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Resistance to Glycopeptide Antibiotics in the Teicoplanin Producer Is Mediated by *van* Gene Homologue Expression Directing the Synthesis of a Modified Cell Wall Peptidoglycan▽

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Glycopeptide resistance has been studied in detail in enterococci and staphylococci. In these microorganisms, high-level resistance is achieved by replacing the C-terminal D-alanyl-D-alanine of the nascent peptidoglycan with D-alanyl–D-lactate or D-alanyl–D-serine, thus reducing the affinities of glycopeptides for cell wall targets. Reorganization of the cell wall is directed by the expression of the *van* gene clusters. The identification of *van* gene homologs in the genomes of several glycopeptide-producing actinomycetes suggests the involvement of a similar self-resistance mechanism to avoid suicide. This report describes a comprehensive study of self-resistance in *Actinoplanes teichomyceticus* ATCC 31121, the producer of the clinically relevant glycopeptide teicoplanin. *A. teichomyceticus* ATCC 31121 showed a MIC of teicoplanin of 25 μg/ml and a MIC of vancomycin of 90 μg/ml during vegetative growth. The *vanH*, *vanA*, and *vanX* genes of *A. teichomyceticus* were found to be organized in an operon whose transcription was constitutive. Analysis of the UDP-linked peptidoglycan precursors revealed the presence of UDP-glycomuramyl pentadepsipeptide terminating in D-alanyl–D-lactate. No trace of precursors ending in D-alanyl–D-alanine was detected. Thus, the *van* gene complex was transcribed and expressed in the genetic background of *A. teichomyceticus* and conferred resistance to vancomycin and teicoplanin through the modification of cell wall biosynthesis. During teicoplanin production (maximum productivity, 70 to 80 μg/ml), the MIC of teicoplanin remained in the range of 25 to 35 μg/ml. Teicoplanin-producing cells were found to be tolerant to high concentrations of exogenously added glycopeptides, which were not bactericidal even at 5,000 μg/ml.

Glycopeptide antibiotics are produced by actinomycetes and inhibit the synthesis of bacterial cell wall by blocking peptidoglycan assembly. They bind to the D-alanyl–D-alanine (D-Ala–D-Ala) C terminus of the nascent peptidoglycan and prevent it from being utilized in the following cross-linking reactions catalyzed by transglycosylases and transpeptidases (18, 38, 44). The structurally related glycopeptides vancomycin and teicoplanin have been used in clinical settings since 1958 and 1988, respectively. These drugs are still extensively used against multiresistant enterococci and methicillin-resistant staphylococci. Concern about vancomycin-resistant enterococci has been increasing during the last decade, and highly vancomycin resistant *Staphylococcus aureus* isolates have recently appeared in clinical specimens (10, 11).

Two resistance phenotypes, VanA and VanB, have been extensively studied in enterococci and are considered of main importance, since the genes responsible for resistance (*van* genes) are inducible and transferable and confer high-level resistance to vancomycin (at concentrations up to 1,000 μg/ml or more) (42). The *vanA* gene cluster also confers high-level resistance to teicoplanin (4, 9, 15, 16). Other resistant phenotypes among enterococci (VanC, VanD, VanE, and VanG) showing a lower level of resistance to vancomycin and none or poor resistance to teicoplanin have been described (reviewed in reference 42). In VanA, VanB, and VanD enterococci, *van* genes direct the synthesis of peptidoglycan precursors terminating in the depsipeptide D-alanyl-D-lactate (D-Ala–D-Lac), whereas in VanC, VanE, and VanG strains, the C-terminal end is D-alanyl–D-serine (D-Ala–D-Ser) in place of the usual D-Ala-D-Ala. The modification of the target drastically reduces affinity for the glycopeptide, thus producing resistance (38). The key enzymes in VanA and VanB enterococci are a dehydrogenase (VanH encoded by the *vanH*/*vanH*$_B$ gene) that converts pyruvate into D-lactate (9, 16), a ligase (VanA or VanB encoded by the respective *van* gene) that joins D-alanine and D-lactic acid (9, 16, 34), and a D,D-dipeptidase (VanX encoded by the *vanX*/*vanX*$_B$ gene) that cleaves the D-Ala–D-Ala produced by the normal peptidoglycan synthesis pathway (16, 45, 51). The genes *vanH*, *vanA*/*vanB*, and *vanX* are organized in a cluster and are often associated with other genes encoding additional but not essential functions (1, 3, 15, 17, 50). A two-component regulatory system, composed of a membrane-associated sensor kinase (VanS or VanS$_B$) and a cytoplasmic response regulator that acts as a transcriptional activator (VanR or VanR$_B$), regulates the expression of *vanH*, *vanA*/*vanB*, and *vanX* (5).

