

D-Alanyl-D-alanine Ligases and the Molecular Mechanism of Vancomycin Resistance

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Bacteria resist hypotonic shock and cell lysis through the biosynthesis of peptidoglycan-containing cell walls. This rigid polymer consists of a repeating disaccharide unit of *N*-acetylglucosamine-(β 1-4)-*N*-acetylmuramic acid to which a pentapeptide is linked through the muramyl lactyl ether carboxylate (Figure 1B). The pentapeptide is composed of L-Ala- γ -D-Glu-(a dibasic amino acid: L-Lys or *m*-diaminopimelic acid)-D-Ala-D-Ala. Depending on the bacterial species and growth conditions, roughly 50-90% of the free amines of the dibasic amino acid are recruited to form an amide cross-link with the penultimate D-Ala of another pentapeptide, either directly or through the intervention of a peptide linker such as Gly₅. The net result of this transpeptidation reaction is the formation of a rigid framework which insulates the organism from external osmotic pressures.

The basic structural unit (peptidyl disaccharide) is synthesized in the cytosol and translocated across the cellular membrane where it is added externally to the growing polymer chain. The first steps in peptidoglycan biosynthesis involve the synthesis of UDP-*N*-acetylmuramic acid from UDP-*N*-acetylglucosamine (Figure 2). This transformation requires two enzymes. The first is the chemically interesting transfer of an enolpyruvyl moiety from phosphoenolpyruvate, analogous to the well-studied enolpyruvylshikimate 3-phosphate (EPSP) synthase enzyme involved in shikimate biosynthesis¹ catalyzed by MurZ, an enzyme which has recently been cloned and overproduced in this lab.² The second reaction is the NADPH-dependent reduction of the enol to give UDP-*N*-acetylmuramic acid. The first three amino acids of the pentapeptide (L-Ala, D-Glu, *m*-DAP) are linked to UDP-*N*-acetylmuramic acid by a series of cytosolic ATP-dependent amide-forming enzymes known as adding enzymes;³ each of these enzymes releases ADP and inorganic phosphate. While detailed mechanistic analysis is lacking for the purified adding enzymes, our work on the enzymes involved in the D-alanine pathway (described below) suggests that peptide bond formation in this ATP to

Gerard D. Wright earned B.Sc. (1986) and Ph.D. (1990) degrees (with J. Honek) from the University of Waterloo, Ontario, Canada. He currently holds an NSERC postdoctoral fellowship in C.T.W.'s lab. His work on the mechanism of bacterial antibiotic resistance reflects an ongoing interest in the molecular mechanisms of antimicrobial agents.

Christopher T. Walsh received an A.B. degree in biology from Harvard College (1985) and a Ph.D. in the life sciences from The Rockefeller University (1970). He has held faculty positions in biology and chemistry at MIT where he was chairman of the Chemistry Department from 1980 to 1986. He became chairman of the Department of Biological Chemistry and Molecular Pharmacology at Harvard Medical School in 1987 and is also currently president of the Dana Farber Cancer Institute. The underlying theme in the Walsh group has been the elucidation of the mechanisms of interesting biological problems using a multidisciplinary approach while rationalizing from a decidedly chemical viewpoint.

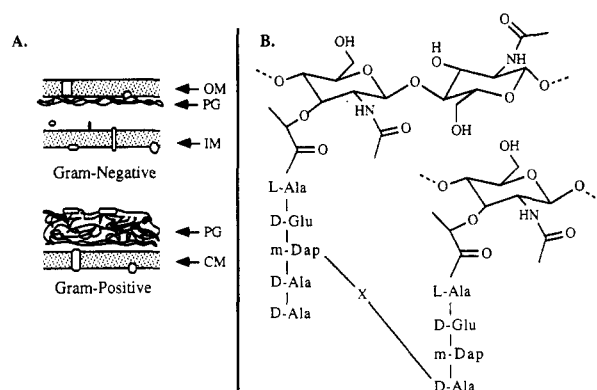


Figure 1. Bacterial cell wall organization. Panel A diagrams the two different types of cell wall structures. Gram-negative organisms envelope the peptidoglycan (PG) layer and inner cellular membrane (IM) with a lipid-containing outer membrane (OM), thereby preventing access of glycopeptide antibiotics to the PG surface. On the other hand, the PG layer of Gram-positive organisms is located directly above the cellular membrane (CM) and is accessible to glycopeptides. Panel B shows the structure of the repeating peptidoglycan unit. The peptidyl disaccharide is composed of *N*-acetylglucosamine and *N*-acetylmuramic acid. The peptide portion is linked through the carboxylate of the muramyl lactyl ether via an amide bond. The basic amino acid can be involved in an intramolecular cross-link with the penultimate D-Ala of a neighboring chain either directly or through a peptide linker (X).

