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Identification of Vancomycin Resistance Protein VanA as a D-Alanine:D-Alanine Ligase of Altered Substrate Specificity†

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ABSTRACT: High-level glycopeptide resistance in *Enterococcus faecium* BM4147 is mediated by a 38-kDa protein VanA, whose amino acid sequence is related to Gram-negative D-alanine:D-alanine (D-Ala-D-Ala) ligases [Dutka-Malen, S., Molinas, C., Arthur, M., & Courvalin, P. (1990) *Mol. Gen. Genet. 224*, 364–372]. We report purification of VanA and demonstrate that it has D-Ala-D-Ala ligase activity but has substantially modified substrate specificity, compared with Gram-negative D-Ala-D-Ala ligases. VanA preferentially condenses D-Ala with D-Met or D-Phe, raising the possibility that its cellular role is to synthesize a modified cell-wall component, which is subsequently not recognized by vancomycin.

The vancomycin group of glycopeptide antibiotics, used clinically to treat life-threatening Gram-positive bacterial infections, functions by complexation of peptidyl-D-Ala-D-Ala termini on the surface of growing bacterial cell walls (Barna & Williams, 1984; Reynolds, 1989). Despite its clinical use for over 30 years, resistance to vancomycin has only recently emerged in strains of *Enterococcus faecium* and *Enterococcus faecalis* (Courvalin, 1990). Glycopeptide resistance is inducible by vancomycin and is associated with high-level expression of a 38–40-kDa protein that is associated with the cytoplasmic membrane (Nicas et al., 1989; Shlaes et al., 1989; Williamson et al., 1989). Subcloning of plasmid pIP816, conferring high-level vancomycin resistance in *E. faecium* strain BM4147 (Leclerq et al., 1988), allowed identification of the *vanA* gene that encodes one such inducible resistance protein designated VanA (Dutka-Malen et al., 1990). Sequencing of the *vanA* gene revealed 28–36% amino acid sequence homology with sequences of D-Ala-D-Ala ligase (Dutka-Malen et al., 1990), an enzyme that catalyzes synthesis of the D-Ala-D-Ala dipeptide for peptidoglycan assembly (Walsh, 1989).

Preliminary studies on the mechanism of resistance have excluded detoxification and impermeability of glycopeptides as possible mechanisms [reviewed in Courvalin (1990)]. In the light of its sequence similarity with Gram-negative D-Ala-D-Ala ligases, the following possible glycopeptide resistance mechanisms involving VanA were anticipated (Dutka-Malen et al., 1990): (i) overproduction of an *N*-acyl-D-Ala-D-Ala derivative, shown in vitro to compete with surface peptidyl-D-Ala-D-Ala residues for vancomycin binding (Nieto et al., 1972); (ii) complex formation with the peptidyl-D-Ala-D-Ala target, preventing binding of vancomycin; (iii) D,D-carboxypeptidase activity cleaving the terminal D-alanine residue, which is essential for binding of glycopeptides; (iv) biosynthesis of a modified peptidyl-D-Ala-D-Ala target from a D-Ala-D-Ala ligase of altered specificity. As an initial step toward elucidation of the molecular basis for vancomycin resistance, we describe purification of VanA, kinetic characterization and comparison with Gram-negative D-Ala-D-Ala ligases, and mechanistic studies to address the anticipated mechanisms.

Materials and Methods

Materials. The following materials were obtained from Sigma Chemical Co.: tris(hydroxymethyl)aminomethane base (Tris), ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT), ampicillin, DL amino acids, D-alanine, D-methionine, D-phenylalanine, D-Ala-D-Ala, D-cycloserine, vancomycin, 6-aminopenicilloic acid, DL-Ala-DL-Phe, and L-Phe-L-Ala. The following materials were obtained from Boehringer Mannheim Biochemicals: phosphoenolpyruvate (PEP), pyruvate kinase/lactate dehydrogenase (PK/LDH), reduced nicotinamide adenine dinucleotide (NADH), 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) was purchased from Amersham. 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). *Salmonella typhimurium* D-Ala-D-Ala ligase A (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

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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 VanA (See Table I). VanA was purified from JM105/pAT214 (Dutka-Malen et al., 1990). All steps were carried out at 4 °C unless otherwise specified. Enzyme activity was monitored by either D-alanine-dependent P_i assay or continuous ADP release coupled assay (Daub et al., 1988). Protein concentration was determined by the method of Bradford (1976). The standard column buffer consisted of 50 mM HEPES, 5 mM $MgCl_2$, 1 mM EDTA, and 1 mM DTT, pH 7.2.

