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The Cytoplasmic Peptidoglycan Precursor of Vancomycin-Resistant *Enterococcus faecalis* Terminates in Lactate

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Vancomycin resistance plasmids in enterococci carry the genes *vanH* and *vanA*, which encode enzymes catalyzing, respectively, the reduction of 2-keto acids to 2-D-hydroxy acids and the addition of D-hydroxy acids to D-alanine. It has therefore been postulated that resistant cells produce peptidoglycan precursors that terminate in the depsipeptide D-alanine–2-D-hydroxy acid rather than the dipeptide D-alanine–D-alanine, thus preventing vancomycin binding (M. Arthur, C. Molinas, T. D. H. Bugg, G. D. Wright, C. T. Walsh, and P. Courvalin, Antimicrob. Agents Chemother. 36:867–869, 1992). In the present work, a cytoplasmic peptidoglycan precursor was isolated from vancomycin-resistant *Enterococcus faecalis* and analyzed by mass spectrometry, which suggested the structure UDP-*N*-acetyl-muramyl-L-Ala-D-Glu-L-Lys-D-Ala-D-lactate.

Glycopeptide antibiotics such as vancomycin act by binding to the D-alanyl–D-alanine terminus of stem pentapeptides present in bacterial peptidoglycan (11, 12). Since the D-alanyl–D-alanine terminus is ubiquitous among eubacterial species producing peptidoglycan, in the absence of a simple barrier to vancomycin binding such as the outer membrane of gram-negative bacteria, fundamental alterations in peptidoglycan synthesis would be required in order for the bacterium to evade the activity of vancomycin. Thus, the emergence of resistance had been considered highly unlikely. However, glycopeptide resistance has recently been recognized among clinical isolates of several gram-positive species (7, 8, 10, 13).

Highly vancomycin-resistant enterococci (VanA phenotype) harbor plasmids on which a cluster of genes conferring resistance reside: a two-component regulatory system (*vanR* and *vanS*), the structural genes *vanA* and *vanH*, and a gene of unknown function, *vanX* (2, 3, 5). The *vanH* gene encodes an enzyme that reduces 2-keto acids to D-2-hydroxy acids; *vanA* encodes an enzyme that shows amino acid homology to D-alanine–D-alanine ligase but which has an altered specificity, catalyzing addition of D-2-hydroxy acids as well as D-amino acids to D-alanine (3).

In vitro studies using analogs of peptidoglycan stem pentapeptide have shown that the C-terminal D-alanine is essential for formation of a stable complex with vancomycin (11). Replacement of the terminal D-alanine by glycine or other D-amino acids reduces binding 10- to 1,000-fold, and substitution of hydroxy acids reduces binding to an even greater extent (3, 11). Therefore, it has been postulated that vancomycin-resistant enterococci produce a stem tetrapeptide terminating in a D-2-hydroxy acid rather than D-alanine, preventing vancomycin binding (3). This hypothesis is supported by the observations that addition of DL-lactate or DL-hydroxybutyrate to the medium restored resistance to a strain in which *vanH* was insertionally inactivated (1). Furthermore, addition to the medium of high concentrations of glycine or D-amino acids that may be incorporated into pentapeptide, and that are bound by vancomycin to a greater degree than D-2-hydroxy acids, diminishes the vancomycin MIC of resistant cells (14).

In the present study, to determine whether vancomycin-resistant enterococci in fact utilize such an altered peptidoglycan precursor and to identify the moiety substituted for D-alanine, cytoplasmic precursors were harvested from resistant and susceptible cells, and the structures were identified by mass spectrometry.

Enterococcus faecalis 221 (vancomycin MIC, 512 μg/ml) was obtained by introduction of the previously described glycopeptide resistance plasmid pHKK100 (6) into the susceptible *E. faecalis* strain JH2-2. Cytoplasmic pools of UDP-linked peptidoglycan precursors were extracted by a modification of the technique of Mengin-Lecreulx et al. (9). Cultures were grown in brain heart infusion (Difco Laboratories, Detroit, Mich.) to mid-logarithmic phase (with 10 μg of vancomycin per ml for cultures of strain 221) and chilled rapidly. Cells were harvested by centrifugation, boiled for 10 min, cooled, and treated with cold trichloroacetic acid (final concentration, 5%). Precipitated material was removed by centrifugation, and the supernatant fluid was separated by gel filtration (Sephadex G-25; Pharmacia, Alameda, Calif.), with elution with water. Hexosamine-containing fractions were identified by the assay of Ghuysen et al. (4), combined, and lyophilized.