ADP + P_i cleavage mode probably occurs by activation of the C-terminal amino acid carboxylate as the acyl phosphate followed by nucleophilic displacement of the phosphate by the amino group of the incoming amino acid. The C-terminal D-Ala-D-Ala unit of the UDP-muramyl pentapeptide is formed first as the dipeptide by such an ATP-utilizing D-Ala-D-Ala ligase and then linked to the UDP-*N*-acetylmuramyl tripeptide by the D-Ala-D-Ala adding enzyme⁴ (step 6 of Figure 2).

Antibiotics which interrupt peptidoglycan biosynthesis have enjoyed preeminence in antimicrobial chemotherapy. The majority of these antibiotics exploit the vulnerability of the extracellular polymer assembly steps; for example, the cross-linking transpeptidation is a major target for the β -lactam antibiotics.^{5,6} The cytosolic steps involved in peptidoglycan biosynthesis

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Table I. Properties of D-Ala-D-Ala Ligases

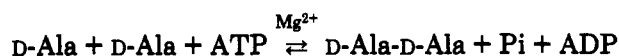
enzyme	source	MW	K_m D-Ala1 (N-term) (mM)	K_m D-Ala2 (C-term) (mM)	K_m ATP (μ M)	k_{cat} (min^{-1})
DdlA ¹²	<i>S. typhimurium</i>	39 300	0.002	0.54	38	640
DdlA ¹⁴	<i>E. coli</i>	39 300	0.006	0.55	38	440
DdlB ¹⁴	<i>E. coli</i>	32 800	0.003	1.2	40	1010
VanA ³⁴	<i>Enterococcus faecium</i>	37 500	3.4	38	116	295

have been less widely targeted by antibiotics, with the exception of such agents as phosphonomycin⁷ and D-cycloserine,⁸ which are not widely used clinically. Yet D-Ala-D-Ala biosynthesis is an attractive target for antibacterial compounds since this dipeptide is common to both Gram-negative and Gram-positive organisms and has no eukaryotic counterpart. The C-terminal D-Ala-D-Ala is also the structural unit which is recognized by the glycopeptide antibiotics such as vancomycin, which find extensive clinical use in the treatment of infections caused by Gram-positive bacteria.⁹ Bacterial D-Ala metabolism is therefore of considerable interest.

D-Ala processing for peptidoglycan biosynthesis requires three enzymes: alanine racemase, D-Ala-D-Ala ligase, and the D-Ala-D-Ala adding enzyme.⁴ The elucidation of the mechanisms of the enzymes involved in the D-alanine pathway has been the focus of this laboratory for over a decade. This Account describes recent work on the D-Ala-D-Ala ligases and their relationship to vancomycin resistance in enterococci.

D-Ala-D-Ala Ligases

Neuhaus demonstrated 30 years ago that biosynthesis of the D-Ala-D-Ala dipeptide required Mg^{2+} , a monovalent cation such as K^+ , and ATP in a partially purified *Streptococcus faecalis* preparation, according to the following equation:^{10,11}



These studies determined that the preferred N-terminal residue was D-Ala but that D-aminobutyrate and D-Ser could also be incorporated less efficiently. In contrast, the C-terminal site was less discriminating in that D-aminobutyrate, D-Ser, D-Thr, D-Nva, and D-Gly were accepted, although D-Ala was again the preferred substrate. Cloning of the gene *ddlA* and overexpression of the resultant protein from *Salmonella typhimurium* permitted the first purification to homogeneity of a D-Ala-D-Ala ligase.¹² Interestingly, insertional mutants in the *ddlA* gene resulted in strains which were still viable, suggesting either that D-Ala-D-Ala ligase is not essential or that there exists a second ligase encoding gene. Identification of two chromosomal alanine racemases, *alr* and *dadB*, which encode anabolic and catabolic racemases, respectively, lent support to the

