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Glycopeptide Resistance *vanA* Operons in *Paenibacillus* Strains Isolated from Soil

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The sequence and gene organization of the *van* operons in vancomycin (MIC of >256 μg/ml)- and teicoplanin (MIC of ≥32 μg/ml)-resistant *Paenibacillus thiaminolyticus* PT-2B1 and *Paenibacillus apiarius* PA-B2B isolated from soil were determined. Both operons had regulatory (*vanR* and *vanS*), resistance (*vanH*, *vanA*, and *vanX*), and accessory (*vanY*, *vanZ*, and *vanW*) genes homologous to the corresponding genes in enterococcal *vanA* and *vanB* operons. The $vanA_{PT}$ operon in *P. thiaminolyticus* PT-2B1 had the same gene organization as that of *vanA* operons whereas $vanA_{PA}$ in *P. apiarius* PA-B2B resembled *vanB* operons due to the presence of *vanW* upstream from the *vanHAX* cluster but was closer to *vanA* operons in sequence. Reference *P. apiarius* strains NRRL B-4299 and NRRL B-4188 were found to harbor operons indistinguishable from $vanA_{PA}$ by PCR mapping, restriction fragment length polymorphism, and partial sequencing, suggesting that this operon was species specific. As in enterococci, resistance was inducible by glycopeptides and associated with the synthesis of pentadepsipeptide peptidoglycan precursors ending in D-Ala-D-Lac, as demonstrated by D,D-dipeptidase activities, high-pressure liquid chromatography, and mass spectrometry. The precursors differed from those in enterococci by the presence of diaminopimelic acid instead of lysine in the peptide chain. Altogether, the results are compatible with the notion that *van* operons in soil *Paenibacillus* strains and in enterococci have evolved from a common ancestor.

The glycopeptide antibiotics vancomycin and teicoplanin are drugs of primary importance for the treatment of hospital infections caused by multiresistant gram-positive bacteria such as *Staphylococcus aureus*, enterococci, and *Clostridium difficile*. Glycopeptides act by inhibiting cell wall synthesis (29), and resistance is due to the synthesis of peptidoglycan precursors with low affinity for these antibiotics (3, 32). Six types of *van* operons conferring glycopeptide resistance have been described in enterococci based on gene sequence and organization (25, 31). The various operons are designated according to the name of the gene, which encodes either a D-Ala:D-Lac (*vanA*, *vanB*, and *vanD*) or a D-Ala:D-Ser (*vanC*, *vanE*, and *vanG*) ligase for synthesis of peptidoglycan precursors with low affinity for glycopeptides. The operons coding for a D-Ala:D-Lac ligase contain genes for a two-component regulatory system (*vanR* and *vanS*), three resistance genes (*vanH*, *vanA* or *vanB* or *vanD*, and *vanX*), an accessory gene (*vanY*), and other genes with unknown functions (*vanW* or *vanZ*).

The most common glycopeptide resistance gene clusters in clinical enterococci are *vanA* and *vanB*. Both operons are associated with transposable elements, i.e., *vanA* with Tn*1546* (2) and *vanB* with Tn*1547* (28) and Tn*1549*-Tn*5382* (7, 13). The recent acquisition of the *vanA* gene cluster by *S. aureus* (8) confirms that these genes are able to spread across bacterial genera. Previous indications of intergenic horizontal transfer of *van* operons were provided by the finding of *vanA* in *Bacillus circulans* (18), *Oerskovia turbata*, and *Arcanobacterium haemolyticum* (26) and of *vanB* in *Streptococcus bovis* (27) and anaerobic bacilli (4). All these bacteria were isolated from clinical specimens, mainly stools, suggesting that dissemination of glycopeptide resistance may occur in the intestinal microflora of patients.

It has been proposed that *van* operons originate from glycopeptide-producing organisms (14, 20, 23). This hypothesis is based on the observation that the glycopeptide producers contain resistance gene clusters homologous to those in human-pathogenic bacteria. However, these clusters have relatively low similarity with the *vanA* and *vanB* operons (from 54 to 64% predicted amino acid identity), lack the two-component regulatory systems, and do not appear to be transferable under laboratory conditions. Finding of the *vanF* operon in *Paenibacillus popilliae* (24) and the recent recovery of genes homologous to *vanA* in other *Paenibacillus* species (15) have raised interest about a possible role of this genus in the dissemination of glycopeptide resistance.