The precursor extracts were chromatographically separated on-line with a Beckman System Gold high-performance liquid chromatograph and a Sciex API triple quadrupole mass spectrometer equipped with an Ionspray interface, which can resolve 1 Da at up to 2,000 Da. The tripeptide precursor UDP-*N*-acetyl-muramyl-L-Ala-D-Glu-L-Lys, isolated from *E. faecalis* JH2-2 grown in the presence of cycloserine (120 μg/ml), was used as a reference material in these studies. A component with a molecular weight of 1,150 present in the precursor extract from *E. faecalis* 221 was detected. The standard pentapeptide precursor (molecular weight, 1,149) was present in preparations of the susceptible strain JH2-2 (data not shown).

Fragmentation patterns of precursors from strain 221 were consistent with substructures of a modified tetrapeptide

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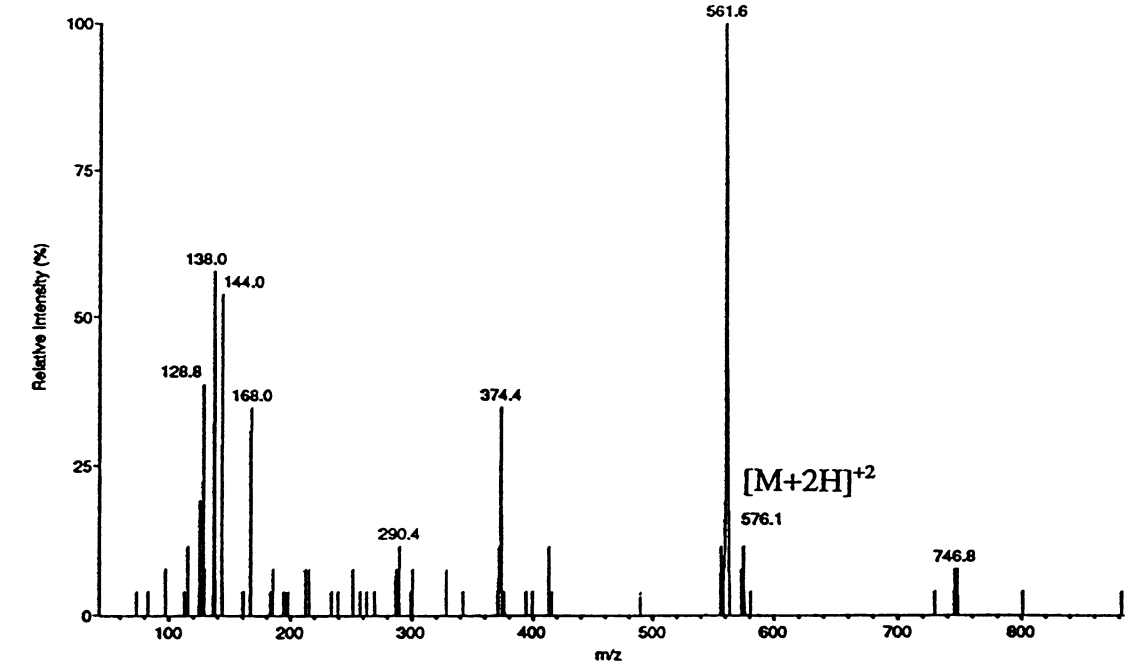