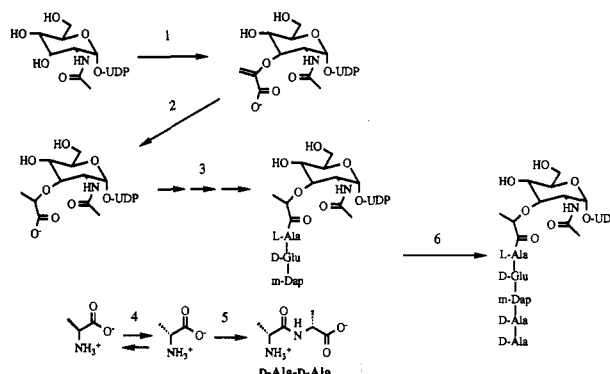


Figure 2. Intracellular biosynthetic events involved in UDP-N-acetylmuramyl pentapeptide synthesis. Step 1: Formation of UDP-N-acetylglucosamine 3-enolpyruvate. Step 2: Formation of UDP-N-acetylmuramic acid. Step 3: Formation of UDP-N-acetylmuramyl tripeptide by ATP-dependent adding enzymes. Step 4: Alanine racemase. Step 5: D-Ala-D-Ala ligase. Step 6: D-Ala-D-Ala adding enzyme.

latter hypothesis.¹³ Indeed, subsequent cloning of ligase genes in *Escherichia coli* has revealed two loci, *ddlA* and *ddlB*, which have 90% and 36% respective homology with the *Salmonella* enzyme.¹⁴ The latter gene had been cloned previously and was located in a cluster of genes implicated in peptidoglycan biosynthesis and mapped to minute 2 of the *E. coli* chromosome.¹⁵ The physiological significance for the presence of two distinct ligases remains unknown. They may mirror the Ala racemases and play catabolic and anabolic roles. If this is true, the identification of *ddlB* in the murein biosynthetic cluster on the *E. coli* genome provides circumstantial evidence for an anabolic activity for this enzyme. Alternatively, one copy may serve as a "backup" and would provide testimony for the crucial activity of this enzyme.

DdlA and DdlB have been overexpressed and purified, and their catalytic properties have been determined^{12,14} (Table I). Kinetic analysis revealed that the N-terminal D-Ala binds enzyme first and with higher affinity than the C-terminal amino acid ($K_{mN-term}/K_{mC-term} \approx 0.003-0.010$). Studies on the kinetic mechanism have revealed an ordered Ter-Ter kinetic mechanism for the *Salmonella* DdlA enzyme where binding of ATP is followed by the amino acids and release of inorganic phosphate, D-Ala-D-Ala, and ADP.¹⁶

The mechanism of D-Ala-D-Ala formation has also been explored by isotope exchange experiments.¹⁶ Positional isotope exchange has shown bridge to non-bridge oxygen exchange using $[\gamma\text{-}^{18}\text{O}_4]\text{-ATP}$ only in the presence of D-Ala, which is consistent with direct

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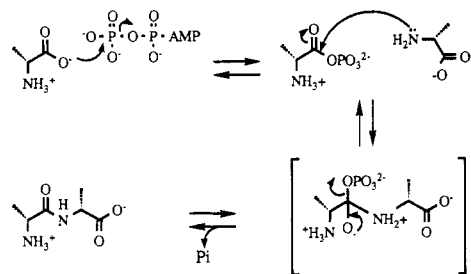
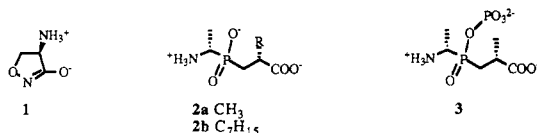


Figure 3. Proposed mechanism of D-Ala-D-Ala ligase.