In this study, we have characterized the organization of the *van* operons in two glycopeptide-resistant *Paenibacillus* strains isolated from soil and known to harbor putative D-Ala:D-Lac ligase genes flanked by *vanH*- and *vanX*-like genes (15). The two operons were closely related to *vanA* on the basis of both gene sequence and organization. Glycopeptide resistance was inducible by vancomycin and teicoplanin and resulted from the synthesis of peptidoglycan precursors containing diaminopimelic acid and ending in D-Ala-D-Lac.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains *Paenibacillus thiaminolyticus* PT-2B1 and *Paenibacillus apiarius* PA-B2B were isolated from soil and identified

TABLE 1. Oligodeoxynucleotides used for TAIL and long PCR

Primer	Sequence (5′ to 3′)	Position[a]
Tpf1	GTTTCAAAAGGATACGTGGC	7419/7438
Tpr1	GCACATGACATCCAAATCCC	5625/5606
Tpf2	CTTTATCGATTAGACACGGG	7482/7501
Tpr2	ATGTCGCGCAGTACCTTGCC	5548/5529
Tpf3	CAAAATCGCAGACGTTTGCG	7587/7606
Tpr3	ATAATCGGCAACGCTATCCG	5438/5419
Ad1	TGWGNAGWANCASAGA	NA[b]
Lpf1	TCCAGAGAAGGATATGAC	5017/5034
Lpf2	GAACTGGCATTTCGCAAGGC	1998/2017
Lpr1	GCCCCCATTTCTTGGTAAAG	8604/8585
Lpf3	GCTCCCATCATAGTCAAT	−190/−173
Lpr2	ACTGCGTTTTCAGAGCCTTT	6839/6820
Patf	TATCCTACGTGGATAAGCGG	2948/2967
Patr	GGGCCAAACTTGAGCACGAT	9178/9159

[a] Nucleotide numbering begins at the start site (+1) of the transposase gene preceding the $vanA_{PT}$ operon in *P. thiaminolyticus* PT-2B1 (accession no. DQ018710).

[b] NA, not applicable.

by sequencing 16S rRNA genes (15). Reference strains *P. apiarius* NRRL B-4299 and NRRL B-4188 were isolated from dead bees and obtained from the collection of the National Center for Agricultural Utilization Research, U.S. Department of Agriculture. Strains were grown at 30°C in brain heart infusion (BHI) broth or on BHI agar (Difco Laboratories, Detroit, Mich.). The MICs of vancomycin and teicoplanin were determined by Etest (AB Biodisk, Solna, Sweden) after 48 h of incubation on Mueller-Hinton agar at 28°C.

TAIL PCR. Thermal asymmetric interlaced (TAIL) PCR (19) was used to clone the 5′ and 3′ regions flanking the *vanHAX* clusters in strains PT-2B1 and PA-B2B and to determine their sequence. Three PCR steps were performed with a specific primer targeting the known sequence and arbitrary degenerate primer AD1 (Table 1 and Fig. 1). The target sequences of the anchor primers used in the second and third steps were selected at decreasing distance from the ends of the known sequence in order to obtain PCR products of slightly decreasing size. Total DNA obtained by use of the High Pure PCR template kit (Roche Diagnostics, Mannheim, Germany) was used as a template in the first PCR. The PCR mixture contained 0.15 μM specific primer (Table 1), 5 μM AD1 primer, 200 μM of each deoxynucleoside triphosphate, 1× PCR buffer with 22.5 mM $MgCl_2$, and 2.5 U of the enzyme mix supplied by the Expand Long Template PCR system (Roche Diagnostics). PCR conditions were as previously described (9). The products obtained from the first and second PCRs were diluted 10^5 and 10 times, respectively, before being used as DNA templates in the following PCR. The DNA bands corresponding to the second and third PCRs, which had the length decrease expected from the positions of the specific primers, were purified using the QIAGEN PCR purification kit (QIAGEN S.A., Courtaboeuf, France) and sequenced.

Cloning of the TAIL PCR products into *Escherichia coli*. The purified PCR products were cloned in plasmid PCR2.1 into *E. coli* TOP10F′ using the TA cloning kit (Invitrogen Corporation, Carlsbad, CA). White colonies were isolated from BHI agar containing ampicillin (50 μg/ml) and 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-Gal; 80 μg/ml). Plasmid DNA was isolated according to the method of Birnboim and Doly (6) and digested with EcoRI (Invitrogen Corporation) to screen for the presence of an insert.

Nucleotide sequencing. Plasmid DNA or PCR products were labeled using a dye-labeled ddNTP Terminator Cycle sequencing kit (Beckman Coulter UK Ltd.) and sequenced with a CEQ 2000 automated sequencer (Beckman). Sequences obtained from cloned TAIL PCR products were confirmed by sequencing PCR products obtained from total DNA using specific primers.

Computer analysis of sequences. Sequences were aligned, translated, and analyzed using DNA Strider 1.3 (CEA/Saclay, Gif-sur-Yvette, France). Comparison with known genes and proteins was carried out using BlastN and BlastX, available at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST/). Nucleotide identities and fractional GC contents were calculated using EMBOSS Align (gap open 10 and gap extend 0.5) and EMBOSS Geecee, respectively (http://www.ebi.ac.uk/emboss/).