JM105/pAT214 was grown at 37 °C in 1 L of Luria broth (LB) containing ampicillin to an 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 (3.9 g) was resuspended in 20 mL of 100 mM HEPES (pH 7.2) containing 300 mM NaCl, 5 mM $MgCl_2$, 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 50% 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 containing 300 mM NaCl. Fractions containing D-Ala-D-Ala ligase activity were pooled, and the pooled enzyme was found to be approximately 80% pure (see Table I). This enzyme was used for the experiments described in this paper. Half of this pool was further purified by elution on the AcA54 column in column buffer with no added salt. The best fraction from this column contained homogeneous VanA of specific activity 7.9 units/mg. An N-terminal sequence M-N-R-I-K-V-A-I-L-F was obtained for purified VanA (carried out by Dr. W. Lane, Harvard Microchemistry Facility), which is identical with the predicted N-terminal amino acid sequence (Dutka-Malen et al., 1990).

Kinetic Assays of VanA (See Table IIA). Kinetic assays were carried out by using the continuous ADP release coupled assay at 340 nm (Daub et al., 1988), except that the assay buffer contained 100 mM Tris (pH 8.6), 10 mM $MgCl_2$, and 10 mM KCl. The two D-alanine binding constants K_1 and K_2 were determined by the method of Neuhaus (1962b). Kinetic parameters for the (aminoalkyl)phosphinate slow-binding inhibitor (R_1 = R_2 = Me, racemic) were determined as previously described (McDermott et al., 1990).

Kinetic parameters of VanA as a D-Ala-X ligase (see Table IIB) were determined by using the same assay. Where X is not D-alanine, the K_m for D-alanine was determined at a fixed concentration of 5 mM X. K_m for X was determined at a fixed concentration of 5 mM D-alanine from the rate of mixed dipeptide synthesis, calculated by subtraction of the background rate of D-Ala-D-Ala synthesis (i.e., rate of reaction with no X added). Under saturating concentrations of D-alanine and X, the same value of v_{max} was observed in each case. No dipeptide formation was observed in the absence of D-alanine.

Thin-Layer Chromatographic Assays of VanA (See Figure 1). Assays (25 μL) contained 100 mM Tris (pH 8.6), 10 mM $MgCl_2$, 10 mM KCl, 0.2 mM [^{14}C]-D-Ala (5 nmol, 40 μCi/μmol), 6 mM ATP, enzyme (5–10 μg), and additional amino acids or mixtures of amino acids (10 mM total). Reactions were incubated for 16 h at room temperature, and 10 μL of each reaction was spotted onto a cellulose thin-layer chromatography plate, eluted for 3 h in 1-butanol–acetic acid–water (12:3:5), dried, and exposed to film overnight. Amino acids giving rise to new spots, A–D, synthesized by VanA (see Figure 1) were identified by subsequent assays with single amino acids as D-norleucine (A), D-norvaline (B), D-methionine (C), and D-phenylalanine (D). Mixed dipeptide formation was also observed to some extent with D-valine, D-leucine, D-isoleucine, D-threonine, and D-tryptophan. No higher order products (D-Ala-D-Ala-D-Ala or mixed tripeptides) were observed in any experiment.

HPLC Analysis of Product Dipeptides. Separations were carried out on a Waters C_{18} reverse-phase column with 25 mM ammonium bicarbonate as buffer A and 100% methanol as buffer B. Peptides were eluted at 1.0 mL/min for 15 min with buffer A, followed by a 0–25% buffer B gradient over 30 min. Dipeptide standard DL-Ala-DL-Phe gave two peaks of equal intensity at 23.0 and 31.0 min, corresponding to the two diastereoisomers present. L-Phe-L-Ala (enantiomeric with D-Phe-D-Ala) gave a single peak at 27.0 min. Injection of an incubation of [^{14}C]-D-alanine and D-phenylalanine with VanA followed by scintillation counting of 1-mL fractions gave a single peak of radioactivity at 23.0 min, which coeluted with one of the two DL-Ala-DL-Phe peaks. Since VanA is specific for D amino acids, the product is therefore identified as D-Ala-D-Phe.