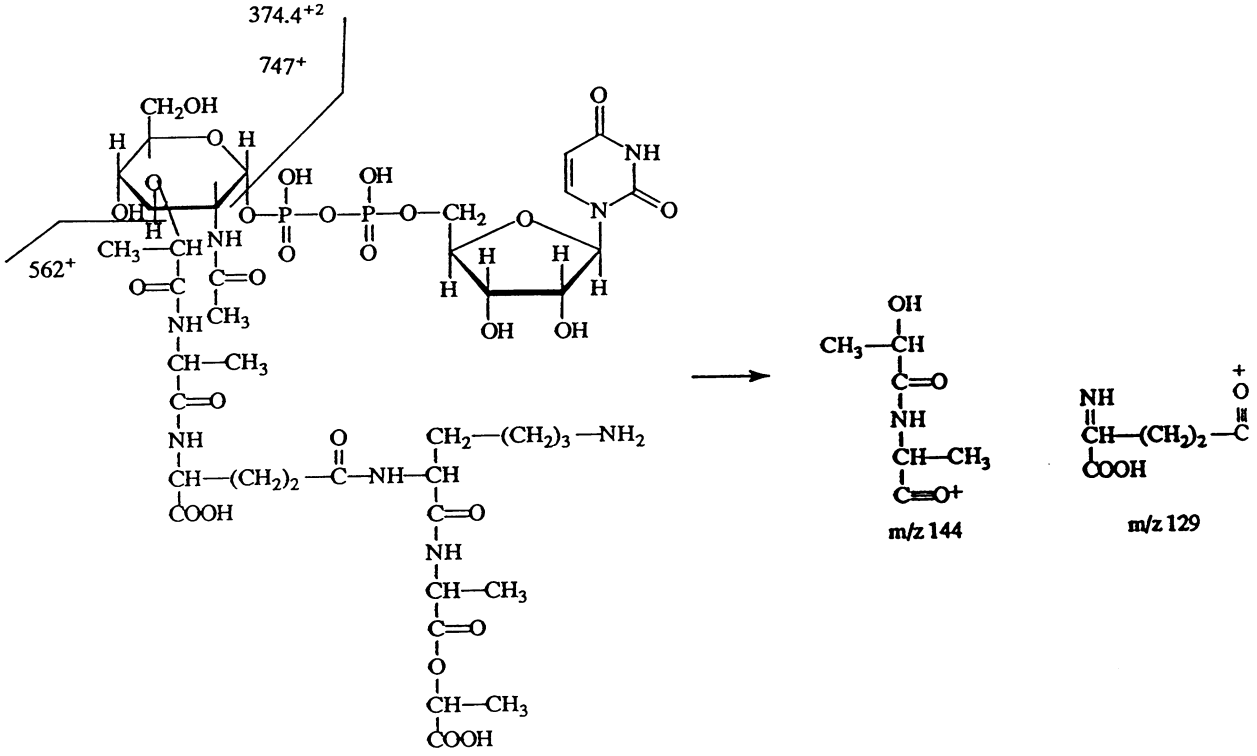

FIG. 1. Tandem mass spectrometry daughter spectrum of the MH_2^{2+} ion of modified tetrapeptide at *m/z* 576 (upper panel) and structures of the modified precursor and daughter fragments (lower panel).

precursor. The full-scan mass spectrum of the modified tetrapeptide showed a protonated molecular ion at *m/z* 1,151 and a doubly charged ion (MH_2^{2+}) at *m/z* 576, which indicates a molecular weight of 1,150 (data not shown), consistent with the incorporation of D-lactate in place of D-alanine. Additional tandem mass spectrometry structural information was obtained for the MH_2^{2+} ion at *m/z* 576 (Fig. 1). The only doubly charged fragment observed is the ion at *m/z* 374 corresponding to the loss of the UDP core. These data also indicate the presence of the alanine substructure (*m/z* 144) resulting from cleavage at position 3 of the *N*-acetyl-muramyl sugar as well as glutamic acid at *m/z* 129. These data and comparison with the tandem mass spectrometry fragmentation pattern of the UDP-muramyl-tripeptide indicate an alteration of an amino acid near the C terminus of a modified tetrapeptide. Additional evidence for lactate substitution can be found in a weak fragment ion at *m/z* 290, indicative of the loss of the Lys-Ala-lactate substructure from the protonated tetrapeptide fragment at *m/z* 562. These results are consistent with formation of a UDP–*N*-acetyl-muramyl–L-Ala–D-Glu–L-Lys–D-Ala–D-lactate cytoplasmic precursor.

The identification of VanH as a dehydrogenase able to catalyze the reduction of 2-ketoacids to D-2-hydroxyacids suggested that vancomycin-resistant enterococci might utilize peptidoglycan precursors terminating in a depsipeptide rather than the dipeptide D-alanine–D-alanine. Kinetic data from in vitro assays with purified VanH showed that 2-keto acids with small side chains, such as pyruvate and 2-ketobutyrate, were the best substrates (3). However, assays for formation of a D-ala–X product by VanA suggested that substrates with longer side chains were used preferentially by this enzyme, with D-2-hydroxybutyrate and D-2-hydroxyvalerate yielding the lowest K_m values (3). In the present study, mass spectrometry analysis suggests that the substrate used in vivo to terminate peptidoglycan precursors is D-lactate.

Since VanA has been shown to be a membrane-associated protein (3, 10, 13), the alteration in precursor structure presumably might occur at the level of either the cytoplasmic UDP-linked precursor or the membrane-associated lipid precursor. Our findings demonstrate that this alteration occurs at the level of the cytoplasmic, UDP-linked peptidoglycan precursor.

Further investigation of cell wall synthesis in vancomycin-resistant enterococci may yield information regarding the effects of altered precursor structure upon later steps in peptidoglycan synthesis. For example, the observation that some vancomycin-resistant enterococci are hypersusceptible to beta-lactams suggests that not all penicillin-binding proteins are able to cross-link depsipeptide-terminating stems efficiently. Furthermore, identification of the structures of peptidoglycan precursors present in vancomycin-resistant enterococci may enable development of antibiotics that can bind to this altered target.

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