Restriction fragment length polymorphism (RFLP) analysis. Specific primers were designed to amplify the entire operons or portions of them (Table 1; Fig. 1). Long PCR (Expand Long Template PCR system; Roche Diagnostics) was carried out using the following conditions: 2 min of denaturation at 94°C and 30 cycles of 10 s at 94°C, 30 s at 50°C, and 4 to 6 min at 68°C depending on the size of the product, followed by final extension at 68°C for 7 min. The long PCR products were partly sequenced and digested for 1 h at 37°C with NaeI (Biolabs, Saint Quentin Yvelines, France) and DdeI (Invitrogen Corporation) for RFLP analysis.

Mating experiments. Transfer of glycopeptide resistance was attempted from *Paenibacillus* spp. to *Enterococcus faecium* BM4105 and J64/3 resistant to ri-

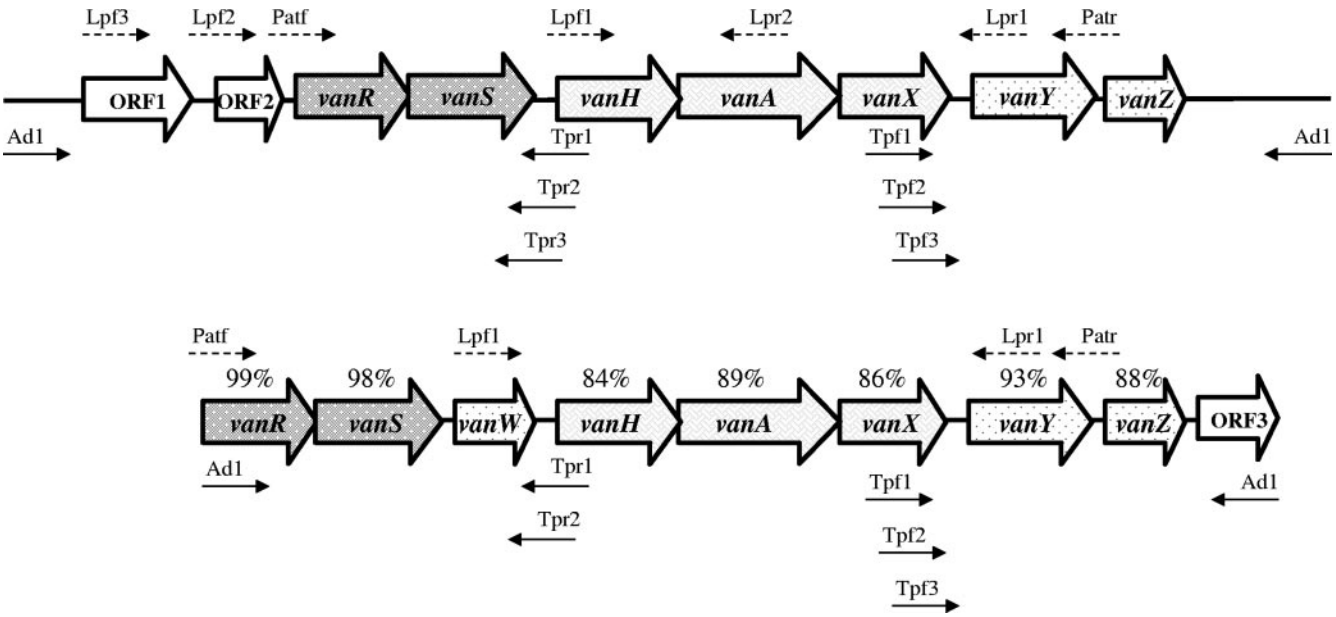

FIG. 1. Schematic representation of the *van* operons in *P. thiaminolyticus* PT-2B1 (top) and *P. apiarius* PA-B2B (bottom) and of the PCR primers used for their characterization. The percentages refer to the levels of identity between the corresponding genes in the two operons. Open arrows represent coding sequences and indicate the direction of transcription. Solid and dashed small arrows indicate the primers used for TAIL and long PCR, respectively.

fampin and fusidic acid. Donor and recipient strains were inoculated on BHI agar or on 0.45-μm-pore-size nitrocellulose filter (Millipore) placed on BHI agar. After 2 days of incubation at 28°C, bacteria were resuspended in 1 ml of BHI broth and plated on Slanetz agar (Oxoid) supplemented with vancomycin (10 μg/ml), rifampin (50 μg/ml), and fusidic acid (10 μg/ml). Longer mating time periods (up to 10 days) and selective enrichment in azide dextrose broth (Merck) supplemented with the three antibiotics were employed for enhancing detection of low-frequency transfer.

Assays of D,D-dipeptidase (VanX) and D,D-carboxypeptidase (VanY) activities. The activities of VanX (cytoplasmic D,D-dipeptidase) and VanY (membrane-associated D,D-carboxypeptidase) were assayed by determining the amount of D-Ala released by hydrolysis of dipeptide D-Ala-D-Ala (6.56 mM) and pentapeptide UDP-MurNAc-L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala (5 mM), respectively. Cytoplasmic and membrane extracts were obtained as previously described (9). Measurement was obtained through coupled indicator reactions using D-amino acid oxidase and horseradish peroxidase (1, 30). Specific activity was defined as the number of nanomoles of product formed at 37°C per minute per milligram of protein contained in the extract.