Preparation of UDPMurNAc-L-Ala-D-Glu-m-DAP-[^{14}C]-D-Ala-[^{14}C]-D-Ala. A reaction mixture (50 μL) containing [^{14}C]-D-alanine (10 nmol, 400 nCi), ATP (4 mM), and *E. coli* D-Ala-D-Ala ligase B (5 μg) in VanA assay buffer was incubated at 25 °C for 1 h. Addition of UDPMurNAc-L-Ala-D-Glu-*m*-DAP (20 nmol), ATP (4 mM), and *E. coli* D-Ala-D-Ala adding enzyme (2 μg) in VanA assay buffer (50 μL) was followed by incubation for 16 h at 25 °C. The mixture was injected onto a Waters C_{18} reverse-phase column and eluted isocratically at 0.5 mL/min in 50 mM ammonium formate (pH 4.4), as described in Flouret et al. (1981), under which conditions UDPMurNAc-tripeptide eluted at 11.2 min and UDPMurNAc-pentapeptide at 32.0 min. The peak containing ^{14}C-labeled UDPMurNAc-pentapeptide was collected and lyophilized. Reinjection of this material gave a peak of radioactivity at 32.0 min, which coeluted with authentic unlabeled UDPMurNAc-pentapeptide.

^{14}C-Labeled UDPMurNAc-pentapeptide (0.25 nmol, 20000 dpm) was incubated with VanA (8 μg) and ATP (4 mM) in VanA assay buffer (total volume 25 μL) with and without the following additions: 10 mM D-alanine, 10 mM D-methionine, and 10 mM D-phenylalanine. After 1 and 16 h at 25 °C, 5 μL of each reaction was eluted on a cellulose plate as described above. Regions of the plate corresponding to D-alanine were excised and counted. No counts above background were detected in any sample. A 10-μL quantity of the incubation with D-phenylalanine was reinjected onto the HPLC column, and all the ^{14}C label still eluted at 32.0 min, indicating that no modification of the pentapeptide had taken place.

RESULTS

Purification of VanA. VanA was purified by a four-step procedure from *E. coli* strain JM105 containing recombinant plasmid pAT214 (Dutka-Malen et al., 1990), which expressed

Table I: Purification of VanA from JM105/pAT214

	vol (mL)	protein[a] (mg/mL)	total protein (mg)	act.[b] (units/mL)	sp act. (units/mg)	purification (x-fold)
crude extract	20.0	7.5	150	12.2	1.6	1.0
$(NH_4)_2SO_4$ ppt	5.7	22.2	127	62.5	2.8	1.7
AcA54 gel filtration	45.0	0.875	39.4	5.48	6.3	3.9
low-salt gel filtration (best fraction)[c]	8.0	0.256	2.0	2.03	7.9	4.9

[a] Determined by the Bradford protein assay (Bradford, 1976). [b] Determined by the coupled spectrophotometric assay (Daub et al., 1988). One unit is defined as the activity required to convert 1 μmol of substrate to product per minute. [c] Carried out on half of the first gel filtration pool. Data are for the best fraction only.

Table II

(A) Kinetic Properties of VanA as a D-Ala-D-Ala Ligase

substrate/inhibitor		*S. typh.* DdlA[a]	*E. coli* DdlB[a]	VanA
D-alanine	k_{cat} (min^{-1})	644	1018	295
	K_1 (μM)	1.9	3.3	3400
	K_2 (mM)	0.54	1.2	38
ATP	K_m (μM)	38	40	116
D-Ala-D-Ala	K_i (μM)	61 (NC)	70 (NC)	2300 (NC)
D-cycloserine	K_i (μM)	14 (C)	27 (C)	730 (C)
(aminoalkyl)-phosphinate	K_i (μM)	250 (C)	28 (C)	430 (C)
	k_{inact} (min^{-1})	2.5	2.3	4.9