Chart I. Inhibitors of D-Ala-D-Ala Ligase



nucleophilic attack of the D-Ala carboxylate oxygen on the γ -phosphate of ATP. Furthermore, molecular isotope exchange experiments using [^{14}C]-D-Ala-D-Ala has allowed measurement of the backward reaction by monitoring the release of [^{14}C]-D-Ala only in the presence of inorganic phosphate. These studies support a mechanism of activation of the carboxylate of the N-terminal D-Ala by phosphorylation to give the acyl phosphate (Figure 3). Nucleophilic attack by the amino group of the C-terminal amino acid on the activated carboxylate yields a phosphorylated tetrahedral adduct that decomposes to the dipeptide.

The key role of D-Ala-D-Ala ligase in peptidoglycan assembly suggests that this enzyme should be an effective target for the design of antibacterial agents. The natural product, D-cycloserine (1) (Chart I), was shown to be a competitive inhibitor of D-Ala-D-Ala biosynthesis in *Staphylococcus aureus* by Strominger's group in 1960.¹⁷ D-Ala-D-Ala ligase inhibition by this compound was further elaborated by Nehaus and Lynch, who confirmed competitive inhibition with partially purified ligase from *Strep. faecalis* and demonstrated a k_i of 22 μM .¹⁰ The low potency of D-cycloserine as well as problems with toxicity have limited the clinical usefulness of this compound.¹⁸ More recently, (aminoalkyl)phosphinate analogues of D-Ala-D-Ala (2) have shown promising results as potent D-Ala-D-Ala ligase inhibitors. The heptyl analogue (2b) has been shown to be an ATP-dependent slow-binding inhibitor of *S. typhimurium* DdlA, according to the general equation



E^*I was found to be a long-lived complex ($t_{1/2}$ for regain of activity = 8 h) consisting of a mix of $E \cdot I \cdot P_i \cdot \text{ADP}$ and $E \cdot I \cdot \text{ATP}$.¹⁹ Inhibition of DdlA with the methyl analogue (2a) also showed slow-binding inhibition, this time with a remarkable $t_{1/2\text{regain}}$ of 17 days!²⁰ The stability of the inhibitor-bound complex has allowed elucidation of the nature of E^*I by ^{31}P rotational resonance solid-state

NMR.²⁰ This methodology is based on magic angle spinning techniques and allows measurement of homonuclear dipolar coupling from which the interatomic distance may be calculated using line shape for directly bonded atoms or magnetization transfer for through-space interactions.²¹ Couplings consistent with a new P-O-P linkage were observed when 2a was allowed to inactivate DdlA in the presence of ATP, and a P-P distance of $2.7 \pm 0.2 \text{ \AA}$ was calculated for the species in the E^*I complex. These assignments are consistent with the phosphoryl-phosphinate adduct (3) as the long-lived bound inhibitor. It would arise by phosphoryl transfer of the γ -phosphate of ATP onto the phosphinate oxygen of 2a in the enzyme active site. The resultant phosphino-phosphate (3) strongly resembles the proposed tetrahedral intermediate (Figure 3) and may show specific structural complementarity and stabilization in the active site. Compound 3 is released some 10^8 – 10^9 -fold more slowly than the normal product. The astonishing potency of these inhibitors is unfortunately not reflected in a reasonable antimicrobial index, presumably because of poor transport into the cell.¹⁸

Vancomycin Resistance

The glycopeptide vancomycin is synthesized by a soil microorganism, *Amycolatopsis orientalis*, which was collected in a Borneo jungle in 1956. This antibiotic has found extensive clinical use for the last quarter century and is the drug of choice for treatment of infections due to methicillin-resistant *Staphylococcus aureus* (MRSA) and Gram-positive organisms in patients allergic to β -lactam antibiotics.⁹ Early work demonstrated that vancomycin exerted its antibiotic effect by interfering with peptidoglycan biosynthesis.²² Further studies by Perkins²³ followed some years later by a series of elegant ^1H NMR experiments by D. H. Williams' group^{24–26} have demonstrated that vancomycin binds to the C-terminal D-Ala-D-Ala of the muramyl pentapeptide exposed at the cell surface. Binding is mediated by a series of five hydrogen bonds between the dipeptide backbone and the glycopeptide (Figure 4). The net result of this interaction is the inhibition of the peptidoglycan transglycosylase and the transpeptidase. The first enzyme adds the *N*-acetylglucosamine-*N*-acetylmuramyl pentapeptide to the growing cell wall, while the second catalyzes the cross-linking of the peptide moieties and is sensitive to β -lactam antibiotics. The glycopeptide antibiotics presumably act by sterically shielding the muramyl peptide from these enzymes.