Peptidoglycan precursors. UDP-MurNAc-L-Ala-γ-D-Glu-*meso*-A_2pm-D-Ala-D-Ala (UDP-MurNAc-pentapeptide) was obtained as previously described (11). UDP-MurNAc-L-Ala-γ-D-Glu-*meso*-A_2pm-D-Ala (UDP-MurNAc-tetrapeptide) was generated by removal of the C-terminal D-alanine of UDP-MurNAc-pentapeptide by action of the D,D-carboxypeptidase from *Actinomura* sp. strain R39 as described previously for the lysine-containing UDP-MurNAc-tetrapeptide (5).

Extraction and quantification of the UDP-MurNAc peptide precursors. The UDP-MurNAc peptide precursors from mid-log-phase cells grown without and with vancomycin (32 μg/ml) were extracted according to the method of Reynolds et al. (33) and analyzed by high-pressure liquid chromatography (HPLC) as previously described (21).

Amino acid analysis. Amino acid and amino sugar compositions were determined with a Hitachi model L8800 analyzer (ScienceTec, Les Ulis, France) equipped with a 2620MSC-PS column (80 × 4.6 mm). Prior to analysis samples were hydrolyzed in 6 M HCl at 95°C for 16 h.

Mass spectrometry analysis. Matrix-assisted laser desorption ionization–time of flight mass spectra were recorded on a PerSeptive Voyager-DE STR instrument (Applied Biosystems, Foster City, CA) in the reflectron mode with delayed extraction. The samples were prepared as follows: 1 μl of a solution of compound in water at 20 pmol/μl was deposited on the plate and mixed with 1 μl of a 10-mg/ml solution of 2,5-dihydroxybenzoic acid in 0.1 M citric acid. After evaporation, desorption and ionization were obtained by pulses from a 337-nm nitrogen laser. Spectra were recorded in the negative ion mode at an acceleration voltage of −20 kV and an extraction delay time of 200 nanoseconds. A mixture of UDP-MurNAc, UDP-MurNAc-L-Ala-D-Glu, and UDP-MurNAc-pentapeptide was used as an external calibrant.

Nucleotide sequence accession numbers. The sequences were submitted to GenBank and assigned the following accession numbers: DQ018710 ($vanA_{PT}$ operon in PT-2B1) and DQ018711 ($vanA_{PA}$ operon in PA-B2B).

RESULTS

Glycopeptide resistance phenotypes. *P. thiaminolyticus* PT-2B1 and *P. apiarius* PA-B2B were resistant to high concentrations (MIC of >256 μg/ml) of vancomycin. An atypical growth response by PT-2B1 was observed with Etest, since the strain grew at high vancomycin concentrations (up to 256 μg/ml) but showed a thin inhibition zone at concentrations between 3 and 4 μg/ml. A similar observation was made by disk agar diffusion, the strain growing at the point of contact with the 30-μg vancomycin disk but displaying a thin zone of inhibition at a distance from the disk (Fig. 2). PA-B2B displayed reduced growth at vancomycin concentrations above 4 μg/ml in the Etest but grew in liquid medium containing higher concentrations of the antibiotic. The MIC of teicoplanin was higher for PT-2B1 (>256 μg/ml) than for PA-B2B (32 μg/ml).

Transfer of glycopeptide resistance from PT-2B1 or PA-B2B to *E. faecium* BM4105 and J64/3 could not be obtained, even when the mating mixtures were incubated for 10 days and selective enrichment was used for detection of transconjugants.