An intriguing hypothesis is that bacterial pathogens have actually acquired antibiotic resistance mechanisms from antibiotic producers, although there is no conclusive evidence which proves gene transfer (19, 39, 40). In fact, *van*-like genes with a high level of homology and an organization similar to

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those of the pathogenic enterococci have been identified in glycopeptide-producing actinomycetes (28, 30–33), in which they might play a role in avoiding self-inhibition during antibiotic production. *van*-like genes are present in glycopeptide producers, such as *Amycolatopsis* spp., which are producers of vancomycin, chloroeremomycin, ristocetin, and avoparcin (30); in *Streptomyces toyocaensis* NRRL15009, which is the producer of A47934 (43); and in the teicoplanin producer *Actinoplanes teichomyceticus* ATCC 31121 (48); but an understanding of their active role in determining resistance is only at the beginning. *van*-like genes are physically linked to the gene clusters for the biosynthesis of A47934 (43) and teicoplanin (*tcp* cluster) (48, 49), but this does not seem to be the case in the other glycopeptide producers so far characterized (14). When the *vanA* gene of *Streptomyces toyocaensis* ($vanA_{st}$; *vanA* homolog) was disrupted, the mutant showed reduced resistance to A47934 but continued to produce this compound with a delay of ca. 16 h in comparison with that for the parental strain (43). One end of the *tcp* cluster contains seven genes likely involved in teicoplanin resistance: *murF2*; the *vanH*, *vanA*, and *vanX* genes of *A. teichomyceticus* ($vanH_{at}$, $vanA_{at}$, and $vanX_{at}$, respectively); the sensory kinase and the response regulator of a putative two-component signal transduction system (orthologous to the *vanS* and *vanR* genes of enterococci); and a short-chain dehydrogenase of unknown function (29, 49). These seven genes of *A. teichomyceticus* were cloned in *Streptomyces coelicolor*, but their effect on the teicoplanin resistance of the recipient strain was marginal (47, 48). On the other hand, *van*-like genes conferring resistance to vancomycin but not to teicoplanin were found in *S. coelicolor* itself, even though this strain lacks the genetic potential to produce glycopeptides (8, 20).

The purpose of this paper is to clarify the role of *van* genes in the resistance mechanisms used by the teicoplanin producer *A. teichomyceticus* during the phases of vegetative growth and the production of teicoplanin. Besides the genetic evidence for the presence of *van*-like genes, no insight into the resistance phenotype of *A. teichomyceticus* has so far been reported. The complex life cycle, the need for properly tailored microbiological methods in handling this rare actinomycete, and the lack of suitable genetic manipulation tools have probably hampered, until now, a comprehensive understanding of resistance comparable to that achieved for pathogenic enterococci.

MATERIALS AND METHODS

Strains and growth conditions. The mycelium of the teicoplanin producer strain *A. teichomyceticus* ATCC 31121 was maintained as a lyophilized master cell bank. Mycelium from the master cell bank was streaked onto slants of salt medium (SM; glucose [Sigma-Aldrich, St. Louis, MO], 10 g/liter; Bacto peptone [Difco, Detroit, MI], 4 g/liter; Bacto yeast extract [Difco], 4 g/liter; $MgSO_4 \cdot 7H_2O$ [Sigma-Aldrich], 0.5 g/liter; KH_2PO_4 [Sigma-Aldrich], 2 g/liter; K_2HPO_4 [Sigma-Aldrich], 4 g/liter; deionized water up to 1 liter) solidified with agar (15 g/liter; Sigma-Aldrich). After growth, the mycelium from a slant was homogenized in 10 ml of isotonic saline and inoculated in SM, grown for 96 h at 28°C with aeration, and stored as working cell bank (WCB) in 1.5-ml cryovials at −80°C for further experiments. The WCB was used to inoculate all the cultures for the experiments described here. Unless otherwise specified, the cultures were grown in medium (SM or SM agar) in which the production of teicoplanin did not occur, as demonstrated by high-pressure liquid chromatography (HPLC) analysis of the culture extracts, as reported below. These cultures/media are defined here as vegetative to distinguish them from productive cultures/media which did support teicoplanin production.

For DNA/RNA extraction, 2 ml WCB was inoculated into 100 ml of SM in a 500-ml baffled flask to which 5 ml sterile glass beads was added, and the flask was incubated at 28°C for 72 h on a rotary shaker at 200 rpm. Mycelium from this seed culture was transferred into 100 ml fresh SM to give an inoculum density of 10 g/liter (wet weight), and growth was allowed for 24 h at 28°C and 200 rpm. Vancomycin (Sigma-Aldrich) or teicoplanin (Sanofi-Aventis, Paris, France) was added, when required, at subinhibitory concentrations of 30 μg/ml and 4 μg/ml, respectively.

DNA/RNA extraction. Genomic DNA of *A. teichomyceticus* ATCC 31121 was extracted by a previously described method (24) from an SM vegetative culture grown to the exponential phase (24 to 48 h). RNA was extracted from mycelium induced for 1, 2, 4, 8, and 24 h with vancomycin or teicoplanin. Control samples to which glycopeptide was not added were extracted at the same time intervals. Mycelium was incubated in 2 ml of Tris-EDTA buffer containing 10 mg/ml lysozyme (Sigma-Aldrich) for 10 min; then, RNA isolation was carried out by following the protocol for the TRIzol reagent (Invitrogen, Carlsbad, CA). For further purification, an RNeasy Midi kit (QIAGEN, Hilden, Germany) was used according to the supplier's instructions. Residual DNA from RNA samples was removed by incubation with 10 U RNase-free DNase I (Roche, Basel, Switzerland) at 37°C for 1 h. The DNase I was then extracted with phenol and chloroform-isoamyl alcohol (24:1; Sigma-Aldrich). Finally, the RNA was recovered by ethanol-sodium acetate precipitation and centrifugation and was stored at −80°C in diethyl pyrocarbonate (Sigma-Aldrich)-treated water at a final concentration of 1 μg/μl.