One of the most attractive aspects of vancomycin treatment has been its virtual immunity to resistance mechanisms. Gram-negative organisms are resistant by virtue of the impermeability of their outer membrane to glycopeptides, which therefore prevents interaction with the peptidoglycan layer (Figure 1A). On the other

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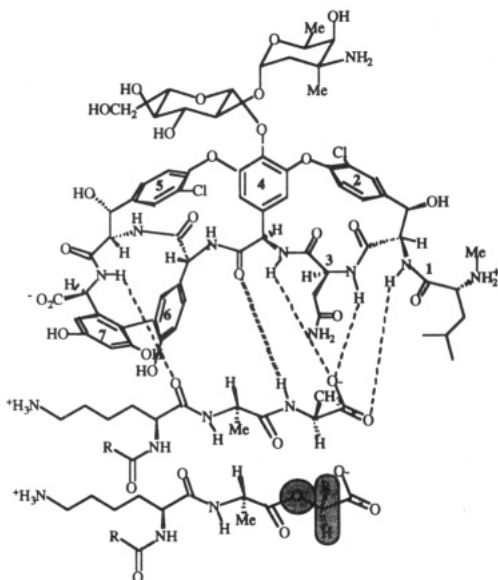
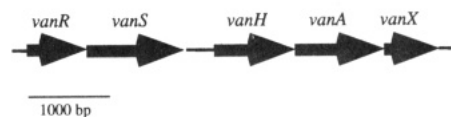


Figure 4. Structure of vancomycin peptide complex. Vancomycin binds Acyl-D-Ala-D-Ala through a series of hydrogen bonds. Resistance to this antibiotic could come about by increasing the size of the C-terminal R group or by the synthesis of a depsipeptide.

hand, Gram-positive organisms, in which the peptidoglycan layer is more exposed, are susceptible to vancomycin and other glycopeptide antibiotics. However, there are some Gram-positive families such as the leuconostocs, lactobacilli, and pediococci which show intrinsic resistance to high levels of vancomycin by as yet unknown mechanisms; fortunately, these organisms are rarely pathogenic.^{27,28} The recent emergence of vancomycin-resistant enterococci has therefore been greeted with apprehension in both the clinical and medicinal chemical fields.

Enterococci are components of the mammalian intestinal flora and therefore pose acute risks during abdominal surgery. They are also associated with urinary tract infections, bacteremia, and endocarditis.⁹ Furthermore, these bacteria are among the three leading causative agents in hospital-acquired (nosocomial) infections.²⁹ The enterococci have acquired resistance to low and high levels of vancomycin in recent years, the first cases emerging in Europe in the mid-1980s. The group of P. Courvalin at the Institut Pasteur has cloned the genes responsible for inducible and constitutive vancomycin resistance in enterococci. Inducible high-level resistance to vancomycin was found to be borne by a transferable plasmid and to require five genes: *vanS*, *vanR*, *vanH*, *vanA*, and *vanX*³⁰ (Chart II). Cell extracts of vancomycin-resistant *Enterococcus faecium* were also found to be associated with the production of large quantities of the 39-kDa protein, VanA.^{28,31,32} Cloning and sequencing of the gene, *vanA*, revealed significant homology to the D-Ala-D-Ala ligases

Chart II. Organization of the *van* Gene Cluster in *Enterococcus faecium*



(28–36%).³³ Purification of the enzyme revealed further similarities with chromosomal Gram-negative ligases (Table I), including molecular weight (37 kDa) and D-Ala K_m values reflecting low- and high-affinity sites.³⁴ However, some striking differences were noted: specifically, the K_m values for the N-terminal and C-terminal D-alanines were roughly 3 and 2 orders of magnitude higher for VanA compared to the chromosomal ligases!