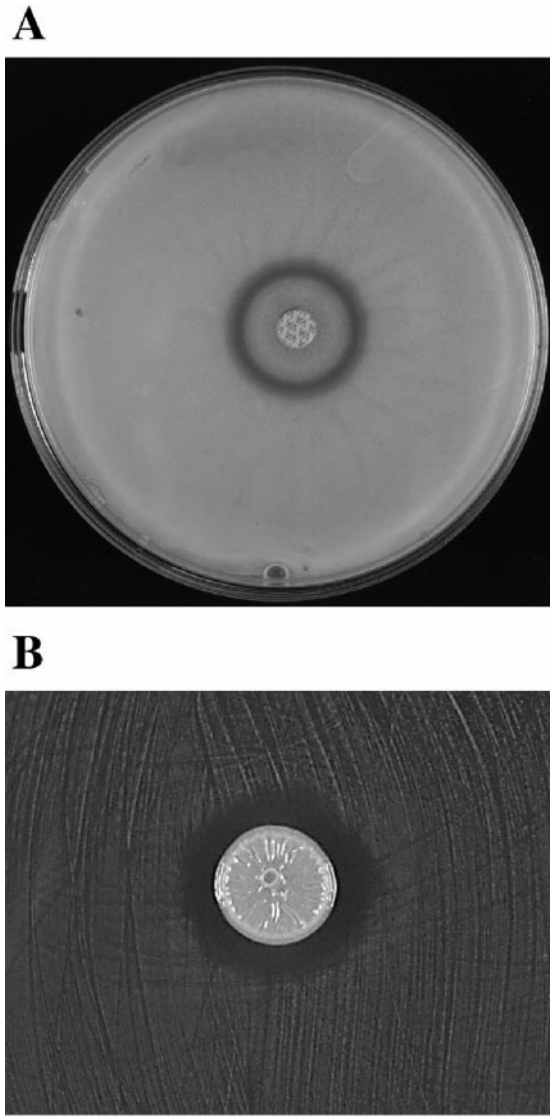

FIG. 2. Phenotypic characteristics of *P. thiaminolyticus* PT-2B1. A. Atypical inhibition zone centered on a 30-μg vancomycin disk. B. Inhibition of *E. faecium* BM4105 by a colony of PT-2B1.

Strain PT-2B1 inhibited the growth of *E. faecium* as indicated by the absence of growth around colonies of the donor strain (Fig. 2).

Organization of the *van* operon in PT-2B1. Two TAIL PCR products of approximately 7 kb each were obtained from the upstream and downstream regions of the *vanHAX* cluster in PT-2B1 (Fig. 1). Sequencing revealed the same organization as in enterococcal *vanA* operons, with the *vanHAX* resistance gene cluster preceded by genes (*vanR* and *vanS*) for a two-component regulatory system and followed by a gene (*vanY*) coding for a putative D,D-carboxypeptidase. The percentage of identity to the corresponding genes in the *vanA* operon of Tn*1546* in *E. faecium* BM4147 (2) varied between 83% and 94% (Fig. 3). Homology with the *vanA* operon of Tn*1546* was also observed in the *vanS-H* (85%) and *vanX-Y* (74%) intergenic regions. As in Tn*1546*, *vanS* overlapped with *vanR* over

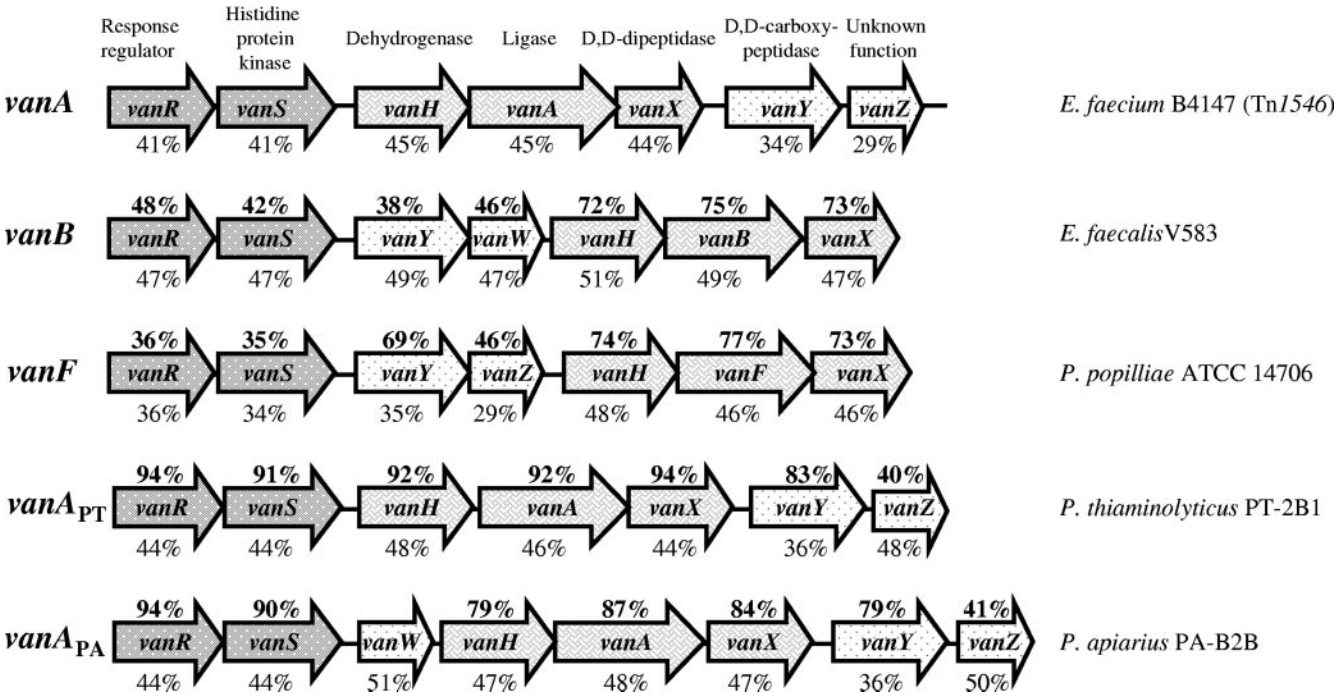

FIG. 3. Organization of *vanA* operons (*E. faecium* B4147, accession no. M97297), *vanB* (*E. faecalis* V583, accession no. U35369), *vanF* (*P. popilliae* ATCC 14706, accession no. AF155139), $vanA_{PT}$ (*P. thiaminolyticus* PT-2B1, accession no. DQ018710), and $vanA_{PA}$ (*P. apiarius* PA-B2B, accession no. DQ018711). For every gene, identity to the corresponding gene of the *vanA* operon in Tn*1546* and the GC content are indicated above and below the gene, respectively. Arrows indicate extent of the genes and direction of transcription.