(B) Kinetic Properties of VanA as a D-Ala-X Ligase[b]

product	K_1 (D-Ala) (mM)	K_2 (X) (mM)
D-Ala-D-Ala	3.4	38
D-Ala-D-Nva	ND	33
D-Ala-D-Nle	ND	15
D-Ala-D-Met	5.4	9.0
D-Ala-D-Phe	6.4	6.0

[a] Data for *S. typhimurium* DdlA and *E. coli* DdlB are as previously reported (Zawadzke et al., 1991). NC = noncompetitive; C = competitive. [b] Assays were carried out as described under Materials and Methods. ND = not determined.

the VanA protein to 20% of soluble cell protein (see Table I). Upon purification it was found that VanA possessed D-Ala-D-Ala ligase activity, as expected from its sequence similarity with Gram-negative D-Ala-D-Ala ligases and from genetic complementation tests (Dutka-Malen et al., 1990). VanA was liable to precipitation under low salt conditions, consistent with the fact that it is associated with the cytoplasmic membrane (Nicas et al., 1989; Shlaes et al., 1989; Williamson et al., 1989).

Kinetic Characterization of VanA. Purified VanA was characterized kinetically by using an ADP release coupled assay developed for D-Ala-D-Ala ligase (Daub et al., 1988), and a number of differences between VanA and D-Ala-D-Ala ligase were found (see Table IIA). The turnover number of VanA was 2-fold lower than those of Gram-negative D-Ala-D-Ala ligases DdlA and DdlB (Zawadzke et al., 1991), and the K_m values for the first and second D-alanine substrates were 1000-fold and 50-fold higher, respectively. VanA was inhibited by known inhibitors of D-Ala-D-Ala ligase: a reversible competitive inhibitor D-cycloserine and an (aminoalkyl)phosphinate time-dependent slow-binding inhibitor (McDermott et al., 1990). However, the K_i values for these inhibitors were 10–100-fold higher than for DdlA and DdlB. The size of the binding constants for VanA suggested that the D-alanine binding sites of VanA were modified, which prompted us to examine closely the substrate specificity of VanA.

