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Molecular Basis for Vancomycin Resistance in *Enterococcus faecium* BM4147: Biosynthesis of a Depsipeptide Peptidoglycan Precursor by Vancomycin Resistance Proteins VanH and VanA†

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ABSTRACT: Vancomycin resistance in *Enterococcus faecium* BM4147 is mediated by vancomycin resistance proteins VanA and VanH. VanA is a D-alanine:D-alanine ligase of altered substrate specificity [Bugg, T. D. H., Dutka-Malen, S., Arthur, M., Courvalin, P., & Walsh, C. T. (1991) *Biochemistry 30*, 2017–2021], while the sequence of VanH is related to those of α-keto acid dehydrogenases [Arthur, M., Molinas, C., Dutka-Malen, S., & Courvalin, P. (1991) *Gene* (submitted)]. We report purification of VanH to homogeneity, characterization as a D-specific α-keto acid dehydrogenase, and comparison with D-lactate dehydrogenases from *Leuconostoc mesenteroides* and *Lactobacillus leichmanii*. VanA was found to catalyze ester bond formation between D-alanine and the D-hydroxy acid products of VanH, the best substrate being D-2-hydroxybutyrate (K_m = 0.60 mM). The VanA product D-alanyl-D-2-hydroxybutyrate could then be incorporated into the UDPMurNAc–pentapeptide peptidoglycan precursor by D-Ala-D-Ala adding enzyme from *Escherichia coli* or by crude extract from *E. faecium* BM4147. The vancomycin binding constant of a synthetic modified peptidoglycan analogue *N*-acetyl-D-alanyl-D-2-hydroxybutyrate (K_d > 73 mM) was >1000-fold higher than the binding constant for *N*-acetyl-D-alanyl-D-alanine (K_d = 54 μM), partly due to the disruption of a hydrogen bond in the vancomycin–target complex, thus providing a molecular rationale for high-level vancomycin resistance.

The recent emergence of bacterial resistance to the glycopeptide family of antibiotics in strains of *Enterococcus faecium* and *Enterococcus faecalis* (Courvalin, 1990) has prompted considerable interest in the mechanism of resistance: both because of the increasing use of the glycopeptide vancomycin in treatment of life-threatening Gram-positive bacterial infections and because of the unusual mode of action of the glycopeptide antibiotics. Vancomycin does not appear to penetrate the cell membrane or interact with cellular proteins but functions by complexation of peptidyl-D-Ala-D-Ala termini on the bacterial cell surface (Barna & Williams, 1984), thereby preventing transglycosylation and cross-linking of the peptidoglycan layer (Reynolds, 1989; Nagarajan, 1991). In view of the apparent ubiquity of the D-Ala-D-Ala terminus of bacterial peptidoglycan, it is difficult to envisage a simple mechanism for vancomycin resistance, which may explain the lack of bacterial resistance for over 30 years of clinical use. How then is this recently emergent vancomycin resistance achieved?

High-level vancomycin resistance in *E. faecium* BM4147, a class A vancomycin-resistant strain (Schlaes et al., 1991), is associated with production of a 38-kDa membrane-associated protein VanA, whose amino acid sequence has been determined (Dutka-Malen et al., 1990). Sequence similarity was found with Gram-negative D-Ala-D-Ala ligases, which are cytoplasmic enzymes responsible for synthesis of the D-Ala-D-Ala dipeptide for peptidoglycan assembly (Walsh, 1989). VanA has been purified, and shows D-Ala-D-Ala ligase activity, but has substantially modified substrate specificity, compared with Gram-negative D-Ala-D-Ala ligases (Bugg et al., 1991). VanA is able to synthesize a number of mixed dipeptides including D-Ala-D-Met and D-Ala-D-Phe in preference to D-Ala-D-Ala, suggesting that its cellular role may be synthesis of a D-Ala-X dipeptide, which is incorporated into peptidoglycan and is able to be cross-linked but is not recognized by vancomycin.

Sequencing of an open reading frame adjacent to the *vanA* gene on plasmid pIP816 that is also required for resistance has identified a further vancomycin resistance protein named VanH (Arthur et al., 1991). The amino acid sequence of VanH shows 19–30% sequence identity with sequences of three D-specific α-keto acid reductases: D-hydroxyisocaproate dehydrogenase, D-3-phosphoglycerate dehydrogenase, and D-erythronate phosphate dehydrogenase. This raises the pos-

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Table I: Purification of VanH from JM83/pSD8

	vol (mL)	act.[a] (unit/mL)	protein[b] (mg/mL)	tot protein (mg)	sp. act. (units/mg)	purification (fold)
crude extract	20.0	0.39	19.4	388	0.020	1.0
25–60% $(NH_4)_2SO4$	7.5	0.97	26.0	195	0.037	1.86
AcA54 gel filtration	48.0	0.28	1.50	72	0.187	9.3
2′,5′-ADP agarose	15.0	0.40	0.03	0.45	13.5	673
Mono-Q FPLC	7.5	0.61	0.009	0.0675	67.8	3,390

[a] Determined by NADH-dependent reduction of pyruvate at pH 5.6 (see Materials and Methods). One unit is defined as the activity required to convert 1 μmol of substrate to product per minute. [b] Determined by Bradford protein assay (Bradford, 1976).

sibility that VanH synthesizes a D-α-hydroxycarboxylic acid, which may be an alternative substrate for VanA. If VanA is able to condense a D-hydroxy acid instead of a D-amino acid with D-alanine, binding of the subsequently resulting peptidoglycan precursor to vancomycin would be directly affected, since one of the hydrogen bonds formed between vancomycin and *N*-acetyl-D-Ala-D-Ala is with the NH of the terminal D-alanine residue (Barna & Williams, 1984). In order to examine this mechanistic proposal, we have purified VanH and report characterization of VanH as a D-specific α-keto acid reductase, coupling with VanA, and experimental support for this mechanism of vancomycin resistance.