23 bp and *vanA* with *vanH* over 8 bp. Due to the high similarity with *vanA* operons in both gene sequence and organization, the cluster was designated $vanA_{PT}$. All the genes had the same length as the corresponding genes in BM4147, except for $vanS_{PT}$, which had a 6-bp insertion at the beginning of the gene, and $vanY_{PT}$, which was shorter (885 versus 912 bp). A sequence homologous to *vanZ* (40% identity) was located downstream from $vanY_{PT}$. The region upstream from $vanR_{PT}$ contained two open reading frames, ORF1 and ORF2. ORF1 (1,263 bp) encoded a hypothetical protein with 39% identity and 58% similarity to a putative transposase in *Lactococcus lactis* (accession no. AAC72261) (34). The predicted amino acid sequence encoded by ORF2 (405 bp) had 35% identity and 48% similarity with an acetyltransferase of the GCN5-related *N*-acetyltransferase (GNAT) superfamily (37) present in the genome of *Bacillus cereus* (accession no. AAP09041) and of other *Bacillus* spp.

Organization of the *van* operon in PA-B2B. TAIL PCR products of approximately 3 and 4 kb were obtained from the regions upstream and downstream from the *vanHAX* cluster in PA-B2B (Fig. 2). The operon in this strain was a hybrid between *vanA* and *vanB* operons based on the relative gene organization: *vanW* was located upstream from *vanHAX* as in *vanB* operons whereas *vanY* was positioned downstream from *vanHAX* as in *vanA* operons (Fig. 3). However, the operon was designated $vanA_{PA}$ since the sequence of the genes was closer to that in *vanA* operons. The percentages of identity to the corresponding genes in the *vanA* operon of Tn*1546* varied between 79% and 94% (Fig. 3). The $vanW_{PA}$ gene was 75% identical to *vanW* in the *vanB* operon of reference *Enterococcus faecalis* V583 (10). The typical overlaps between *vanR* and *vanS* and between *vanH* and *vanA* were also present in this operon. Similarly to $vanS_{PT}$, a 12-bp insertion was found at the beginning of $vanS_{PA}$ relative to *vanS* in Tn*1546*, and $vanY_{PA}$ was followed by a putative $vanZ_{PA}$ (Fig. 3). The $vanR_{PA}$ and $vanS_{PA}$ regulatory genes had sequences nearly identical to those of $vanR_{PT}$ and $vanS_{PT}$ whereas the remaining genes in $vanA_{PA}$ were less closely related to the corresponding genes in $vanA_{PT}$ (Fig. 1). The region downstream from $vanY_{PA}$ ended with a partial open reading frame (ORF3) homologous to *btrU* (79% identity), a gene in the aminoglycoside butirosin biosynthetic operon of *B. circulans* (accession no. CAD41946) (22).

PCR mapping and RFLP analysis of long PCR products. PCR products of the expected size were obtained using specific primers (Table 1 and Fig. 1). Primers Patf and Patr allowed amplification of the entire operons in PT-2B1 and PA-B2B as well as in the two reference *P. apiarius* strains, NRRL B-4299 and NRRL B-4188. The *van* operons in the three *P. apiarius* strains had the same size and were indistinguishable based on RFLP analysis (data not shown) and partial sequencing of the long PCR products.

D,D-Peptidase activities. Vancomycin and teicoplanin induced D,D-dipeptidase (VanX) and D,D-carboxypeptidase (VanY) activities in both strains (Table 2). No baseline enzymatic activity could be detected in the absence of antibiotic. There was good correlation between the D,D-peptidase and the nature of late peptidoglycan precursors. In particular, VanY activity was associated with an increase in UDP-MurNAC-tetrapeptide.

TABLE 2. D,D-Dipeptidase (VanX) and D,D-carboxypeptidase (VanY) activities in cytoplamic and membrane extracts from *Paenibacillus* strains[a]

Strain and glycopeptide	Glycopeptide concn (μg/ml)	Activity (nmol/min/mg protein)	
		Cytoplamic extract (VanX activity)	Membrane extract (VanY activity)
PA-B2B			
Vancomycin	4	108 ± 21	14 ± 4
	64	142 ± 7	19 ± 9
Teicoplanin	8	24 ± 2	1 ± 0.5
	32	86 ± 9	5 ± 2
PT-2B1			
Vancomycin	2	1 ± 0.5	37 ± 10
	64	13 ± 4	81 ± 2
Teicoplanin	4	1.5 ± 0.6	1 ± 0.5
	32	22 ± 4	53 ± 8

[a] The reported mean values were calculated based on three separate measurements.