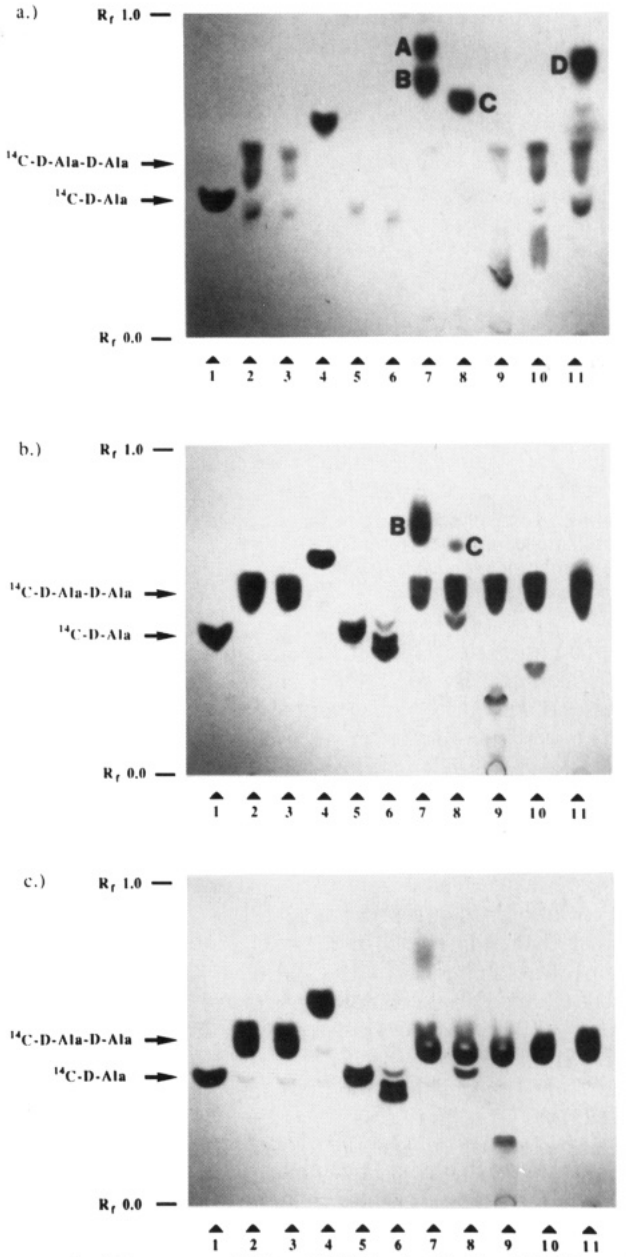

FIGURE 1: Substrate specificity of (a) VanA, (b) *E. coli* DdlB, and (c) *S. typhimurium* DdlA as assessed by mixed dipeptide formation with [^{14}C]-D-alanine. Assays were carried out as described under Materials and Methods. Additions to each assay: (1) no enzyme; (2) enzyme only; (3) +DL-Ala; (4) +D-aminobutyrate; (5) +Gly; (6) +DL-Ser; (7) +DL-Ile/DL-Leu/DL-Val/DL-Nle/DL-Nva; (8) +DL-Met/DL-Thr/DL-Pro/DL-Cys; (9) +DL-Lys/DL-Arg/DL-His/DL-Orn/*meso*-diaminopimelate; (10) +DL-Asp/DL-Glu/DL-Asn; (11) +DL-Phe/DL-Tyr/DL-Trp/DL-dopamine. Spots A–D synthesized by VanA correspond to D-Ala-D-Nle (A), D-Ala-D-Nva (B), D-Ala-D-Met (C), and D-Ala-D-Phe (D).

Substrate Specificity of VanA. The ability of VanA to synthesize a mixed dipeptide containing D-alanine was tested by incubation of enzyme with [^{14}C]-D-alanine and a series of mixtures of amino acids, followed by thin-layer chromatography and autoradiography (see Figure 1). The results show that, in contrast to the Gram-negative D-Ala-D-Ala ligases DdlA and DdlB, VanA is able to synthesize a mixed dipeptide with a number of hydrophobic amino acids, which were identified on subsequent assays as D-norvaline, D-norleucine, D-methionine, and D-phenylalanine. No reaction was observed with the L isomers of these amino acids. Addition of these amino acids to assays of VanA containing 5 mM D-alanine resulted in stimulation of enzyme activity, due to mixed di-

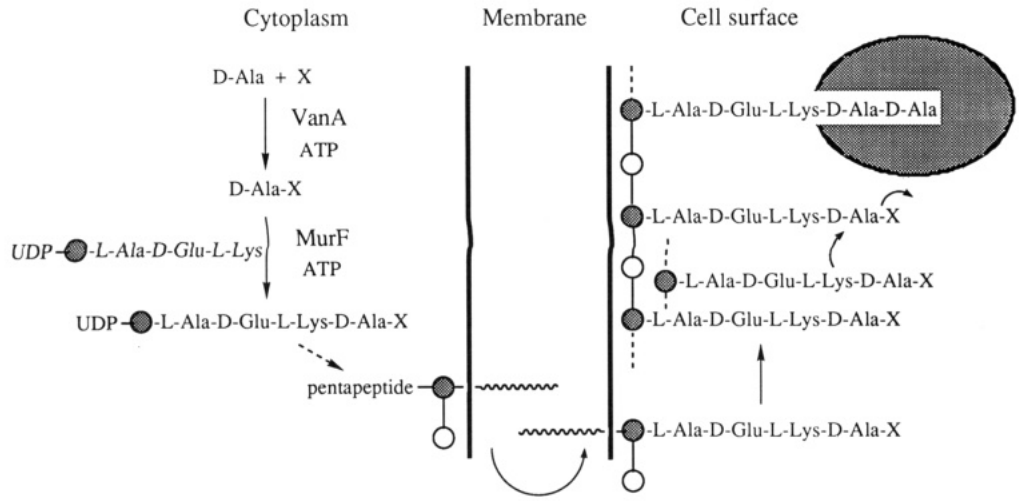

FIGURE 2: Schematic figure of the proposed target modification mechanism involving VanA. MurF is a D-Ala-D-Ala adding enzyme; the cross-hatched circle represents an *N*-acetylmuramyl residue; the open circle represents an *N*-acetylglucosamine residue. The symbol inside the membrane represents the undecaprenyl lipid carrier. Depicted on the cell surface are the binding of peptidyl-D-Ala-D-Ala by vancomycin and the cross-linking reaction of a modified peptidoglycan terminus.

peptide formation. No activity was observed in the absence of D-alanine. By subtraction of background D-Ala-D-Ala synthesis, K_m values were measured for the novel amino acids, which were 4–6-fold lower than the K_m for D-alanine of 38 mM (see Table IIB).