Materials and Methods

Materials. The following materials were obtained from Sigma Chemical Corp.: tris(hydroxymethyl)aminomethane base (Tris), ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT), ampicillin, D-alanine, vancomycin, D-lactate, L-lactate, 2-ketocarboxylic acids, DL-2-hydroxycarboxylic acids, *Leuconostoc mesenteroides* D-lactate dehydrogenase, *Lactobacillus leichmanii* D-lactate dehydrogenase, and rabbit muscle L-lactate dehydrogenase. The following materials were obtained from Boehringer Mannheim Biochemicals: nicotinamide adenine dinucleotides (NADH, NAD^+, NADPH), ATP, and isopropyl thiogalactoside (IPTG). 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was purchased from U.S. Biochemicals. [U-^{14}C]-D-alanine (40 mCi/mmol) and [1-^{14}C]pyruvic acid (35 mCi/mmol) were purchased from Amersham. Protected amino acids, *N*,*N*′-diacetyl-L-lysyl-D-alanyl-D-alanine and *N*,*N*′-diacetyl-L-lysyl-D-alanyl-D-lactate were from Bachem Bioscience. Isoprenyl chloroformate was from Fluka. Other chemicals and solvents were of reagent grade. Samples of UDPMurNAc–tripeptide and UDPMurNAc–pentapeptide were provided by Dr. J. van Heijenoort (University of Paris). VanA (Bugg et al., 1991), *Salmonella typhimurium* D-Ala-D-Ala ligase (Knox et al., 1989), and *Escherichia coli* D-Ala-D-Ala ligase B (Zawadzke et al., 1991) were prepared as previously described. *E. coli* D-Ala-D-Ala adding enzyme was purified from *E. coli* strain JM105 transformed with recombinant plasmid pTB3 containing the *murF* gene under the control of the *tac* promoter (T. D. H. Bugg and C. T. Walsh, unpublished results).

Purification of VanH (see Table I). VanH was purified from JM83/pSD8, which contains the *vanH* gene under the control of the *lac* promoter (S. Dutka-Malen, unpublished results). All steps were carried out at 4 °C unless otherwise specified. Enzyme activity was monitored by decrease in absorbance of NADPH at 340 nm in the presence of 10 mM pyruvate in 50 mM sodium phosphate buffer (pH 5.6). Protein concentration was determined by the method of Bradford (1976). The standard column buffer consisted of 50 mM Tris (pH 7.4), 1 mM EDTA, and 1 mM DTT.

JM105/pSD8 was grown at 37 °C in 1 L of Luria Broth (1% tryptone, 1% NaCl, 0.5% yeast extract) containing ampicillin (100 μg/mL) to A_{595} of 0.6, whereupon 100 mM IPTG (10 mL) was added to induce the *lac* promoter. Cells were then grown for a further 5 h at 37 °C and harvested by centrifugation at 8000 *g* for 10 min. The cell pellet (4.4 g) was resuspended in 20 mL of 100 mM Tris (pH 7.4) containing 1 mM EDTA and 5 mM DTT and was passed twice through a French press at 1000 psi. Cell debris was removed by centrifugation at 25000*g* for 30 min.

Powdered ammonium sulfate was gradually added to the supernatant to a final concentration of 25% saturation, and the solution was stirred for 1 h. The solution was cleared by centrifugation at 12000*g* for 30 min, and ammonium sulfate was added to the supernatant to a concentration of 60% saturation. After the solution was stirred for a further 1 h, the precipitate was removed by centrifugation at 12000*g* for 30 min. The precipitate was resuspended overnight in 5 mL of column buffer.

The resuspended pellet was loaded onto an Ultrogel AcA54 gel filtration column (2.5 × 108 cm) and eluted at 60 mL/h with column buffer. Active fractions were pooled, and the pool was loaded onto an adenosine 2′,5′-diphosphate agarose column (Sigma; 1.6 × 10 cm) and eluted at 1.0 mL/min with a 300-mL gradient of 0–2 M KCl in column buffer. Enzyme activity eluted at 0.6 M KCl: active fractions were pooled and dialyzed overnight into Tris column buffer. The pool was then loaded onto an HR 5/5 Mono-Q column (Pharmacia) and eluted at 0.5 mL/min with a 100-mL gradient of 0–0.5 M KCl in column buffer. Enzyme activity eluted in a single sharp peak at 140 mM KCl, giving 68 μg of VanH of specific activity 68 units/mg which was homogeneous by SDS–PAGE. An N-terminal sequence M-N-N-I-G-I-T-V-Y-G was obtained from an electroblot of VanH onto PVDF membrane (carried out by Dr. W. Lane, Harvard Microchemistry Facility), which is identical to the predicted N-terminal amino acid sequence (Arthur et al., 1991).

Kinetic Analysis of VanH and D-Lactate Dehydrogenases. VanH and D-lactate dehydrogenases from *L. mesenteroides* and *L. leichmanii* were assayed by the decrease in absorbance of NADPH at 340 nm ($\epsilon = 6.2 \times 10^3$ M^{-1} cm^{-1}) in 50 mM sodium phosphate buffer (pH 5.6) with varying concentrations of α-keto acid substrates. pH 5.6 was found to be the optimum pH for the forward reaction of VanH. K_m values were determined by varying substrate concentration from $0.2K_m$ (minimum 2 μM) to $5K_m$ (maximum 80 mM), assays being carried out in duplicate and K_m and k_{cat} being determined by Lineweaver–Burk plots.

Determination of Stereospecificity of VanH. A 200-μL mixture of VanH (0.05 unit), pyruvate (5 μmol), and NADH (5 μmol) in 50 mM potassium phosphate buffer (pH 5.6) was incubated for 24 h at 25 °C, heated for 5 min at 70 °C, spun for 2 min at 13000*g*, filtered through a plug of activated charcoal, and stored at 4 °C. The mixture was then injected onto an Interaction ION-300 Organic Acids HPLC column and eluted in 5 mM H_2SO_4 at 0.5 mL/min. Pyruvic acid was found to elute at 27 min and lactic acid at 38 min. Lactic acid

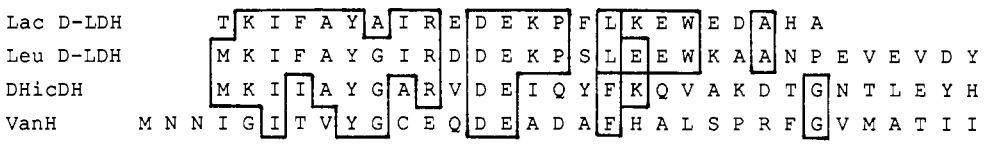


FIGURE 1: Sequence alignment of N-terminal sequences of *L. leichmanii* and *L. mesenteroides* D-lactate dehydrogenases (Lac D-LDH and Leu D-LDH, respectively) with D-hydroxyisocaproate dehydrogenase (DHicDH) and VanH. Identical conserved residues are boxed.

product was collected, neutralized with NaOH, and added to an assay monitored at 340 nm containing 5 mM NAD^+ in 100 mM HEPES buffer (pH 8.6), to which was added either *L. mesenteroides* D-lactate dehydrogenase or rabbit muscle L-lactate dehydrogenase. No increase in absorbance was observed with L-lactate dehydrogenase, but an increase in absorbance of 0.023 was observed with D-lactate dehydrogenase.