HPLC and mass spectrometry of peptidoglycan precursors. The crude cell wall extract from PT-2B1 was composed of ca. 25% A_2pm-containing peptidoglycan. The main precursor peak detected in the HPLC profile of the cytoplasmic extract from untreated cells was eluted at 33 min (Fig. 4) and identified as A_2pm-containing UDP-MurNAc-pentapeptide by its coelution with an authentic sample under two different HPLC conditions.

In the extract from vancomycin-treated PT-2B1 cells, the UDP-MurNAc-(A_2pm) pentapeptide peak was practically absent and replaced by two major peaks eluted at 18 and 50 min, respectively (Fig. 4). Both peaks were recovered and purified again by HPLC. The 18-min peak coeluted with an authentic sample of A_2pm-containing UDP-MurNAc-tetrapeptide under two different HPLC conditions. This identification was confirmed by mass spectrometry which led to an $[M-H]^-$ ion with an *m/z* ratio of 1,121.13 in agreement with an A_2pm-containing UDP-MurNAc-tetrapeptide, $C_{38}H_{60}N_8O_{27}P_2$, having a monoisotopic mass of 1,122.30 g/mol.

Analysis of the 50-min peak (0.9 Mur, 1 A_2pm, 1.1 Glu, and 1.5 Ala) was compatible with a precursor containing at least a MurNAc-(A_2pm)tetrapeptide moiety. Mass spectrometry analysis led to an $[M-H]^-$ ion with an *m/z* ratio of 1,193.40, which was in agreement with a lactic acid-containing UDP-MurNAc-pentadepsipeptide, $C_{41}H_{64}N_8O_{29}P_2$, having a monoisotopic mass of 1,194.32 g/mol. It was noteworthy that a low level of this UDP-MurNAc-pentadepsipeptide was detectable in cells grown without vancomycin (Fig. 4). Furthermore, both UDP-MurNAc-tetrapeptide and UDP-MurNAc-pentadepsipeptide were also found to be predominant peaks in an extract from vancomycin-treated PA-2B1.

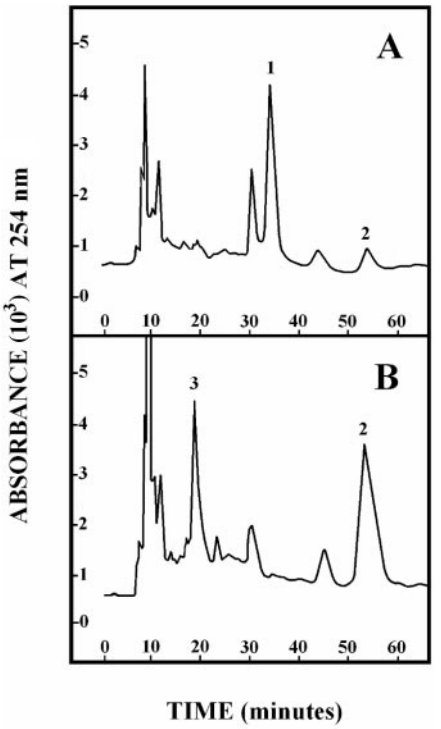

FIG. 4. HPLC analysis of peptidoglycan precursors of *P. thiaminolyticus* PT-2B1 grown without (A) or with (B) vancomycin (32 μg/ml). Samples (one-fifth of the extracts) were applied to a μ-Bondapak C_{18} column (300 × 3.9 mm), and isocratic elution was performed with 0.05 M ammonium phosphate (pH 4.4) at a flow rate of 0.5 ml/min. The main peaks detected by absorbance at 254 nm were identified as UDP-MurNAc-pentapeptide (1), UDP-MurNAc-pentadepsipeptide (2), and UDP-MurNAc-tetrapeptide (3) and quantitated by their uridine content. Peak 1 in panel A, 1.2 nmol; peak 2 in panel A, 0.2 nmol; peak 2 in panel B, 1.5 nmol; peak 3 in panel B, 0.9 nmol.

DISCUSSION

This study shows that the genetic and biochemical basis of glycopeptide resistance in *Paenibacillus* from soil is the same as in enterococci and in other human-pathogenic bacteria. In particular, the glycopeptide resistance operons in *Paenibacillus* have primary sequences and gene organizations very similar to those of enterococcal *vanA* operons (Fig. 3). Furthermore, as in clinical isolates, resistance is inducible by glycopeptides (Table 2) and results from synthesis of peptidoglycan precursors terminating in D-Ala-D-Lac (Fig. 4). The pentadepsipeptide precursors differ from those in glycopeptide-resistant enterococci by the presence of diaminopimelic acid instead of lysine in the peptide chain. To the best of our knowledge, this is the first report of D-Ala-D-Lac-ending pentadepsipeptide precursors containing diaminopimelic acid. Diaminopimelic acid has been previously shown to be a normal constituent of peptidoglycan in various *Paenibacillus* species (17, 38, 39).

