The sequence and gene organization of the \textit{van} operons in vancomycin (MIC of \(\geq 256\) \(\mu\)g/ml)- and teicoplanin (MIC of \(\geq 32\) \(\mu\)g/ml)-resistant \textit{Paenibacillus thiaminolyticus} PT-2B1 and \textit{Paenibacillus apiarius} PA-B2B isolated from soil were determined. Both operons had regulatory (\textit{vanR} and \textit{vanS}), resistance (\textit{vanH}, \textit{vanA}, and \textit{vanX}), and accessory (\textit{vanY}, \textit{vanZ}, and \textit{vanW}) genes homologous to the corresponding genes in enterococcal \textit{vanA} and \textit{vanB} operons. The \textit{van}_{\textit{PT}} operon in \textit{P. thiaminolyticus} PT-2B1 had the same gene organization as that of \textit{vanA} operons whereas \textit{van}_{\textit{PA}} operon in \textit{P. apiarius} PA-B2B resembled \textit{vanB} operons due to the presence of \textit{vanW} upstream from the \textit{vanHAX} cluster but was closer to \textit{vanA} operons in sequence. Reference \textit{P. apiarius} strains NRRL B-4299 and NRRL B-4188 were found to harbor operons indistinguishable from \textit{van}_{\textit{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 \textit{van} operons in soil \textit{Paenibacillus} strains and in enterococci have evolved from a common ancestor.
TABLE 1. Oligodeoxynucleotides used for TAIL and long PCR

| Primer | Sequence (5’ to 3’) | Position
<table>
<thead>
<tr>
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<th></th>
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<tbody>
<tr>
<td>Tp1f</td>
<td>GGTTCACAAAGGATACGTGGC</td>
<td>7419/7438</td>
</tr>
<tr>
<td>Tp1f</td>
<td>GCACATGCATCCATAATCCC</td>
<td>5265/5606</td>
</tr>
<tr>
<td>Tp2f</td>
<td>CTATTACGATTAGACACGGG</td>
<td>7482/7501</td>
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<tr>
<td>Tp2f</td>
<td>ATGTCGCGCAGTACCTTGGCC</td>
<td>5548/5529</td>
</tr>
<tr>
<td>Tp3f</td>
<td>CAAAATCGCAGATTTGGGTC</td>
<td>7583/7606</td>
</tr>
<tr>
<td>Tp3f</td>
<td>AATAATCGGACCGTATCCG</td>
<td>5436/5419</td>
</tr>
<tr>
<td>Ad1</td>
<td>TGWGNAGWANASAGA</td>
<td>NA</td>
</tr>
<tr>
<td>Lp1f</td>
<td>TCCAGAAGAGGATATGAC</td>
<td>5017/5034</td>
</tr>
<tr>
<td>Lp2f</td>
<td>GAACTGCGATTTCGCAAGGC</td>
<td>1998/2017</td>
</tr>
<tr>
<td>Lp1f</td>
<td>GCCCCCATTTCTTGGTAAAG</td>
<td>8604/8585</td>
</tr>
<tr>
<td>Lp3f</td>
<td>GCCTCCTATCATGCTATA</td>
<td>-190/-173</td>
</tr>
<tr>
<td>Lp2r</td>
<td>ACTGCGTTTCCAGAGCTTT</td>
<td>6839/6820</td>
</tr>
<tr>
<td>Patf</td>
<td>TATCTACAGTGTGGATAAGCGG</td>
<td>2948/2967</td>
</tr>
<tr>
<td>Patr</td>
<td>GGGCCAAACTTGAGCACGAT</td>
<td>9178/9159</td>
</tr>
</tbody>
</table>

a Nucleotide numbering begins at the start site (+1) of the transposase gene preceding the vanA, 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). PCR conditions were as previously described (9). The products obtained from the first and second PCRs were diluted 10⁷ 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-indolyl-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 30°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 Nael (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-
VanA OPERONS IN PAENIBACILLUS

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. 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 Tn546 in E. faecium BM4147 (2) varied between 83% and 94% (Fig. 3). Homology with the vanA operon of Tn546 was also observed in the vanS-H (85%) and vanX-Y (74%) intergenic regions. As in Tn546, vanS overlapped with vanR over

...
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 Tn1546 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, whereas vanY PT 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.
HPLC and mass spectrometry of peptidoglycan precursors.
The crude cell wall extract from PT-2B1 was composed of ca. 25% A2pm-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 A2pm-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-(A2pm) 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 A2pm-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 A2pm-containing UDP-MurNAc-tetrapeptide, C38H60N8O27P2, 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-(A2pm)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, C41H64N6O25P2, 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.

**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. thiannoyticus* 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

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**TABLE 2.** d,d-Dipeptidase (VanX) and d,d-carboxypeptidase (VanY) activities in cytoplasmic and membrane extracts from *Paenibacillus* strains

<table>
<thead>
<tr>
<th>Strain and glycopeptide</th>
<th>Glycopeptide concn (µg/ml)</th>
<th>Activity (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cytoplasmic extract (VanX activity)</td>
</tr>
<tr>
<td>PA-B2B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vancomycin</td>
<td>4</td>
<td>108 ± 21</td>
</tr>
<tr>
<td></td>
<td>64</td>
<td>142 ± 7</td>
</tr>
<tr>
<td>Teicoplanin</td>
<td>8</td>
<td>24 ± 2</td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>86 ± 9</td>
</tr>
<tr>
<td>PT-2B1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vancomycin</td>
<td>2</td>
<td>1 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>64</td>
<td>13 ± 4</td>
</tr>
<tr>
<td>Teicoplanin</td>
<td>4</td>
<td>1.5 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>22 ± 4</td>
</tr>
</tbody>
</table>

* The reported mean values were calculated based on three separate measurements.

**FIG. 4.** HPLC analysis of peptidoglycan precursors of *P. thiannoyticus* PT-2B1 grown without (A) or with (B) vancomycin (32 µg/ml). Samples (one-fifth of the extracts) were applied to a µ-Bondapak C18 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 urine 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.
P. apiarius strains studied were resistant to glycopeptides and 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, vanANPT and vanA\textsubscript{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 vanANPT 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 vanT in P. popilliae (24) and vanA\textsubscript{PA} 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.

ACKNOWLEDGMENT

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REFERENCES