The unusually high, and undoubtedly physiologically irrelevant, K_m for D-Ala (38 mM) suggested the possibility that VanA may prefer an alternate substrate. Further experiments have revealed that VanA possesses the capacity to synthesize dipeptides of the form D-Ala-D-X with improved K_m in the C-terminal site for hydrophobic residues such as Met and Phe on the order of 5 mM.³⁴ Additional cloning and sequencing upstream of the *vanA* gene revealed an open reading frame, *vanH* (Chart II), which showed homology to α -keto acid dehydrogenases.³⁵ Purification of VanH to homogeneity demonstrated that this 35-kDa protein was a D-specific α -keto acid dehydrogenase, which formed D-lactate and D-2-hydroxybutyrate from pyruvate and α -ketobutyrate, respectively, with high catalytic efficiency ($k_{cat}/K_m = 2.8 \times 10^4$ and $1.4 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, respectively).³⁶

This discovery immediately suggested that VanA may catalyze the synthesis of depsipeptides. Indeed, this was found to be the case, and VanA was shown to synthesize D-Ala-D-lactate and D-Ala-D-hydroxybutyrate with K_m values for D-lactate and D-2-hydroxybutyrate of 7 and 0.6 mM, respectively.³⁶ The chromosomal ligases, DdlA and DdlB, are unable to synthesize depsipeptides; therefore, this activity is intrinsic only to VanA which is associated with vancomycin resistance. Since the C-terminal amide hydrogen of *N*-acyl-D-Ala-D-Ala is involved in a hydrogen bond with the carbonyl oxygen of the (*p*-hydroxyphenyl)glycine residue (amino acid 4) of vancomycin²⁶ (Figure 3), it was expected that a depsipeptide, which no longer possesses the capacity to be a hydrogen-bond donor, should have poor affinity for glycopeptides. The interactions of a number of *N*-Ac-D-Ala-D-X peptides and depsipeptides with vancomycin have been examined. Increasing the size of the C-terminal R group (Figure 3) results in decreased affinity for the drug. Depsipeptides terminating in D-lactate, a D-Ala isostere, also show at least 1000-fold higher K_D values than the amide. This is consistent with an apparent contribution of ~ 4 kcal/mol of binding energy from the interaction of the amide hydrogen with vancomycin. These findings clearly demonstrate that vancomycin resistance can be conferred by the synthesis of murein peptides

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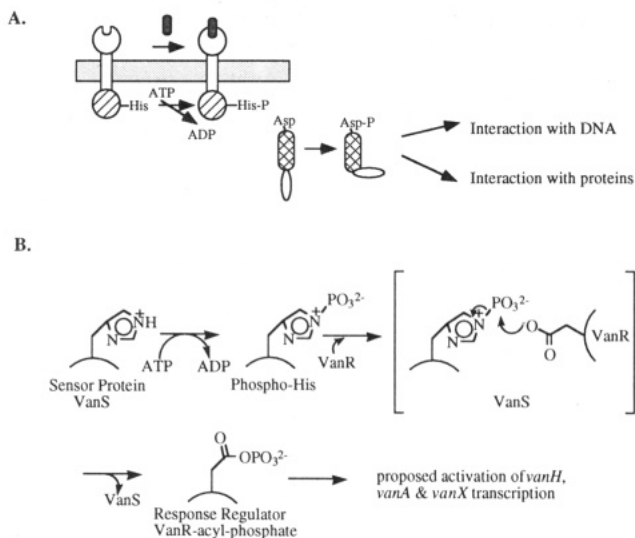


Figure 5. Mechanism of prokaryotic two-component regulatory systems. A: An external stimulus triggers autophosphorylation of a cytosolic His of the sensor protein. The phosphate is transferred to an Asp group of a regulator, which interacts with DNA or protein to effect a response. B: Detail of the proposed mechanism of VanS and VanR.

terminating in D-Ala-D-X, where X is an amino acid with an R group larger than a methyl group or where X is a hydroxy acid (Figure 4).

The obvious question remains: what is the physiological substrate for VanA and VanH? Isolation of cell wall components of vancomycin-resistant organisms has not yet been accomplished and will not be trivial given the lability of the ester linkage, so that direct evidence is lacking. However, experiments in which *vanH* mutants were supplemented with D-hydroxy acids revealed that D-lactate was able to restore vancomycin resistance at a lower concentration than D-hydroxybutyrate.³⁷ This provides indirect evidence that the *van* gene cluster is responsible for the synthesis of D-Ala-D-lactate in vancomycin-resistant *Enterococcus faecium* and direct evidence for the involvement of D-hydroxy acids in vancomycin resistance.