As controls, authentic D-lactate was oxidized uniquely by D-lactate dehydrogenase, and L-lactate by L-lactate dehydrogenase. Identical large scale reductions of pyruvate were carried out using D-lactate dehydrogenase and L-lactate dehydrogenase, giving after HPLC purification lactic acid products which were oxidized by D-lactate dehydrogenase and L-lactate dehydrogenase, respectively. These results imply that VanH carries out a D-specific reduction of pyruvate, to the extent of >96% enantiomeric excess.

Determination of N-Terminal Sequences of D-*Lactate Dehydrogenases.* Samples of D-lactate dehydrogenases from *L. mesenteroides* and *L. leichmanii* (Sigma) were transferred by electroblotting from a 9% SDS–PAGE gel to PVDF membrane, and N-terminal sequences were determined by Dr. W. Lane, Harvard Microchemistry Facility. The N-terminal sequences obtained are shown in Figure 1.

Assays of 2-Hydroxy Acids as Substrates for VanA. k_{cat} values were determined by coupled spectrophotometric assay (Daub et al., 1988), in the presence of 10 mM D-alanine and 50 mM DL-2-hydroxy acid, in VanA assay buffer [100 mM Tris (pH 8.6), 10 mM $MgCl_2$, 10 mM KCl]. K_m values were determined by thin layer chromatographic separation of [^{14}C]D-Ala-X product from [^{14}C]D-Ala-D-Ala at varying concentrations of X, followed by scintillation counting. Assays (25 μL) contained 100 mM Tris (pH 8.6), 10 mM $MgCl_2$, 10 mM KCl, 0.2 mM [U-^{14}C]D-Ala (5 nmol, 40 μCi/μmol), 6 mM ATP, VanA (3.2 μg), and DL-2-hydroxy acid (0.1–80 mM). Reactions were incubated for 1 h at 25 °C, and 10 μL of each reaction was spotted onto a cellulose thin layer chromatography plate (Kodak), eluted for 3 h in a *n*-butanol/acetic acid/water (12:3:5), dried, and exposed to film overnight. Spots visualized by autoradiography corresponding to D-Ala-X product were cut out and scintillation counted. K_m values were calculated by plots of 1/(D-Ala-X product formation) against 1/[X].

A K_m value of 7.1 mM was measured for D-lactate by measurement of the incorporation of radioactivity into D-Ala-[^{14}C]D-Lac at varying concentrations (2–40 mM) of [^{14}C]D-lactate, generated from [^{14}C]pyruvate by *L. mesenteroides* D-lactate dehydrogenase, and a fixed concentration of D-alanine (1 mM).

Preparation of Crude Extract of BM4147 and BM4147-1. Cultures of *E. faecium* strains BM4147 and BM4147-1 (500 mL) were grown in brain heart infusion media at 37 °C for 8 h, BM4147 in the presence of 10 μg/mL vancomycin. Cells were harvested by centrifugation at 8000*g* for 10 min, resuspended in 10 mL of HEPES lysis buffer [100 mM HEPES buffer (pH 7.2) containing 300 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, and 5 mM DTT]. The suspensions were then passed twice through a French press at 1000 psi, and cell debris was pelleted by centrifugation at 25000*g* for 30 min.

Powdered ammonium sulfate was gradually added to the supernatant to a final concentration of 70% saturation, and the solution was stirred for 1 h. The precipitate was collected by centrifugation at 12000*g* for 30 min and stored at 4 °C. When required for assays, aliquots of ammonium sulfate pellets were resuspended in HEPES lysis buffer.

Synthesis of Peptides and Depsipeptides. NAc-D-Ala-D-Ala was prepared by acetylation of commerical D-Ala-D-Ala with freshly distilled acetic anhydride (Nieto & Perkins, 1971).

Dipeptides were prepared from NAc-D-Ala and appropriately protected amino acids by conventional methodologies. Benzyl esters of D-lactate and DL-2-hydroxybutyrate were prepared by the general method of Gisin et al. (1969). Depsipeptides were prepared from Cbz-D-Ala and hydroxy acid benzyl esters using isoprenyl chloroformate as coupling agent in the presence of dimethylaminopyridine and triethylamine in dichloromethane (Zeggaf et al., 1989). Debenzylation was carried out in ethanol under H_2 atmosphere with 10% Pd over charcoal for 3–4 h at 23 °C. After filtration through Celite and concentration at room temperature, the crude compounds were applied to a Sephadex G-10 column (35 × 1 cm) and eluted with water. Depsipeptide containing fractions were lyophilized and further purified by RP-HPLC (Bio-Rad HI Pore 318, 250 × 21.5 mm) using 25 mM NH_4HCO_3 as the mobile phase and collecting fractions at 0 °C followed by lyophilization. The free depsipeptides were stored at −80 °C.

Diastereomers of D-Ala-DL-2-hydroxybutyrate were obtained as separate peaks by RP-HPLC. D-Ala-D-2-hydroxybutyrate was identified by the retention time of the ^{14}C-labeled compound prepared by incubation of [^{14}C]D-Ala with Van A and DL-2-hydroxybutyrate.

D-*Ala*-D-*lactate.* ^{1}H NMR (D_2O) δ: 4.82 (quart., J = 7 Hz, 1H, OCH), 4.09 (quart., J = 7 Hz, 1H, NCH), 1.53 (d, J = 7 Hz, 3H, CH_3), 1.48 (d, J = 7 Hz, 3H, CH_3); FAB mass spectrum: m/z 162 (M + 1); calculated 162.0762, found 162.0760.

D-*Ala*-D-*2-hydroxybutyrate.* ^{1}H NMR (D_2O) δ: 4.7 (t, J = 7 Hz, 1H, OCH), 4.07 (quart., J = 7 Hz, 1H, NCH), 1.75 (quintet, J = 7 Hz, 2H, CH_2CH_3), 1.48 (d, J = 7 Hz, 3H, CH_3), 0.86 (t, J = 7 Hz, 3H, CH_2CH_3); FAB mass spectrum: m/z 176 (M + 1); calculated 176.0918, found 176.0899.