Genetic analyses. DNA manipulations were performed by standard methods (46) unless otherwise specified. The positions reported for PCR and cDNA synthesis primers refer to the sequence in the GenBank database with accession no. AJ605139. Total RNA samples from the different cultivation conditions were accurately quantified with a UV 2100 spectrophotometer (Shimadzu, Kyoto, Japan) and normalized by direct comparison on 1% agarose electrophoresis gels (Sigma-Aldrich). Five micrograms of each RNA sample was reverse transcribed by using the SuperScript first-strand synthesis system for reverse transcription-PCR (RT-PCR; Invitrogen). The first-strand synthesis reaction was primed with random hexamers for 1 h at 37°C. Different cDNA concentrations were then used as the template for amplification of $vanX_{at}$ with primers *vanXFw* (position 14301-5′-TTCGCGTTCGTGGACGAG-3′-position 14319) and *vanXRev* (position 14897-5′-CTACACGATCGGAAAATCAAA-3′-position 14876); $vanA_{at}$ was amplified with primers *vanAFw* (position 13269-5′-CGGCATCATCTTCGGCG-3′-position 13286) and *vanARev* (position 14273-5′-AGGGCCAGCGACACGA T-3′-position 14256); and $vanH_{at}$ was amplified with primers *vanHFw* (position 12057-5′-AGCGCGGACGCCATCCGT-3′-position 12075) and *vanHRev* (position 13256-5′-CCCATGCTGTTGTTCCCT-3′-position 13238). Chromosomal DNA of *A. teichomyceticus* ATCC 31121 was used as a positive control, and total RNA without first-strand synthesis was used to test for genomic DNA contamination. The amplification products were separated on a 1% agarose gel. Semiquantitative PCR of the $vanX_{at}$ gene was performed as reported previously (36). In brief, 1:2 serial dilutions of the RNA reverse transcription product were amplified as described above with primers *vanXFw* and *vanXRev* by using 25 instead of 30 amplification cycles in order to compare the products during the exponential phase of the PCR and to quantify the relative mRNA levels. Samples were separated on a 1% agarose gel, and the relative transcript amount was determined by densitometric quantification with MI Image Analysis software (Kodak, Rochester, NY). In order to ensure that the amount of total cDNA was the same in the different samples, the same set of cDNA dilutions used as the template for the $vanX_{at}$ gene was also used to amplify the 16S rRNA genes.

Reverse transcription of the putative *van* operon encompassing the $vanH_{at}$, $vanA_{at}$, and $vanX_{at}$ genes was carried out by using 5 μg of total RNA as the template and the gene-specific primer *vanXRev*. Different cDNA concentrations were used as the template for PCR amplification with primers *TevanHFw* (position 12886-5′-CACTTCACACCCCGCTCA-3′-position 12904), which maps 844 bp downstream from the $VanH_{at}$ putative start, and *TevanXRev* (position 14639-5′-GCTGTGACCGGACTTGGT-3′-position 14620), which is located 351 bp downstream from the $VanX_{at}$ putative start. The conditions for amplification were as described above. Chromosomal DNA of *A. teichomyceticus* ATCC 31121 was used as the positive control, and total RNA without first-strand synthesis was used to test for genomic DNA contamination. The amplification product was analyzed on a 1% agarose gel.

Sequence homology analysis was performed with the BLAST program, and protein multisequence alignment was performed with the Clustal X program.

Antimicrobial activity assays. The MICs of teicoplanin and vancomycin were determined by the broth microdilution methodology, according to the procedures of the CLSI (formerly the NCCLS) (37), which were adapted to the cultivation conditions and the duplication times of *A. teichomyceticus* ATCC 31121. Vegetative mycelium grown to the exponential phase (24 to 48 h) in SM was harvested by centrifugation; suspended in isotonic saline; and fragmented by

sonication with a VCX 400 sonicator (Sonics and Materials Inc., Newtown, CT) at an intensity of 6 kHz and with the pulse on for 5 s and the pulse off for 3 s for a total interval sufficient to give single unbranched hyphae with sizes ranging from 1 to 5 μm (the sizes were checked with a microscope). The resulting cell suspension was filtered through a 5-μm-pore-size Durapore membrane filter (Millipore, Bedford MA), harvested by centrifugation, suspended in fresh SM at a concentration of 10 g/liter (fresh weight), and dispensed in a set of flasks containing vancomycin or teicoplanin at different concentrations. Growth was allowed for 96 h at 28°C with shaking. The MIC was the first antibiotic concentration that afforded no visible growth after 96 h of incubation.

Induction of glycopeptide resistance was investigated as follows. Mycelium was grown in SM medium for 24 h at 28°C. Then, subinhibitory concentrations of vancomycin (30 μg/ml) or teicoplanin (4 μg/ml) were added to the culture and growth was allowed for a further 24 h. Finally, the vegetative mycelium was sonicated and dispensed in flasks for MIC determination, as described above.