The roles of the other genes required for vancomycin resistance are now being explored in detail. The *vanS* and *vanR* genes show significant homology to the prokaryotic two-component regulatory systems.^{30,38} These pathways are used by organisms to sense and respond to a variety of external stimuli such as nutrient gradients (chemotaxis), phosphate and nitrogen levels, and osmotic pressure. To effect this environmental response, prokaryotes have evolved protein-mediated phosphoryl-transfer chemistry as a method of relaying and responding to information gleaned from their surroundings. A prototypic two-component regulatory system is indicated in Figure 5a with a transmembrane sensor and a cytosolic response regulator. A membrane-bound histidine kinase (sensor protein) undergoes autophosphorylation on its intracellular domain by cytosolic ATP in response to an external stimulus. The phosphoryl histidine form of the sensor protein is then an activated phosphoryl-transfer reagent that, on interaction with a specific partner response regulatory

protein, phosphorylates a conserved aspartate β -carboxylate group. The acyl phosphate form of the regulator then effects some physiological change such as transcriptional regulation or interaction with other proteins (for instance, the phosphorylated chemotactic response regulator, CheY, interacts with the flagellar motor to trigger a change in rotation direction³⁹). The hydrolytic lability of the β -aspartyl phosphate form of the regulator is a built-in clock that turns off the signal. As the acyl phosphate is hydrolyzed, the signal is terminated. If the primary sequence homology between known two-component regulatory proteins and VanR and VanS reflects similarity in function, *vanS* would encode an integral membrane "sensing protein" which has inducible histidine kinase activity. Interaction of VanS with VanR, the response regulator protein, should therefore transfer the phosphate to an aspartate residue of VanR, resulting in signal propagation by an as yet unknown mechanism, perhaps by DNA binding (Figure 5b). We have recently prepared and purified a truncated construct which consists of the predicted cytosolic kinase domain of VanS, and we have also overproduced and purified VanR.⁴⁰ The VanS construct undergoes autophosphorylation from ATP and will then transfer the phosphoryl group intermolecularly to pure VanR.⁴⁰ The role of phospho-VanR in vancomycin resistance is currently under investigation.

The final protein required for high-level vancomycin resistance, VanX, is a 23-kDa protein which does not show sequence similarity to any known protein. The overexpressed and purified protein has provided the opportunity to test a number of potential functions such as α -keto acid synthesis, DNA binding, and phosphatase activities, none with success; the function(s) of VanX remains unknown.⁴¹

Vancomycin-inducible DD-carboxypeptidase activity has recently been noted in enterococci.^{42,43} It has been suggested that a DD-carboxypeptidase could play a role in resistance, presumably by effectively eliminating the target D-Ala-D-Ala terminating peptide by conversion to the tetrapeptide which no longer carries the vancomycin recognition site. The Courvalin group has recently cloned and sequenced a gene, *vanY*, downstream from *vanX* which is associated with DD-carboxypeptidase activity despite no significant primary sequence similarity with known DD-carboxypeptidases.⁴⁴ VanY is associated with cellular membranes, possesses DD-carboxypeptidase and DD-carboxyesterase activities, and is not susceptible to β -lactam antibiotics.⁴⁵ As previously noted, high-level vancomycin resistance requires only the five genes described above; addition of *vanY* does not alter resistance levels nor does *vanY* confer resistance in itself.⁴⁴ Therefore, DD-carboxypeptidase activity does not appear to contribute to vancomycin resistance.