The N-acetylated depsipeptides were prepared by acetylation of the free depsipeptides with acetic anhydride (1.1 equiv) in ethanol in the presence of diisopropylethyl amine (1.1 equiv) or by coupling of *N*-Ac-D-alanine with the appropriate hydroxy acid benzyl ester. The N-acylated depsipeptides were purified by chromatography over Sephadex G-10 followed by RP-HPLC using 0.1% TFA as the mobile phase and eluting with a linear gradient of 0–25% CH_3CN (*N*-Ac-D-Ala-D-lactate) or 0–30% (*N*-Ac-D-Ala-DL-2-hydroxybutyrate).

NAc-D-*Ala*-D-*lactate.* ^{1}H NMR (D_2O) δ: 4.55 (quart., J = 7 Hz, 1H, OCH), 4.42 (quart., J = 7 Hz, 1H, NCH), 2.01 (s, 3H, CH_3CO), 1.53 (d, J = 7 Hz, 3H, CH_3), 1.47 (d, J = 7 Hz, 3H, CH_3); FAB mass spectrum: m/z 204 (M + 1); calculated 204.0867, found 204.0873.

NAc-D-*Ala*-DL-*2-hydroxybutyrate.* ^{1}H NMR (D_2O) δ: 4.95 (m, 1H, OCH), 4.42 (quint., J = 4 Hz, 1H, NCH), 2.02 (s, 3H, CH_3CO), 1.9 (m, 2H, CH_2), 1.46 [d, J = 7 Hz, 3H,

Table II: Kinetic Data for VanH and D-Lactate Dehydrogenases[a]

	VanH			*Leuconostoc mesenteroides* D-lactate DH			*Lactobacillus leichmanii* D-lactate DH		
substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} s^{-1})
pyruvate	40	1.45	2.8×10^4	2680	0.075	2.2×10^7	290	0.14	1.3×10^6
α-ketobutyrate	35	2.6	1.4×10^4	1900	12.5	9.2×10^4	252	34	4.6×10^3
α-ketovalerate	35	40	880	<100[b]			<10[b]		
α-ketoisovalerate	5.2	≈150[c]	35	<100[b]			<10[b]		
α-ketocaproate	2.4	20	120	<100[b]			<10[b]		
α-ketoisocaproate	5.2	31	168	<100[b]			<10[b]		
phenylpyruvate	<2[b]			1150	6.7	1.0×10^5	55	11.7	2.9×10^3
K_m (NADPH) (μM)		≈2[c]			96			190	
K_m (NADH) (μM)		21			8.0			10	
V_{NADH}/V_{NADPH}		0.86			0.71			2.95	

[a] Assays were carried out as described under Materials and Methods. [b] Negligible substrate conversion is observed; the maximum estimate of k_{cat} is given. [c] The calculated K_m value lies outside range of possible substrate concentrations (2 μM–80 mM); the estimated value is ±50%.

CH_3(Ala)], 0.98 (t, J = 7 Hz, 1.5 H, CH_3 (hydroxybutyrate isomer 1), 0.95 (t, J = 7 Hz, 1.5 H, CH_3 (hydroxybutyrate isomer 2); FAB mass spectrum: m/z 218 (M + 1); calculated 218.1023, found 218.1036.

Assays of D-*Ala*-D-*Ala Adding Enzyme.* D-Ala-D-Ala, DL-Ala-DL-Met, DL-Ala-DL-Phe, D-Ala-D-Lac, and D-Ala-D-HBut were assayed as substrates for *E. coli* D-Ala-D-Ala adding enzyme using the phosphate release assay (Duncan et al., 1990). Assays (200 μL) contained 100 mM Tris (pH 8.6), 10 mM $MgCl_2$, 1 mM ATP, 5 nmol of UDPMurNAc-L-Ala-D-Glu-*m*-DAP, and dipeptide substrate (0.05–20 mM) and were incubated for 10 min at 37 °C prior to quenching with color reagent.

[^{14}C]D-Ala-[^{14}C]D-Ala, [^{14}C]D-Ala-D-Abu, [^{14}C]D-Ala-D-Met, [^{14}C]D-Ala-D-Phe, [^{14}C]D-Ala-D-Lac, and [^{14}C]D-Ala-D-HBut were prepared by incubation of [^{14}C]D-Ala with the appropriate amino or hydroxy acid (50 mM) with VanA and 10 mM ATP in VanA assay buffer for 2 h at 25 °C. ^{14}C-Labeled dipeptides were then incubated with *E. coli* D-Ala-D-Ala adding enzyme (4 μg), BM4147 crude extract (100 μg), or BM4147-1 crude extract (100 μg) for 16 h in VanA assay buffer containing 6 mM ATP and 20 nmol of UDPMurNAc-L-Ala-D-Glu-*m*-DAP. Formation of [^{14}C]UDPMurNAc–pentapeptide was observed by paper electrophoresis (Mengin-Lecreulx et al., 1982), eluting with 2% formic acid at 1500 V for 2 h, followed by autoradiography.

Vancomycin Binding Studies. Interaction of N-acetylated peptides and depsipeptides was monitored by UV difference spectroscopy at 283 or 240 nm (Nieto & Perkins, 1971) and by titration of ligand with a ε-*N*-acetyl-N^{α}-dansyl-L-lysyl-D-alanyl-D-alanine:vancomycin complex, as previously described (Popienek & Pratt, 1987).

Results

Purification of VanH. VanH was purified 3000-fold from *E. coli* strain JM83 containing recombinant plasmid pSD8 (S. Dutka-Malen, unpublished results), which expressed VanH at low levels (see Table I). During the purification it was observed that VanH preferentially utilized NADPH as a cofactor, so an ADP agarose column was used as an affinity column, which bound VanH and effected a 73-fold purification in one step. Purified enzyme had specific activity 68 units/mg and showed a single band at 35 kDa by SDS–PAGE.

Characterization of VanH and D-*Lactate Dehydrogenases.* Purified VanH was characterized kinetically, as described under Materials and Methods (see Table II). VanH can utilize either NADPH or NADH as a cofactor and can reduce a number of aliphatic α-keto acids. There is a slight preference for NADPH, which may be rationalized by the presence of a hydrophobic amino acid rather than an acidic amino acid at the final position of the NAD-binding fold in the amino acid sequence (Arthur et al., 1991).