For the determination of the minimal bactericidal concentration, the sonicated vegetative mycelium was inoculated in a set of flasks containing increasing antibiotic concentrations and the flask was incubated at 28°C with shaking for a time interval equal to three generations (approximately 24 h), which was estimated in SM without any antibiotic. Mycelium from each flask was then washed with isotonic saline, diluted, and plated on SM agar plates without added antibiotic. After 15 days at 28°C, the fraction of cells which survived in the presence of the respective antibiotic concentrations in the flask and which were thus able to grow on a plate was determined. Antibiotic concentrations that resulted in a percentage of survivors less than 0.1 were considered bactericidal (27).

Teicoplanin production. Two milliliters of *A. teichomyceticus* WCB was inoculated in SM and grown for 72 h at 28°C on a rotary shaker at 200 rpm. Ten milliliters of this vegetative culture was then transferred to 100 ml of production medium C (glucose [Sigma-Aldrich], 20 g/liter; Bacto yeast extract [Difco], 5 g/liter; $MgSO_4 \cdot 7H_2O$ [Sigma-Aldrich], 1.5 g/liter; $CaCO_3$ [Sigma-Aldrich], 0.5 g/liter; asparagine [Sigma-Aldrich]; 1.5 g/liter; NaCl [Sigma-Aldrich], 0.1 g/liter; $CaCl_2 \cdot 2H_2O$ [Sigma-Aldrich], 0.1 g/liter; deionized water up to 1 liter). At different times of incubation at 28°C and 200 rpm, total teicoplanin was extracted by mixing 1 volume of whole culture and 3 volumes of borate buffer (100 mM H_3BO_3 [Sigma-Aldrich], 100 mM NaOH [Sigma-Aldrich], pH 12). The samples were then centrifuged (16,000 × *g* for 15 min) and the glycopeptide-containing supernatant was filtered through a Durapore membrane filter (pore size, 0.45 μm; Millipore). The teicoplanin bound to the mycelium was extracted as follows. The culture samples were centrifuged (16,000 × *g* for 15 min) in graduated vials and decanted, and 1 volume of packed mycelium was suspended in 3 volumes of borate buffer. The supernatant was then filtered as described above. Glycopeptide production was estimated by HPLC, which was performed on a 5-μm-particle-size Ultrasphere ODS (Beckman Coulter Inc., Fullerton, CA) column (4.6 by 250 mm) with elution at a flow rate of 1 ml/min with a 26-min linear gradient from 25% to 37% phase B. Phase A was 20 mM $HCOONH_4$ (pH 4.5; Sigma-Aldrich) and CH_3CN (Sigma-Aldrich) (95:5 [vol/vol]), and phase B was 20 mM $HCOONH_4$ (pH 4.5) and CH_3CN (5:95 [vol/vol]). Chromatography was performed with a model 1100 HPLC system (Hewlett-Packard, Palo Alto, CA), and UV detection was at 254 nm. Pure samples of teicoplanin (Sanofi-Aventis) were used as the internal standard. For growth estimation, mycelium was collected by centrifugation (3,250 × *g* for 20 min) and was washed with 2 ml isotonic saline. The dry weight was measured after 24 h of incubation in an 80°C oven. For MIC determination during teicoplanin production, 10 g/liter of mycelium was transferred from fermentative medium C to vegetative SM in the presence of increasing teicoplanin concentrations. The effective antibiotic concentration in the MIC flasks was the sum of the teicoplanin concentration added and the concentration of teicoplanin produced and carried over into the mycelium. Fermentations, growth estimations, and MIC determinations were performed in triplicate.

Peptidoglycan precursor extraction and analysis. *A. teichomyceticus* ATCC 31121 peptidoglycan precursors were extracted by a method previously described for *Bacillus cereus* (25), with some modification. Briefly, mycelium grown in vegetative medium to the exponential phase (24 to 48 h) was incubated for 1 h with 150 μg/ml ramoplanin (ca. eightfold the ramoplanin MIC; Biosearch Italia SpA, Gerenzano, Italy) to block final assembly of the cell wall and thus to amplify the cytoplasmic pool of uridine diphospho-linked precursors (6). Then, the cells were harvested, suspended in water (0.1 g [fresh weight] per ml), and boiled for 20 min. After the mycelium was first cooled at room temperature and then in ice, it was centrifuged at 39,000 × *g*. The supernatant was lyophilized and dissolved in 0.1 volume of water adjusted to pH 3 with formic acid. The samples were analyzed by reversed-phase HPLC and electrospray ionization (ESI)-mass spectrometry (MS) on a LCQ-Deca spectrometer equipped with an ion-trap analyzer (Thermo Finnigan, San Jose, CA). The samples were separated on a C_{18} column (5 μm; 4.6 by 250 mm; Phenomenex Luna, Torrance, CA) eluted at a flow rate of 1 ml/min with 2 min with 100% phase A and then a 50-min linear gradient to 100% phase B. Phase A was 2% CH_3CN, 97.9% H_2O, 0.1% HCOOH (vol/vol/vol); and phase B was 95% CH_3CN, 4.915% H_2O, and 0.085% HCOOH (vol/vol/vol). The column temperature was 22°C. The chromatographic UV absorption profile was provided with a photo diode array detector (UV6000; Thermo Finnigan). One-fifth of the detector elution flow was split into the mass spectrometer. The MS spectra were obtained by electrospray ionization both in the positive mode and in the negative mode under the following conditions. Sample inlet conditions were as follows: capillary temperature, 250°C; sheath gas (N_2), 80 LCQ arbitrary units; and auxiliary gas (N_2), 20 LCQ arbitrary units. Sample inlet voltage settings were as follows: positive polarity, 4.5 kV; negative polarity, 2.8 kV; capillary voltage, 4 V; and tube lens offset, 30 V. Helium was used as the buffer and collision gas. MS-MS analyses were performed at a collision energy of 30 keV. All the spectra were acquired in the 150- to 2,000-atomic-mass-unit mass range.