In order to determine the position of the novel amino acid incorporated, an incubation of [^{14}C]-D-Ala and D-Phe with VanA was analyzed by reverse-phase HPLC (see Materials and Methods). The single ^{14}C-labeled product was identified by coelution with dipeptide standards as [^{14}C]-D-Ala-D-Phe, rather than D-Phe-[^{14}C]-D-Ala. The altered specificity of VanA therefore resides in the C-terminal half of the dipeptide product. This observation is consistent with the known substrate specificity of D-Ala-D-Ala ligase from *Streptococcus faecalis*, in which the specificity of the N-terminal D-alanine site is more strict than for the C-terminal site (Neuhaus, 1962a).

Interaction of VanA with UDPMurNAc-pentapeptide. A mechanism involving binding and subsequent modification of the peptidyl-D-Ala-D-Ala target by the inducible resistance protein has been proposed by Al-Obeid et al. (1990a), on the basis of experiments using crude membrane fractions. In order to investigate this mechanism, D-Ala-D-Ala ligase and D-Ala-D-Ala adding enzyme were used to prepare a sample of UDPMurNAc-L-Ala-D-Glu-*m*-DAP-[^{14}C]-D-Ala-[^{14}C]-D-Ala, which was purified by HPLC. Incubation of the labeled pentapeptide with purified VanA in the absence or presence of 10 mM D-alanine, D-methionine, or D-phenylalanine resulted in no release of [^{14}C]-D-alanine, separable by thin-layer chromatography. VanA therefore has no carboxypeptidase or C-terminal amino acid transferase activity that might account for vancomycin resistance. Furthermore, no inhibition of VanA activity was observed upon addition of 0.5 mM pentapeptide, indicating no binding of the cell-wall pentapeptide by VanA, and no reversible or irreversible inhibition of VanA was observed in the presence of 1 mM concentrations of vancomycin or the β-lactams ampicillin or 6-aminopenicilloic acid.

Discussion

In order to characterize the molecular basis for high-level vancomycin resistance in *E. faecium* BM4147, we have purified and characterized the VanA resistance protein. We have shown that it possesses D-Ala-D-Ala ligase activity, rationalizing the sequence similarity with Gram-negative D-Ala-D-Ala ligases and complementation studies for ligase activity (Dutka-Malen et al., 1990). However, it displays markedly lower catalytic efficiency than Gram-negative D-Ala-D-Ala ligases and substantially modified substrate specificity, which can be correlated with one of the possible resistance mechanisms outlined in the introduction. In the light of the results presented in this paper, a number of possible antibiotic resistance mechanisms can be excluded and a tentative mechanism of vancomycin resistance due to VanA proposed.

Overproduction of an *N*-acyl-D-Ala-D-Ala peptide, which could account for glycopeptide resistance, appears to be very unlikely, given the kinetic properties and specificity of VanA, which synthesizes D-Ala-D-Ala at 100-fold lower catalytic efficiency than Gram-negative D-Ala-D-Ala ligases. Furthermore, VanA synthesizes only dipeptides, whereas vancomycin does not bind free D-Ala-D-Ala but requires an acylated N-terminus for efficient complexation (Nieto & Perkins, 1971). In addition, no vancomycin-antagonizing substance has been detected in the culture medium of vancomycin-resistant cells (Nicas et al., 1989).

The observation that there was no inhibition of VanA ligase activity by UDPMurNAc-pentapeptide indicates that VanA would be unable to bind the peptidyl-D-Ala-D-Ala target; hence, it could not protect the target from glycopeptide binding. In addition, analysis of the amino acid sequence of VanA suggests that the protein is not translocated through the membrane (Dutka-Malen et al., 1990).