Although sequence similarity had been observed with D-hydroxyisocaproate dehydrogenase from *Lactobacillus casei* (Lerch et al., 1989), which can reduce a wide range of long-chain aliphatic and aromatic α-keto acids (Hummel et al., 1984), the specificity of VanH was found to favor smaller side chains, the best two substrates being pyruvate and 2-ketobutyrate. This immediately suggested similarity with D-lactate dehydrogenases (D-LDH's), which are found in many species of *Lactobacillus* and *Leuconostoc* Gram-positive bacteria (Garvie, 1980). Therefore, commercially available D-LDH's from *L. mesenteroides* and *L. leichmanii* were also characterized kinetically, and N-terminal sequences were obtained.

The D-LDH enzymes could also utilize both NADPH and NADH, although they both showed a preference for NADH, perhaps reflecting their degradative role, rather than the biosynthetic role of VanH. Both D-LDH enzymes reduced only pyruvate, 2-ketobutyrate, and phenylpyruvate (which VanH did not reduce) and were markedly more specific for pyruvate than VanH. Nevertheless, some sequence similarity was detected between the N-terminal sequences of the D-LDH's, D-hydroxy-isocaproate dehydrogenase, and VanH (see Figure 1), suggesting that these D-specific α-keto acid reductases are evolutionarily related. They are also all of similar size, VanH being 35 kDa, *L. mesenteroides* D-LDH 36 kDa, *L. leichmanii* D-LDH 37 kDa, and *L. casei* D-hydroxy-isocaproate dehydrogenase 38 kDa.

The stereospecificity of VanH was demonstrated by large scale reduction of pyruvate, HPLC purification of the lactic acid product, and NAD^+-dependent oxidation by D-lactate dehydrogenase, but not L-lactate dehydrogenase (see Materials and Methods), implying that VanH is, as expected, D-specific.

Ester Formation by VanA. Having determined that VanH is capable of synthesizing D-2-hydroxycarboxylic acids, a series of authentic DL-2-hydroxycarboxylic acids were tested as substrates for VanA, by conversion of [^{14}C]D-Ala-X product and separation from [^{14}C]D-Ala-D-Ala by thin layer chromatography. By this method VanA was found to be able to form a D-Ala-X product with 2-hydroxybutyrate, 2-hydroxyvalerate, 2-hydroxycaproate, and phenyllactate. Product formation with D-lactate was complicated by the comigration of D-Ala-D-Ala and D-Ala-D-Lac by thin layer chromatography, so [^{14}C]D-lactate was prepared by NADH-dependent reduction of [^{14}C]pyruvate with *L. mesenteroides* D-lactate dehydrogenase. Incubation of unlabeled D-alanine with

Table III: Kinetic Data for α-Hydroxy Acids as Substrates for VanA[a]

product (D-Ala-X)	K_m (D-Ala) (mM)	K_m (X) (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
D-Ala-D-Ala	3.8	38	295	130
D-Ala-D-Lac	3.2	7.1	94	220
D-Ala-D-HBut	4.1	0.60	108	3000
D-Ala-D-HVal	ND	3.2	156	810
D-Ala-D-HCap	ND	11	66	100
D-Ala-D-Phlac	ND	≈60	75	≈20

[a] Assays were carried out as described under Materials and Methods. Abbreviations: Lac, lactate; HBut, 2-hydroxybutyrate; HVal, 2-hydroxyvalerate; HCap, 2-hydroxycaproate; Phlac, phenyllactate; ND, not determined.

[^{14}C]D-lactate gave a radioactive spot comigrating with D-Ala-D-Ala by thin layer chromatography, corresponding to D-Ala-D-Lac, indicating that D-lactate is also a substrate for VanA.

In order to confirm the identity of the depsipeptide products, authentic depsipeptides D-Ala-D-Lac and D-Ala-D-HBut were synthesized chemically, as described under Materials and Methods. These standards showed identical mobility by thin layer chromatography and HPLC with ^{14}C-labeled VanA products, indicating that the modified specificity of VanA is in the C-terminal D-alanine site, as observed for the α-amino acid specificity of VanA (Bugg et al., 1991).

K_m values for the hydroxy acids were measured by separation of [^{14}C]D-Ala-X products by thin layer chromatography, followed by scintillation counting, at a range of substrate concentrations. The results (Table III) indicate that 2-hydroxybutyrate is the best substrate, with a K_m of 0.6 mM—the lowest K_m value observed thus far for VanA. This value is significantly lower than the K_m of 7.1 mM obtained for D-lactate, indicating the longer side chain preference of VanA, also observed for its amino acid specificity (Bugg et al., 1991). The pattern of side chain specificity observed for the α-hydroxy acid series appears to be slightly different from the α-amino acid series, particularly comparing D-phenylalanine (K_m 6.0 mM) and D-phenyllactate, perhaps indicating a slightly different mode of binding for the α-hydroxy acids and α-amino acids. However, measurement of a K_m value for D-aminobutyrate by the thin layer chromatography assay gave a value of 0.77 mM, indicating that the ethyl side chain is preferred in both series.

Observation of Mechanistic Steps in BM4147. Having characterized functional roles for purified VanH and VanA proteins in a vancomycin resistance mechanism, crude extract from vancomycin-resistant *E. faecium* BM4147 and vancomycin-sensitive *E. faecium* BM4147-1 (lacking plasmid pIP816) was prepared. Incubation with NADPH and [^{14}C]pyruvate followed by thin layer chromatography gave rise to enhanced synthesis of [^{14}C]lactate (presumably D-lactate) in BM4147 relative to BM4147-1 (data not shown), which is consistent with the presence of VanH in BM4147.