RESULTS AND DISCUSSION

Organization and transcription of *van* genes in *A. teichomyceticus*. In A. *teichomyceticus* ATCC 31121, *van* gene homologs were found in a region contiguous with the *tcp* cluster (47–49). In the present study we have analyzed by RT-PCR the expression of *van* genes during growth in vegetative medium. Under these conditions, *A. teichomyceticus* did not produce detectable quantities of teicoplanin (HPLC detection limit, 0.1 mg/liter) which could eventually induce the *van* genes and, consequently, interfere with the investigation of gene expression. First, qualitative monitoring of transcription indicated that the $vanA_{at}$ and $vanX_{at}$ genes are always transcribed, independently of whether exogenous vancomycin or teicoplanin is added or not added as an inducer (data not shown). Amplification of the cDNA obtained with primers *TevanHFw* and *TevanXRev* (mapping 373 bp upstream from the stop codon of *vanH* and 350 bp downstream from the start codon of *vanX*, respectively) indicated that the $vanH_{at}$, $vanA_{at}$, and $vanX_{at}$ genes are cotranscribed in an unique polycistronic mRNA (data not shown). Next, the *van* transcript levels of cultures grown either for 8 h or for 24 h in the presence of exogenously added vancomycin (30 μg/ml) or teicoplanin (4 μg/ml) were compared to those of cultures grown in the absence of the glycopeptides by semiquantitative RT-PCR with primers *vanXFw* and *vanXRev*. The subinhibitory glycopeptide concentrations used (previously defined in MIC experiments; see below) did not influence the growth rate of *A. teichomyceticus*. Densitometric analysis of the RT-PCR products obtained from the vancomycin- and teicoplanin-induced cultures showed that for each dilution of the cDNA template, the amounts of the amplification products corresponding to the $vanX_{at}$ transcript were essentially comparable to those achieved in uninduced cultures (data not shown). Vancomycin-induced cultures showed only a slightly (twofold) greater amount of transcript at 8 h, but this difference was no longer apparent after 24 h of induction. These results suggest the constitutive transcription of the *A. teichomyceticus van* cluster.

Mutations in *A. teichomyceticus* VanS suggest impaired activity of the sensor kinase. Constitutive expression of the *van* genes determined by mutation of the VanS sensor kinase has been reported for glycopeptide-resistant enterococci and, recently, for *S. coelicolor* (5, 7, 13, 22). We have taken advantage of the high degree of identity (77%) between the VanS sequences of *S. coelicolor* (EMBL accession no. AL939117.1) and *A. teichomyceticus* (EMBL accession no. AJ632270.1) to

check for the presence and significance of possible mutations in the VanS protein of *A. teichomyceticus*. By sequence alignment, we found two significant amino acid differences, located at positions 215 and 270 (positions 216 and 271, respectively, in *S. coelicolor*). Amino acids L216 and G271, located in the ATPase domain of VanS, have been reported to be crucial for the VanS phosphorylase activity in *S. coelicolor*, since their mutation (L216P and G271V) resulted in constitutive *van* gene expression and subsequent constitutive resistance (22). The differences in these amino acids (L216 and G271 of *S. coelicolor* have the orthologs N215 and R270, respectively, in *A. teichomyceticus*) suggest that *A. teichomyceticus* also has an impaired phosphatase function. In this actinomycete, a mutated VanS homolog conferring constitutive glycopeptide resistance may have been selected as an adaptation to teicoplanin production. Previous results on the marginal effect of cloning of *A. teichomyceticus van* genes in *S. coelicolor* (47) to teicoplanin resistance are likely due to complementation by the fully functional VanS homolog of *S. coelicolor*, which maintains an inducible phenotype of vancomycin resistance and teicoplanin sensitivity.

Resistance to vancomycin and teicoplanin in *A. teichomyceticus*. Detailed studies of the resistance phenotypes of glycopeptide-producing microorganisms are not reported in the literature. The standard method used to determine MICs for mycelial actinomycetes is hampered by the formation of multicellular aggregates and by the copresence of cells in different differentiation phases (i.e., vegetative mycelium, aerial mycelium, and spores) (35). We obtained hyphal preparations that could be converted to dispersed bacterial populations by use of a properly balanced protocol of sonication/filtration that was able to disrupt pellets and detach small hyphal fragments without damaging cell integrity. Under these conditions, *A. teichomyceticus* was resistant to glycopeptides, showing MICs of teicoplanin and vancomycin of 25 μg/ml and 90 μg/ml, respectively. These levels of glycopeptide resistance resemble those reported for intermediately resistant pathogenic enterococci, such as VanD isolates (42). The growth of *A. teichomyceticus* in the presence of teicoplanin or vancomycin at subinhibitory concentrations before MIC determination did not cause any changes in the MICs.