VanA does not appear to function as a D,D-carboxypeptidase, since the purified enzyme has no ability to cleave or exchange the C-terminal D-alanine residue of a synthetic UDPMurNAc-pentapeptide. Al-Obeid et al. (1990a) have detected an inducible D,D-carboxypeptidase activity in crude membrane fractions of vancomycin-resistant strains *E. faecium* D366 and *E. faecalis* A256 and have proposed that this activity is the basis for vancomycin resistance. This carboxypeptidase activity has also been detected in *E. faecium* BM4147 (S. Dutka-Malen, unpublished results), yet purified VanA protein possesses no such carboxypeptidase activity. Strains D366 and A256 most probably produce an inducible resistance protein similar to VanA, since 8 residues out of 11 from an N-terminal sequence obtained for the inducible resistance protein from *E. faecium* D366 (Al-Obeid et al., 1990b) are identical with the N-terminal sequence of VanA. Both the origin and role

of the inducible carboxypeptidase activity therefore remain unexplained, although it is tempting to speculate that it could contribute to resistance as an accessory enzyme by cleaving the terminal D-alanine residues of the "normal" peptidyl-D-Ala-D-Ala termini synthesized by the chromosomally encoded peptidoglycan synthesis pathway.

The kinetic data obtained for VanA suggest that its cellular role is synthesis of a mixed D,D-dipeptide D-Ala-X, rather than D-Ala-D-Ala. The K_m for D-alanine of 38 mM is sufficiently high as to be considered not physiologically relevant, whereas the K_m values for D-methionine and D-phenylalanine are 4–6-fold lower. Subsequent incorporation of D-Ala-X into the cell wall would generate a modified peptidoglycan structure containing a D-Ala-X terminus, raising the possibility that such a modified terminus might not be recognized by vancomycin (see Figure 2). The binding to vancomycin of a number of analogues of Ac_2Lys-D-Ala-D-Ala ($K_d = 6.7 \times 10^{-7}$ M) has been studied by Nieto and Perkins (1971), who found that the analogue Ac_2Lys-D-Ala-D-Leu ($K_d = 1.1 \times 10^{-4}$ M) was bound 150 times less tightly than the corresponding D-Ala-D-Ala terminus, indicating that vancomycin binding is sensitive to substitution of larger side chains. If the affinity for a D-Ala-X terminus is similarly diminished by 100–200-fold, this could be sufficient in vivo to prevent to a large extent binding of vancomycin, but still allow the modified peptidoglycan to cross-link, provided that the modified structures are substrates for the transpeptidase enzymes responsible for cross-linking. In support of this postulate, UDPMurNAc-pentapeptides containing modified amino acids at position 5 have been found to be good substrates for translocation and cross-linking in *Gaffkya homari* (Carpenter et al., 1976).

Incorporation of the D-Ala-X dipeptide into peptidoglycan would be strongly dependent on the specificity of later enzymes in peptidoglycan assembly, particularly D-Ala-D-Ala adding enzyme. Although substrate specificity profiles for this enzyme have indicated that D-Ala-D-Met and D-Ala-D-Phe are both poor substrates for D-Ala-D-Ala adding enzyme from *E. coli* (Duncan et al., 1990; T.D.H. Bugg and C.T. Walsh, unpublished results) and from *S. faecalis* (Neuhaus & Struve, 1965), the specificity of D-Ala-D-Ala adding enzyme from vancomycin-resistant cells has yet to be established. If a novel D amino acid is incorporated into a D-Ala-X dipeptide, X could arise from a broad specificity amino acid racemase or transaminase, the latter of which is known to accept D-methionine and D-phenylalanine as substrates (Yonaha et al., 1975), or from further plasmid-encoded enzymes.

In conclusion, VanA is a D,D-dipeptide ligase that is structurally and functionally related to the D-Ala-D-Ala ligase enzymes found in all bacteria but which displays substantially modified substrate specificity. The altered specificity of VanA is consistent with a mechanism of resistance involving synthesis of a modified peptidoglycan structure from a D-Ala-X dipeptide, not recognizable by vancomycin (see Figure 2). The identity of VanA as a D-Ala-D-Ala ligase of altered substrate specificity can now be used as a basis for the rational design of VanA inhibitors, which could be of clinical use in the future, given the proven transferability of high-level vancomycin resistance (Leclerq et al., 1989).

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