of chloro-eremomycin, ristocetin, vancomycin, and teicoplanin-avoparcin (Fig. 6A), indicating that these organisms harbor similar genes. Second, a PCR screen was developed with degenerate primers complementary to conserved sequences in VanH and VanX. The presence of a *vanHAX* gene cluster should give rise to an amplified product of approximately 2.7 kb. By using total DNA from several glycopeptide-producing organisms, a band of the expected size was observed in all organisms tested, and the sequence represented by this band was confirmed to encode the *vanHAX* cluster by Southern hybridization with the *A. orientalis* C329.2 *van* cluster probe (Fig. 6B). Thus, this gene cluster appears to be ubiquitously present in glycopeptide-producing bacteria.

Implications of the *van* gene cluster in glycopeptide-producing organisms. In addition to the considerable similarity at the amino acid level noted above, the orientation and alignment of the $vanH_{st}$, *ddlM*, and $vanX_{st}$ genes and the $vanH_{aov}$, *ddlN*, and $vanX_{aov}$ genes in the glycopeptide producers is identical to the orientation of the homologous genes in VRE (Fig. 1). There are several implications of these findings. First, the fact that glycopeptide producers possess this gene cluster implies that they use a glycopeptide resistance mechanism which is similar to that found in VRE. Second, the presence of the *vanX* D,D-dipeptidase gene suggests that at some point during growth, the organisms switch from producing the conventional D-Ala-D-Ala peptidoglycan terminus to producing D-Ala-D-Lac. This is supported by our recent isolation of a dedicated D-Ala-D-Ala ligase which is incapable of D-Ala-D-Lac synthesis from *S. toyocaensis* NRRL 15009 cultures that are not producing the A47934 glycopeptide antibiotic (24).

More significant, however, are the evolutionary implications of these results. Phylogenetic analysis reveals that the gene products from *S. toyocaensis* NRRL 15009, *A. orientalis* C329.2, and VRE form distinct subfamilies of D-Ala-D-Ala ligases and D-LDHs. Furthermore, the spatial arrangements of the genes are maintained in both the antibiotic-producing and clinically resistant organisms, consistent with a common ancestry. Additionally, the signature overlap of the 5′ end of the *vanA* and homologous genes and the 3′ end of the *vanH* genes is conserved. Thus, the cumulative evidence indicates that the origin of clinically relevant vancomycin resistance lies within the glycopeptide-producing organisms and not the lactic acid bacteria, which also possess D-LDH and D-Ala-D-Lact ligase enzymes but whose enzymes are of a distinct lineage.

The G+C contents of the *S. toyocaensis* NRRL 15009 and *A. orientalis* C329.2 glycopeptide resistance genes are 65.3 and 63.6%, respectively, while the genes of VRE are less purine rich, with 44 and 49% purines for the VanA and VanB clusters, respectively. This weighs against recent direct transfer of the genes from either *S. toyocaensis* NRRL 15009 or *A. orientalis* C329.2 to enterococci, although other glycopeptide-producing organisms, with different G+C contents, may have served as donor. We note that the G+C contents of the VRE *vanH*, *vanA*/*B*, and *vanX* elements are 5 to 10% higher than those of the adjacent *vanR*, *vanS*, *vanY*, and *vanZ* (or *vanW*) genes, consistent with mobilization of the *vanH-vanA*/*B-vanX* genes as a unit from another source into a context with appropriate control elements. It is also interesting that a difference in G+C content is also found in the flanking DNA of the gene clusters cloned from the glycopeptide-producing organisms, but here the flanking sequences show higher G+C contents: 69.9% for *S. toyocaensis* NRRL 15009 and 68.4% for *A. orientalis* C329.2. Thus, the resistance cluster may have been acquired by these organisms as well. On the basis of G+C content analysis and in the absence of more sequence information from other glycopeptide antibiotic-producing organisms, the source of the *vanHAX* cluster in VRE is not likely to be an actinomycete.

Alternatively, the resistance genes may have been acquired by non-glycopeptide-producing soil organisms as a method of defense, probably originally from a glycopeptide-producing organism, and then mobilized and passed on to other organisms, thus eventually diluting the original G+C content bias. It has been observed in *vanA*-harboring *Oerskovia* (G+C content of 70 to 75 mol%) that two of the three nucleotide mutations present in its version of *vanA* serve to increase the G+C content (30). Whatever the mechanism of mobilization of the *vanHAX* cluster, if it originated in antibiotic-producing organisms, the transfer was not a recent event. However, the use of vast quantities of the glycopeptide antibiotic avoparcin over the past two decades in European agriculture may have provided the selective pressure for this mobilization or the enrichment of resistant organisms in the environment. The fact that enterococci harboring the *vanA* gene cluster on transposons such as Tn*1546* can be isolated from farm environments in Europe (5, 7) but not the United States (10, 26) is telling. Furthermore, the observation that VRE first emerged in Eu-

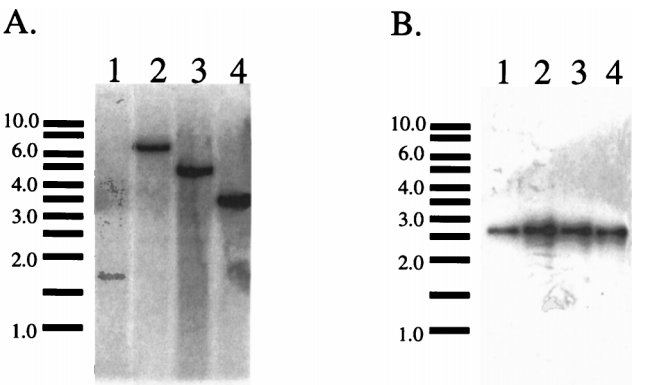

FIG. 6. The presence of the *vanHAX* gene cluster in glycopeptide-producing organisms. (A) Southern blot of complete *Bam*HI digests of *A. orientalis* 18098 (chloro-eremomycin producer) (lane 1), *A. orientalis* subsp. *lurida* (ristocetin producer) (lane 2), *A. coloradensis* subsp. *labeda* (teicoplanin and avoparcin producer) (lane 3), and *A. orientalis* C329.2 (vancomycin producer) (lane 4). The blot was probed with a portion of the *ddlN* gene amplified from *A. orientalis* C329.2. (B) Results of PCR amplification of the *vanHAX* cluster from glycopeptide-producing organism. The lane assignments and the blot probe are the same as described for panel A. Numbers to the left of the blots are in kilobases.

rope may therefore not be a coincidence, although another reservoir for VRE in the United States cannot be ruled out (26). For example, there has been a dramatic rise in the use of vancomycin in clinical settings since the early 1980s, especially in the United States (from 2,000 kg/year in 1984 to 11,200 kg/year in 1996 [20]), and this increase in antibiotic use must be considered when discussing the rise in the prevalence of VRE over the past decade. As an additional cautionary note, it has recently been demonstrated that many antibiotic formulations are contaminated with DNA from producing organisms (41); thus, a possible source for the dissemination of resistance genes exists, possibly as a contaminant in agricultural or clinical glycopeptide antibiotic formulations. The results described here should emphasize that caution must be employed in our use of antibiotics that, while not themselves clinically useful, have the potential to select for donors of resistance genes or preexisting resistant populations.

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