Determination of structures of peptidoglycan precursors. In enterococci the expression of *van* genes redirects cell wall biosynthesis toward the modified UDP–muropentapeptides UDP-MurNAc–L-Ala–D-Glu–L-Lys–D-Ala–D-Lac or UDP-MurNAc–L-Ala–D-Glu–L-Lys–D-Ala–D-Ser (42). To characterize the cell wall precursors in *A. teichomyceticus* ATCC 31121, cell wall assembly was blocked by the addition of ramoplanin, as reported in the literature (2). This depsipeptide antibiotic is structurally unrelated to vancomycin and teicoplanin, inhibits peptidoglycan transglycosylases (21), and is devoid of any effect on the induction of *van* gene expression in *Enterococcus faecalis* and *S. coelicolor* (6, 22). The cell wall precursor pool in ramoplanin-treated cells was analyzed by liquid chromatography (LC)-MS. A major peak was found to elute at a retention time of 17.8 min (Fig. 1B). As untreated cells did not exhibit this peak (Fig. 1A), it thus appeared to be related to a cell wall precursor that accumulated as a result of the effect of ramoplanin. The positive-ion ESI-MS spectrum corresponding to this peak revealed the presence of two quasimolecular ions,

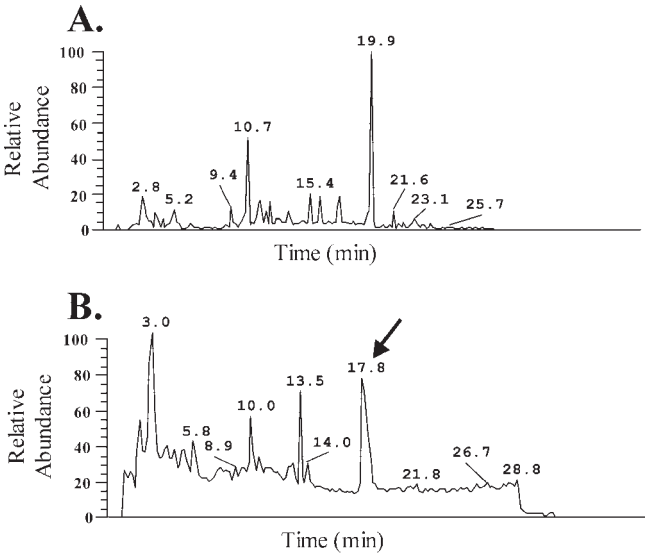

FIG. 1. LC-MS analysis of the cell wall cytoplasmatic precursor pool in ramoplanin-treated mycelium (B) or untreated mycelium (A) of *A. teichomyceticus.* Bacterial cultures were grown, harvested, and extracted as reported in Materials and Methods. Detection was by ESI-MS. A major peak, detectable only in ramoplanin-treated cells at a retention time of 17.8 min (indicated by the arrow), corresponded to the cell wall precursor UDP-*N*-glycolylmuramyl–Gly–D-Glu–*m*Dap–D-Ala–D-Lac (as identified by ESI-MS). No trace of precursors terminating in D-Ala–D-Ala was detected. The other peaks present on the HPLC plot showed MS profiles not related to soluble peptidoglycan precursors.

$[M-H]^+$, at *m/z* 1196.9 and 1212.9 (data not shown). The negative-ion spectrum (Fig. 2A) showed quasimolecular ions, $[M-H]^-$, at *m/z* 1195.2 and 1211.0 and the double-charged ions, $[M-2H]^{2-}$, at *m/z* 597.1 and 605.1, in agreement with the presence of two molecular species with the molecular formulas $C_{40}H_{62}N_8O_{30}P_2$ and $C_{40}H_{62}N_8O_{31}P_2$ and corresponding to calculated monoisotopic molecular masses of 1,196.30 and 1,212.30 Da, respectively. The UDP-*N*-glycolylmuramyl depsipeptide structure UDP-*N*-glycolylmuramyl–Gly–D-Glu–*meso*-diaminopimelic acid (*m*Dap)–D-Ala–D-Lac was attributed to the species showing a quasimolecular ion, $[M-H]^-$, at *m/z* 1195.2 (Fig. 2C). This depsipeptide contains the D-Ala–D-Lac terminus characteristic of the VanA, VanB, and VanD phenotypes (42) and a remaining part consistent with data in the literature (23). In fact, in *Actinoplanes* species and in some other actinomycetes, the glycolyl group is present instead of acetyl groups in muramic acid and the composition of the peptide unit is glycine, glutamic acid, *meso*-diaminopimelic acid (or *meso*-3-hydroxy-diaminopimelic acid), and alanine (23). The difference of 16 mass units between the molecular mass of the two species which coelute by LC-MS at 17.8 min suggests the presence of a hydroxy group on the UDP-*N*-glycomuramyl–Gly–D-Glu–*m*Dap–D-Ala–D-Lac, probably on the *meso*-diaminopimelic acid (indicated by R in Fig. 2C), as also reported for *Actinoplanes* species and in other actinomycetes (23). Additional evidence for the UDP-*N*-glycolylmuramyl–Gly–D-Glu–*m*Dap–D-Ala–D-Lac structure was provided by MS-MS analysis in the negative-ion mode of the quasimolecular ion $[M-H]^-$ corresponding to the UDP-muropentadep-