Vancomycin resistance in enterococci has been sep-

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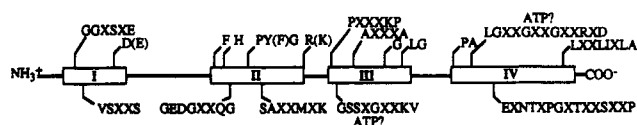


Figure 6. Homology domain structure of D-Ala-D-Ala ligases. Regions of significant similarity between DdlA, DdlB, VanA, and VanC are noted. Conserved residues are indicated. Note the Gly-rich regions in domains III and IV terminating in K and R, respectively, which may be nucleotide binding loops.

arated into three classes.⁴⁶ The first, class A, describes inducible, high-level resistance and is associated with the *van* genes described above. Class B resistance is inducible, but shows low-level resistance. The mechanism for this resistance is unknown; however, the N-terminal 11 amino acids of a 39.5-kDa protein associated with class B resistance have been reported and show similarity to the N-termini of D-Ala-D-Ala ligases.³² The third group, class C, describes constitutive, low-level resistance to vancomycin. The gene associated with this class in *E. galinarum*, *vanC*, has recently been cloned and sequenced and encodes a protein of a predicted molecular weight of 37.5 kDa.⁴⁷ The deduced primary sequence has approximately 30% similarity with the Gram-negative ligases and 38% with VanA, therefore suggesting that VanC also has D-Ala-D-Ala ligase activity and perhaps also depsipeptide synthesis capacity. Surprisingly, transfection of the *vanA* gene into a *vanC* knockout strain did not rescue vancomycin resistance, a result which presents some interesting mechanistic questions which will be answered only when a sufficient quantity of VanC is available for detailed analysis.

Structure of D-Ala-D-Ala Ligases

The availability of diffraction grade crystals of *S. typhimurium* DdlA⁴⁸ and more recently *E. coli* DdlB⁴⁹ has permitted progress toward determining a three-dimensional structure of a D-Ala-D-Ala ligase. Alignment of ligase sequences indicates four regions of primary structure homology³³ (Figure 6). Polyclonal antibodies to DdlB and VanA do not cross-react or recognize *S. typhimurium* DdlA, indicating that the regions of identical primary sequence are not antigenic. Nonetheless, limited proteolysis studies on DdlA, DdlB, and VanA have revealed that domains III and IV are separated by a protease-accessible loop consistent with

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a similar three-dimensional structure.⁵⁰ Cleavage at this loop is accompanied by a loss of activity, indicating that both domains are required for enzymatic dipeptide formation.

Using the criterion of glycine-rich regions which incorporate a basic amino acid (Arg or Lys) as a paradigm for the location of nucleotide binding loops,⁵¹ two putative ATP binding regions can be located on the consensus ligase structure. The first is located at the N-terminus of domain III and the second at the N-terminus of domain IV. Photoaffinity labeling studies using [³²P]-8-azido-ATP have demonstrated that this label competes at the ATP binding site of DdlB and VanA.⁵⁰ Labeling studies performed both prior to and following limited proteolysis reveal that the majority of the label is associated with an N-terminal fragment corresponding to domains I, II, and III. The lack of discernible incorporation in domain IV argues against an ATP binding role for this region. Furthermore, the ability of the proteolyzed C-terminal domain to recognize and bind [³²P]-8-azido-ATP suggests that the ATP binding site is contiguous within this fragment.

The locations of the D-Ala binding sites remain unknown. As noted above, kinetic studies suggest two binding sites: a high-affinity N-terminal site and a lower affinity C-terminal site. Identification of residues important to substrate binding and catalysis is currently being pursued by mutational analysis. Identification of important amino acids will be essential for future inhibitor design.

Conclusions and Future Prospects

The D-Ala-D-Ala ligases are essential to bacterial peptidoglycan biosynthesis; a number of these have been cloned and purified, their mechanisms have been probed, and potent in vitro inhibitors have been produced. Furthermore, changes in ligase substrate specificity have been directly correlated with resistance to vancomycin. Specifically, the synthesis of depsipeptide-containing peptidoglycan precursors by VanA attenuates vancomycin binding to the peptidyl disaccharide of bacterial cell walls, resulting in resistance to this drug. Given the unique substrate specificity and catalytic chemistry of VanA, it should be possible to prepare specific inhibitors of this enzyme. In particular, analogues of D-cycloserine and (aminoalkyl)phosphinates which reflect the substrate promiscuity of VanA should show inhibition and may be useful against vancomycin-resistant organisms.

Registry No. Vancomycin, 1404-90-6; D-Ala-D-Ala ligase, 9023-63-6.

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