Incubation of crude extract with [^{14}C]D-alanine, ATP, and three α-hydroxy acids followed by thin layer chromatography gave the chromatogram shown in Figure 2. The lower part of the chromatogram is smeared, presumably due to conversion of [^{14}C]D-alanine to [^{14}C]L-alanine by alanine racemase and further metabolism of L-alanine by other enzymes in the extract. Nevertheless, it is possible to discern in lanes 6, 7, and 8 the production with BM4147 extract of spots corresponding to D-Ala-D-HBut, D-Ala-D-HVal, and D-Ala-D-HCap, consistent with the presence of VanA in BM4147. The reason for the weak appearance in lane 6 of D-Ala-D-HBut, which

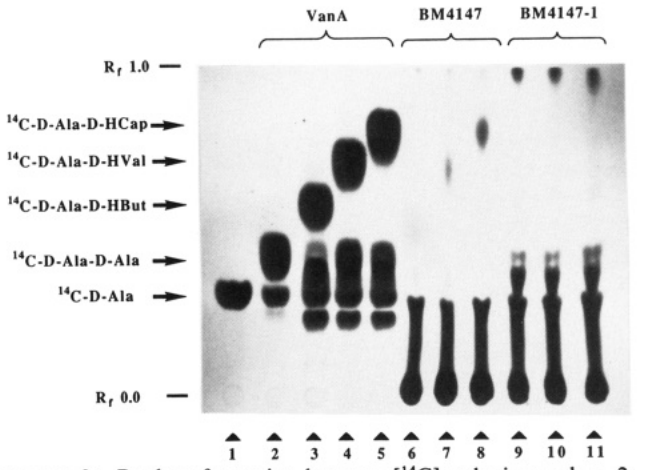


FIGURE 2: Product formation between [^{14}C]D-alanine and DL-2-hydroxycarboxylic acids with VanA and crude extract from *E. faecium* BM4147 and BM4147-1. Assays were carried out as described under Materials and Methods. Incubations 2–5 contain VanA, 6–8 contain BM4147 crude extract (100 μg), and 9–11 contain BM4147-1 crude extract (100 μg). Incubations 3, 6, and 9 contain DL-2-hydroxybutyrate (HBut), 4, 7, and 10 contain DL-2-hydroxyvalerate (HVal), and 5, 8, and 11 contain DL-2-hydroxycaproate (HCap). No enzyme was added to incubation 1. The spots at R_f 0.6 in lanes 9–11 comigrate with pyruvic acid, which could be formed from D-alanine by D-alanine dehydrogenase. The identity of the spots at R_f 1.0 in lanes 6–11 is not known.

Table IV: Substrate Specificity Data for *E. coli* D-Ala-D-Ala Adding Enzyme[a]

substrate	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
D-Ala-D-Ala	0.054	1080	3.3×10^5
D-Ala-D-Met	21	34	27
D-Ala-D-Phe	32	55	29
D-Ala-D-Lac	0.095	830	1.5×10^5
D-Ala-D-HBut	2.6	550	3.5×10^3

[a] Assays were carried out as described under Materials and Methods.

is produced most rapidly by VanA, is unclear, but it could be due to the presence of D-specific esterases or peptidases in the extract. In lanes 9, 10, and 11 there is no evidence of synthesis of D-alanyl esters by BM4147-1 extract, but [^{14}C]D-Ala-D-Ala is produced, whereas very little D-Ala-D-Ala synthesis is observed with BM4147 using the same amount of protein. This preliminary observation suggests that the chromosomal D-Ala-D-Ala ligase activity in BM4147 is inhibited or repressed to a significant extent.

Specificity of D-Ala-D-Ala Adding Enzyme. If the D-Ala-X VanA products are to be incorporated into peptidoglycan they must be substrates for D-Ala-D-Ala adding enzyme, which synthesizes UDPMurNAc–pentapeptide from UDPMurNAc–tripeptide and D-Ala-D-Ala (Walsh, 1989). Accordingly, a series of synthetic dipeptides and depsipeptides were assayed kinetically against *E. coli* D-Ala-D-Ala adding enzyme (Table IV). Depsipeptides D-Ala-D-Lac and D-Ala-D-HBut are both turned over rapidly by the enzyme, implying that the D-Ala-D-Ala amide bond is not critical for substrate recognition. D-Ala-D-HBut has a K_m value of 2.6 mM, which is much higher than that of D-Ala-D-Lac, but its catalytic efficiency of 3.5×10^3 M^{-1} s^{-1} is still comparable with the catalytic efficiencies of the preceding enzymes VanH and VanA. The dipeptides D-Ala-D-Met and D-Ala-D-Phe, which are alternative VanA products (Bugg et al., 1991), are turned over at very low catalytic efficiencies by the *E. coli* enzyme, which may not be sufficiently high for these to be considered likely intermediates in vivo.

In order to assess the specificity of D-Ala-D-Ala adding enzyme from *E. faecium* BM4147, ^{14}C-labeled dipeptides, and

Table V: Vancomycin Binding Experiments[a]

compound	K_d (mM)
NAc-D-Ala-D-Ala	0.054
NAc-D-Ala-D-Phe	>15[b]
NAc-D-Ala-D-ABut	>18[b]
NAc-D-Ala-D-Lac	>45[b]
NAc-D-Ala-D-HBut	>73[b]
N,N'-diacetyl-L-Lys-D-Ala-D-Ala	0.021
N,N'-diacetyl-L-Lys-D-Ala-D-Lac	>38[b]

[a] Binding assays were carried out using the UV binding assay of Nieto and Perkins (1971). [b] Little or no binding was observed at the highest ligand concentration (experimental error 10%). The lower limit quoted for K_d is five times the maximum concentration of ligand at which no binding is observed. Abbreviations: ABut, 2-aminobutyrate.

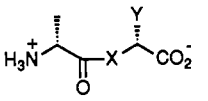
depsipeptides were generated using VanA and were incubated with crude extract from *E. faecium* BM4147 and BM4147-1 (see Materials and Methods). In each case [^{14}C]MurNAc–pentapeptide was detected by paper electrophoresis, although product formation was weaker for [^{14}C]D-Ala-D-Met and [^{14}C]D-Ala-D-Phe. Thus, although the substrate specificity of *E. faecium* D-Ala-D-Ala adding enzyme has not been quantitatively assessed, it does not appear to be qualitatively different from the *E. coli* enzyme, and there is no evidence or apparent requirement for a vancomycin-inducible D-Ala-D-Ala adding enzyme in BM4147.

Specificity of Vancomycin Binding. In order to model the binding of peptidyl-D-Ala-X peptidoglycan termini by vancomycin, we have synthesised a number of *N*-acetyl-D-Ala-X dipeptides and depsipeptides and have assayed their binding to vancomycin using a UV binding assay (Nieto & Perkins, 1971). Peptides terminating in D-Ala showed good interaction with vancomycin (K_d values 54 and 21 μM for NAc-D-Ala-D-Ala and *N,N'*-diacetyl-L-Lys-D-Ala-D-Ala, respectively), while for peptides with non-D-Ala C-termini (D-Abu, D-Met, D-Phe) and depsipeptides terminating in D-Lac or D-HBut little or no binding to vancomycin was observed under the conditions used (Table V). The error in these UV studies was estimated to be 10% allowing a lower limit for K_d to be established for peptides and depsipeptides with poor affinity for vancomycin. Similar data were also obtained using a fluorescence binding assay (Popieniek & Pratt, 1987) (not shown). Our results therefore indicate that substitution of a larger side chain in the C-terminal position gives rise to >300-fold lower binding by vancomycin, and substitution of the amide NH for O gives >1000-fold lower binding, consistent with previous binding studies (Nieto & Perkins, 1971; Popieniek & Pratt, 1987).