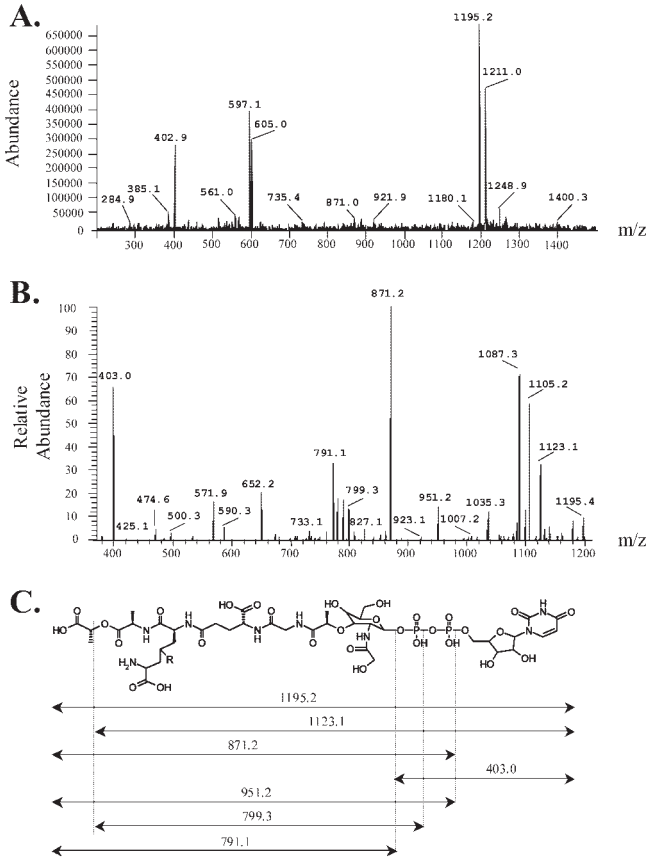

FIG. 2. (A) Full-scan mass spectrum (negative ion current) of the peak eluted at 17.8 min. The molecular ion $[M-H]^-$ at *m/z* 1195.2 corresponded to the UDP-*N*-glycolylmuramyl–Gly–D-Glu–*m*Dap–D-Ala-D-Lac and double-charged $[M-H]^{2-}$ at *m/z* 597.1. UDP-*N*-glycolylmuramyl–Gly–D-Glu–*m*-3-hydroxy-Dap–D-Ala–D-Lac is identifiable by the molecular ion $[M-H]^-$ at 1211.0 and double-charged $[M-2H]^{2-}$ at *m/z* 605.0. The ion at *m/z* 402.9 corresponded to UDP formed directly in the MS source. (B) MS-MS analysis of the quasimolecular ion $[M-H]^-$ at *m/z* 1195.2. (C) Chemical structure of the UDP-*N*-glycolylmuramyl–Gly–D-Glu–*m*Dap–D-Ala–D-Lac (R = H) or of the UDP-*N*-glycolylmuramyl–Gly–D-Glu–*m*-3-hydroxy-Dap–D-Ala–D-Lac (R = OH) and structural assignments for the main fragment ions. Note that the masses of the observed fragment ions (numbers above arrows) are 1 Da smaller than the masses of the neutral structures due to the loss of H^+.

sipeptide mentioned above (Fig. 2B). Significant fragments are evidenced in Fig. 2C. Fragmentation of the molecular ion at *m/z* 1195.2 gave a product ion at *m/z* 1123.1, corresponding to the loss of 72 mass units equivalent to a lactate (Lac) fragment and thus corresponding to the sequence UDP-*N*-glycolylmuramyl–Gly–D-Glu–*m*Dap–D-Ala. The fragmentation ions at *m/z* 871.2 and 791.1 corresponded to the loss of UMP or UDP from the UDP-muropentadepsipeptide, giving P–*N*-glycolylmuramyl–Gly–D-Glu–*m*Dap–D-Ala–D-Lac and *N*-glycolylmuramyl–Gly–D-Glu–*m*Dap–D-Ala–D-Lac, respectively. The ion at *m/z* 403.0 corresponded to UDP, while the ions at *m/z* 1105.2 and 1087.3 were attributed to losses of lactic acid and lactic acid plus water, respectively, from the quasimolecular ion. Among the ions with a relative abundance of less than 30% (Fig. 2B), it was possible to assign those at *m/z* 951.2 and 799.3,

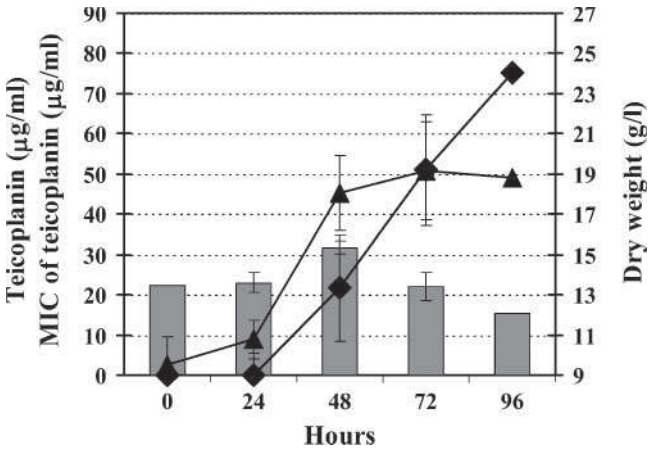

FIG. 3. Teicoplanin MICs (thick bars) in *A. teichomyceticus* mycelium prepared from fermentation flasks during teicoplanin production. The levels of production of teicoplanin (reported as mg/liter ◆) and growth (reported as g/liter [dry weight] of mycelium ▲) at the different fermentation intervals are indicated. The results are the averages of three independent experiments. Bars represent ± 1 standard deviation.

which were attributed to the loss of uridine and UMP plus lactate, respectively.