Occurrence of *van* operons in members of the genus *Paenibacillus* has been reported in the biopesticide *P. popilliae* ATCC 14706 (24). However, the level of identity with enterococcal operons is markedly lower than those reported in this study and the organization of the *vanF* operon in *P. popilliae* differs from those of *vanA* and *vanB* because of the presence of *vanZ* and *vanY* between the regulatory and the resistance genes (Fig. 3). In contrast, the $vanA_{PT}$ operon in *P. thiaminolyticus* had the same organization as the *vanA* operon (Fig. 3). Furthermore, as opposed to *vanF*, the similarity of $vanA_{PT}$ and $vanA_{PA}$ with enterococcal *vanA* operons was not limited to the resistance genes but extended to *vanR* and *vanS* with respect to both sequence and GC content (Fig. 3).

Irrespective of their sources and times of isolation, the three

P. apiarius strains studied were resistant to glycopeptides and harbored indistinguishable operons. Based on current knowledge, it is unclear if glycopeptide resistance is acquired or intrinsic in *Paenibacillus* species. Unrelated *P. popilliae* isolates have been recently shown to be vancomycin resistant and to carry *vanF* operons (12). However, in a previous study (16), *P. popilliae* strains from Central and South America were reported to be susceptible to vancomycin and genetically divergent from North American isolates, which are vancomycin resistant and include the type strains for the species. The *Paenibacillus* genus has been only recently established (35), and thus, there is scarce information on the phylogenetic diversity within the species. Based on the results of this study it can be concluded that, if resistance is not intrinsic, it has been likely acquired a long time ago by certain genetic lineages, as indicated by the recovery of highly conserved genetic determinants among unrelated *P. apiarius* strains (NRRL B-4188, NRRL B-4299, and PA-B2B) isolated from different sample types (dead bees and soil), countries (United States, United Kingdom, and Denmark), and times (1973, 1975, and 2002).

The highly conserved structure of the *van* operons among various members of the same *Paenibacillus* species raises questions on the function of these gene clusters in environmental bacteria. The *van* operons could protect *Paenibacillus* from glycopeptides produced by actinomycetes in soil. However, it is not known whether glycopeptide production is common in soil and which concentrations can be achieved in situ. Alternatively, the *van* operons could be involved in another biological function and induced by physico-chemical factors other than glycopeptide antibiotics. Genome sequencing of various *Bacillus* species has revealed the presence of a cluster of genes homologous to *vanR*, *vanS*, and *vanY* in the chromosome of these bacteria (Integrated Genomics, www.ergo-light.com), but the role of these genes remains unknown. It has been proposed that the VanY-like D,D-carboxypeptidase in *Bacillus* spp. contributes to sporulation and germination (36). The predicted amino acid identities of putative VanR, VanS, and VanY in *B. cereus* ATCC 14579 (accession no. RZC01688) with the corresponding proteins in *P. thiaminolyticus* PT-2B1 are 43%, 32%, and 55%, respectively.

The close similarity of $vanA_{PT}$ and $vanA_{PA}$ operons with enterococcal *vanA* supports the hypothesis that these gene clusters have evolved from a common ancestor. Most likely, *vanA* operons originated in soil organisms and were subsequently acquired by enterococci. Two arguments suggest that *vanA* operons occurred in *Paenibacillus* before they occurred in enterococci: (i) glycopeptide resistance was first detected in enterococci in 1984 whereas the two *P. apiarius* reference strains studied were isolated in the early 1970s, when the use of glycopeptides was limited in clinical practice, and (ii) while glycopeptide resistance is associated with mobile genetic elements in enterococci, resistance in *Paenibacillus* is apparently chromosomal and intrinsic or at least acquired in very ancient times.

We do not provide any definitive evidence on the chromosomal location of the $vanA_{PT}$ and $vanA_{PA}$ operons. However, the open reading frames located upstream from $vanA_{PT}$ (ORF2) and downstream from $vanA_{PA}$ (ORF3) were homologous to chromosomal genes in other gram-positive bacteria. The chromosomal location of the operons is also supported by the fact that no plasmid of sufficient size to contain the operons was detected in the two strains (data not shown) and by lack of in vitro transfer of glycopeptide resistance.

Despite their similarity to enterococcal *vanA* and *vanB* operons, $vanA_{PT}$ and $vanA_{PA}$ were apparently not associated with any of the transposons previously described in enterococci of the VanA or VanB type (2, 7, 13, 28). Analysis of the region upstream from $vanA_{PT}$ revealed the presence of an open reading frame (ORF1) homologous to a putative transposase gene in *L. lactis* (34). Open reading frames encoding putative transposases have been described upstream from other *van* operons in gram-positive bacilli, such as *vanF* in *P. popilliae* (24) and $vanA_{BA}$ in *B. circulans* (18). However, mobility of the genes has not been demonstrated under laboratory conditions. Thus, further investigation is needed to determine whether *van* operons in bacilli are associated with functional transposable elements and to investigate the possible mechanisms of transfer from bacilli to enterococci.

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