Discussion

The identification of vancomycin resistance protein VanA as a D-alanine:D-alanine ligase of altered substrate specificity immediately suggested that the mechanism of glycopeptide resistance involved biosynthesis of a novel D-Ala-X dipeptide by VanA which could be incorporated into peptidoglycan but could not be recognized by vancomycin (Bugg et al., 1991). The precise identity of X was, however, unclear, due to the limitations of availability of other D-amino acids and the substrate specificity of later enzymes involved in peptidoglycan assembly. Sequencing of the resistance gene *vanH* and sequence similarity with D-specific α-keto acid reductases (Arthur et al., 1991) has suggested that X might not be a D-amino acid but a D-hydroxy acid, which if ligated with D-alanine by VanA could generate a novel depsipeptide peptidoglycan precursor.

Purification and characterization of VanH has confirmed that it is indeed a D-specific α-keto acid reductase, which



	DdlA/B	VanA
X	*NH*	NH, *O*
Y	H, *Me*, Et	H, Me, *Et*, Pr, Bu, Bz

FIGURE 3: Dipeptide product specificity of VanA compared with D-Ala-D-Ala ligases DdlA and DdlB (Zawadzke et al., 1991). The preferred substituent in each case is indicated in italics.

appears to be related to D-lactate dehydrogenases found in *Leuconostoc* and *Lactobacillus* Gram-positive bacteria. Interestingly, it is precisely these bacteria that possess intrinsic resistance to glycopeptides (Nicas et al., 1989), of unknown mechanism, suggesting a possible connection between intrinsic chromosomal glycopeptide resistance and recently emergent plasmid-mediated glycopeptide resistance.

The finding that the D-hydroxy acid products of VanH are substrates for VanA establishes a functional link between VanH and VanA and provides the crux of the proposed vancomycin resistance mechanism, since the change from dipeptide to depsipeptide product specificity effectively removes the amide NH of the terminal D-alanine which subsequently interacts with vancomycin. Thus there are *two* changes in specificity between Gram-negative D-Ala-D-Ala ligases and VanA (see Figure 3): the side-chain specificity of the C-terminal D-alanine, observed both the α-amino acid series (Bugg et al., 1991) and the α-hydroxy acid series, favoring an ethyl side chain in both series; and the ability of VanA to form an ester bond rather than an amide bond. This is a remarkable change in specificity, since the α-amino group ($pK_a \approx 9.5$) would be expected at physiological pH ranges to be several orders of magnitude more nucleophilic than the α-hydroxy group ($pK_a \approx 14$). None of the *E. coli* or *S. typhimurium* D-Ala-D-Ala ligases show any capacity to use the hydroxy acids as nucleophiles. The molecular basis for this change in specificity remains to be determined, but it is reasonable to speculate that there is a negatively charged active site residue which binds (and perhaps deprotonates) the α-amino group of the C-terminal D-alanine in D-Ala-D-Ala ligase but which is absent or mutated in VanA. One piece of data consistent with this hypothesis is the higher pH_{opt} for VanA of 8.6 as opposed to 7.8 for DdlA.

Comparing the side-chain specificities of VanH and VanA, the most likely candidate for X is D-2-hydroxybutyrate, for which the k_{cat}/K_m for VanA is 3.0×10^3 M^{-1} s^{-1}. This is considerably higher than the value for D-Ala-D-Phe synthesis of 8.2×10^2 M^{-1} s^{-1} but is still less than the k_{cat}/K_m for D-Ala-D-Ala synthesis by DdlA of 2.0×10^4 M^{-1} s^{-1}. This disparity may explain the high degree of overexpression of VanA in *E. faecium* BM4147 (Dutka-Malen et al., 1990), which may be necessary if VanA carries a substantial amount of flux through peptidoglycan synthesis in vancomycin-resistant cells. This overexpression may be explained by the presence of a highly optimized Shine–Dalgarno site preceding the *vanA* gene.

Having shown that it is possible for purified VanH and VanA to synthesize a D-alanyl ester such as D-Ala-D-hydroxybutyrate, we have also demonstrated that the same transformations are found in crude extract from vancomycin-resistant *E. faecium* BM4147 but not in vancomycin-sensitive *E. faecium* BM4147-1. The preliminary observation that D-Ala-D-Ala ligase activity is very low in extract from

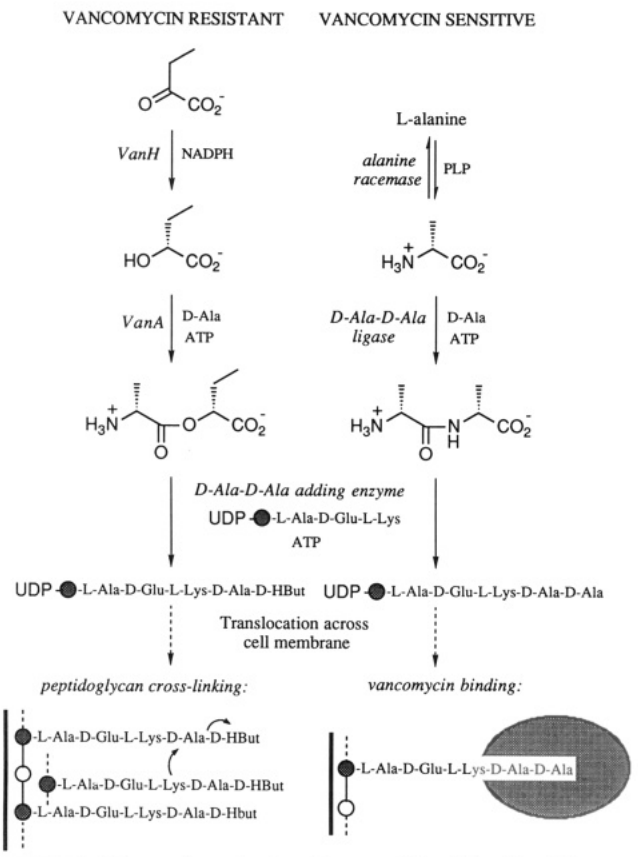


FIGURE 4: Proposed mechanism for assembly of depsipeptide peptidoglycan precursor in *E. faecium* BM4147, compared with normal steps of peptidoglycan assembly. The cross-hatched circle represents MurNAc and HBut represents hydroxybutyrate.