The LC/MS profiles of ramoplanin-treated cells cultured either in glycopeptide-free vegetative medium or in vegetative medium to which vancomycin or teicoplanin was added were virtually identical (data not shown). In all these preparations, the only UDP-muropentapeptide recovered was the one terminating with the depsipeptide D-Ala–D-Lac. No traces of D-Ala–D-Ala UDP-*N*-glycolylmuramylpentapeptide (expected $[M-H]^-$ at *m/z* 1194) were detected (Fig. 2). This further confirms that the *van* genes are constitutively expressed in *A. teichomyceticus*. We cannot rule out the possibility that peptidoglycan precursors terminating in D-Ala–D-Ala are also synthesized, but in this case they should be very efficiently and completely converted to the D-Ala–D-Lac-terminating ones. These data, together with those on the constitutive transcription of *van* genes in RT-PCR experiments and with the MICs of either vancomycin or teicoplanin, define a constitutive phenotype of glycopeptide resistance in *A. teichomyceticus* with features similar to those described in VanD enterococci. In fact, VanD enterococci are intermediately resistant to vancomycin and teicoplanin, their *van* genes are constitutively transcribed, and the UDP-muropentapeptide terminates with D-Ala–D-Lac independently from the presence of glycopeptides in the cultivation medium (41).

Teicoplanin production and glycopeptide resistance. Resistance could be coregulated with endogenous antibiotic production in antibiotic-producing microorganisms, and an increase of self-resistance during antibiotic production has been reported previously (12). The proximity of the *van* genes to the *tcp* cluster in *A. teichomyceticus* may imply some mechanism of coregulation (47–49). Figure 3 shows the correlations among growth, teicoplanin production, and MIC at different times of *A. teichomyceticus* cultivation in the antibiotic production medium. The teicoplanin MIC at the beginning of fermentation was the same as that in vegetative cultures (25 μg/ml), and then

it showed a slight, transient increase (33 μg/ml) at the end of the exponential phase of growth and finally decreased during the stationary phase, in association with maximal antibiotic production. At 48 h after inoculation (exponential phase of growth), teicoplanin was produced at about 20 μg/ml and reached 70 to 80 μg/ml at 96 h of fermentation (stationary phase of growth).

Because glycopeptides are reported to exert bactericidal actions in pathogens (26), the minimal bactericidal concentrations of teicoplanin and vancomycin were determined. In *A. teichomyceticus* more than 20% of the population survived 24 h of exposure to teicoplanin at 5,000 μg/ml, and treatment with the same vancomycin concentration allowed 50% survival. The control antibiotic kanamycin showed, instead, bactericidal action at much lower concentrations (data not shown).

It can be concluded that teicoplanin as well as vancomycin is not bactericidal for *A. teichomyceticus*. Thus, this microorganism fails to grow in the presence of more than 25 μg/ml of teicoplanin but could resume growth if the antibiotic is removed. The maximum level of teicoplanin production (80 μg/ml) in submerged cultures is higher than the MIC, but teicoplanin production occurs in the late exponential-stationary phase of growth, when scarce mycelium proliferation is reported. Under these physiological conditions, the activity of cell wall biosynthesis per se is reduced. The mechanisms of *van*-mediated resistance appear to be involved in the phase of active growth, when the levels of teicoplanin production are usually at sub-MICs. As the strain enters into the stationary phase, resistance is no longer required and the nonproliferating cells are tolerant to high concentrations of their own product. When Pootoolal and coworkers (43) knocked out the *vanA*-like gene conferring A47934 resistance to the producer strain *S. toyocaensis* NRRL15009, the mutant retained the ability to produce the antibiotic, but its production was delayed for more than 16 h. Probably, also in this case, antibiotic production was delayed to a period when cells have ceased growing and are predicted to become insensitive to antibiotic action.

Analysis of the temporal succession of growth and resistance, antibiotic production, and tolerance in *A. teichomyceticus* brings our attention back to the complex life cycle of this nonmotile saprophytic filamentous soil actinomycete, which grows by colonizing solid substrates with vegetative hyphal mass and then differentiates into aerial structures with motile spores that assist species spread and persistence. In nature, antibiotic production starts when the growth of the vegetative mycelium slows down as a result of nutrient exhaustion and secondary structures evolve at the expense of the nutrients released by the breakdown of vegetative cells at the center of the colony. It has been proposed that antibiotics defend the food source from the threat of competitors, without damaging the cells that differentiate into spores, which should tolerate high local concentrations. Meanwhile, the expression of antibiotic resistance genes may be an advantage to mycelium that is still growing at the borders of the colony, where the antibiotic diffuses at low concentrations from the center. In conclusion, resistance allows the growth and spread of vegetative mycelium, while antibiotic production protects the center of the colony, which has switched to the sporulating phase.

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