BM4147 is consistent with some form of down-regulation of the chromosomal D-Ala-D-Ala ligase(s), which would facilitate entry of depsipeptides into peptidoglycan biosynthesis by removing the competing D-Ala-D-Ala. We have also shown that D-Ala-D-HBut is a substrate for the next enzyme of peptidoglycan assembly, D-Ala-D-Ala adding enzyme, both with purified *E. coli* enzyme and with BM4147 crude extract. While this does not prove that a depsipeptide peptidoglycan precursor is produced in vivo, the ability to make such precursors is provided by the enzyme activities of VanH and VanA, whose corresponding *vanH* and *vanA* genes are both required for resistance (Arthur et al., 1991).

If a depsipeptide peptidoglycan precursor is produced in vancomycin-resistant cells, the question arises whether such a precursor could be cross-linked. Rasmussen and Strominger (1978) have shown that Ac_2-L-Lys-D-Ala-D-lactate is a good substrate for D-alanine carboxypeptidases, which are highly analogous to the transpeptidases which cross-link peptidoglycan. It is therefore not unreasonable to suppose that normal cross-linking can occur using depsipeptide peptidoglycan precursors (Figure 4). It will be of interest to determine which specific PBP's are active on depsipeptide peptidoglycan precursors, in view of the increased penicillin susceptibility of vancomycin-resistant strains (Courvalin, 1990) and the existence of a vancomycin-inducible DD-carboxypeptidase activity (Al-Obeid et al., 1990).

The proposed resistance mechanism (Figure 4) ultimately depends on the fact that a depsipeptide peptidoglycan precursor is not recognized by vancomycin. We have shown that synthetic analogues of general structure *N*-acetyl-D-Ala-D-X show poor interaction with vancomycin as determined by UV spectroscopy. Increasing the side-chain size to an ethyl or

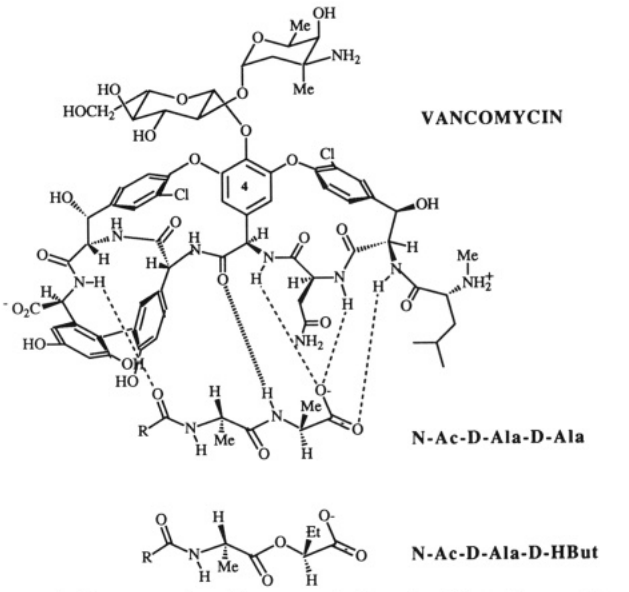


FIGURE 5: Representation of vancomycin:*N*-acyl-D-Ala-D-Ala complex (Barna & Williams, 1984) and comparison with structure of *N*-acyl-D-Ala-D-HBut. Hydrogen bonds are represented by dashed lines. The hydrogen bond disrupted in the vancomycin:*N*-acyl-D-Ala-D-HBut complex is indicated in bold. Ring 4 of vancomycin is indicated (see Discussion).

benzyl group (aminobutyrate and Phe) decreased the affinity of the *N*-acyl dipeptides for vancomycin. NMR studies have demonstrated that the C-terminal D-Ala methyl group is positioned directly over an aromatic ring (ring 4) (Williams & Butcher, 1981) and is "capped" by the amino sugar vancosamine (Kannan et al., 1988) thereby effectively excluding side chains with more steric bulk than a methyl group. In addition, replacement of the NH of the terminal D-alanine, which forms a hydrogen bond with vancomycin, with an oxygen atom, which cannot hydrogen bond (Figure 5) (NAc-D-Ala-D-Lac) also resulted in decreased affinity for vancomycin, indicating the importance of this crucial hydrogen bond. Finally, NAc-D-Ala-D-HBut, which combines steric bulk and loss of the amide NH, did not demonstrate detectable affinity for vancomycin as expected.

The substrate specificity of VanA could therefore account for vancomycin resistance by synthesis of either a D-Ala-D-X dipeptide (Bugg et al., 1991) or a D-Ala-X depsipeptide, which could be incorporated into peptidoglycan but is not recognized by vancomycin. However, the fact that both the *vanA* and *vanH* genes are required for resistance in *E. faecium* BM4147 (M. Arthur and P. Courvalin, manuscript in preparation), together with the observed substrate specificity of D-Ala-D-Ala adding enzyme (Table IV), suggests that in this strain a depsipeptide peptidoglycan precursor is produced. If the X component of D-Ala-X is indeed D-2-hydroxybutyrate, as our results suggest, then further studies may reveal whether the source of the 2-ketobutyrate substrate for VanH is chromosomally encoded or plasmid encoded and may shed light on the mechanism by which vancomycin resistance is induced (by vancomycin) and regulated. It may be possible to isolate and characterize depsipeptide peptidoglycan precursors from vancomycin-resistant cell walls, but the lability of the ester bond ($t_{1/2} \approx 48$ h at pH 7.0, 25 °C; data not shown) is such that it might not survive isolation procedures, in which case tetrapeptides would be observed.

On the basis of the substrate specificities of VanH and VanA, we therefore predict that the peptidoglycan precursors of vancomycin-resistant strains have a novel chemical structure, which is not recognized by vancomycin. It remains to be

determined whether there is any connection between this proposed mechanism for high-level resistance in *E. faecium* BM4147 and the mechanisms of intrinsic glycopeptide resistance in *Leuconostoc* and *Lactobacillus* strains and low-level resistance in *Enterococcus* strains. An understanding of the molecular basis for glycopeptide resistance could then lead to the development of antiresistance agents and perhaps the development of novel "irresistible" glycopeptides capable of binding peptidyl-D-Ala-D-HBut.

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