Vancomycin resistance in enterococci: reprogramming of the D-Ala–D-Ala ligases in bacterial peptidoglycan biosynthesis

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Vancomycin binds to bacterial cell-wall intermediates to achieve its antibiotic effect. Infections of vancomycin-resistant enterococci are, however, becoming an increasing problem; the bacteria are resistant because they synthesize different cell-wall intermediates. The enzymes involved in cell-wall biosynthesis, therefore, are potential targets for combating this resistance. Recent biochemical and crystallographic results are providing mechanistic and structural details about some of these targets.

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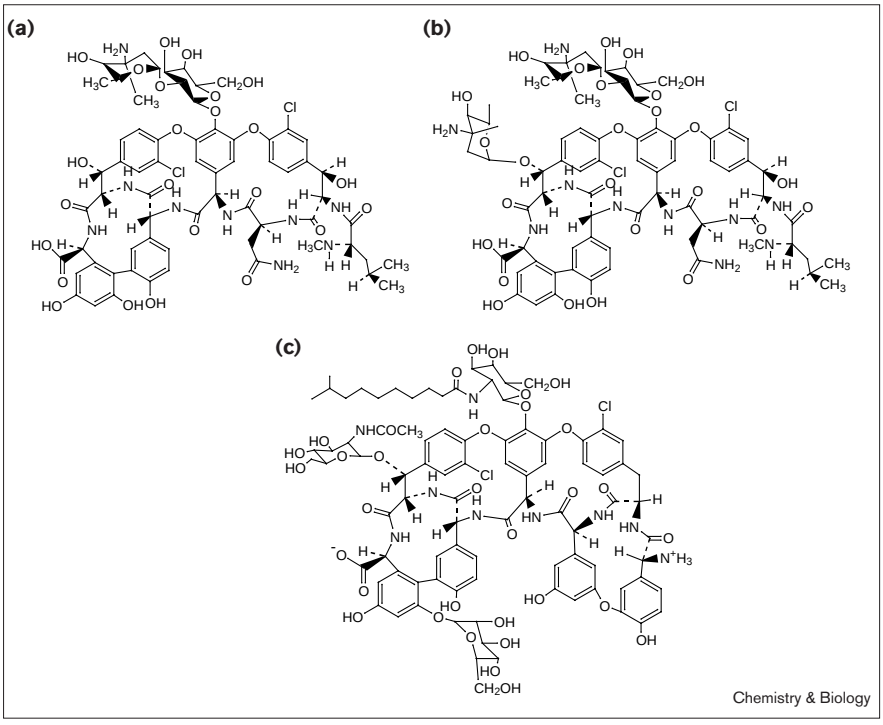
Introduction

Vancomycin belongs to the glycopeptide class of antibiotics, which are effective against gram-positive organisms. In contrast, gram-negative bacteria are intrinsically resistant to glycopeptides because of their impermeable outer membrane. Glycopeptides are composed of a heptapeptide backbone that is substituted with five to seven aromatic rings and different sugars. Vancomycin contains two hexoses and five aromatic rings; chloroeremomycin has an additional sugar molecule (Figure 1a,b). A second glycopeptide antibiotic approved for human use, teicoplanin, contains three sugars and seven aromatic rings (Figure 1c).

In contrast to penicillin, which directly binds to and inhibits the bifunctional transglycosylases/transpeptidases involved in cell-wall biosynthesis, vancomycin binds to the substrate of these enzymes, the D-alanyl–D-alanine (D-Ala–D-Ala) terminus of the lipid-PP-disaccharide-pentapeptide (Figure 2a). Binding of vancomycin to this target is thought to sterically prevent the subsequent action of both activities of the transglycosylase/transpeptidase: adding disaccharyl pentapeptide units to the growing peptidoglycan (PG) strand and then cross-linking peptides within and between PG strands on the external face of the cytoplasmic membrane (Figure 2b). This failure to form cross-links between peptidoglycan intermediates lowers the rigidity of the cell wall and renders these bacteria susceptible to osmotic lysis [1–5].

During the past decade, vancomycin has become the front-line therapy for treating problematic infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA), and by enterococci in patients after surgery. The specter of vancomycin-resistant MRSA looms [6–9], whereas vancomycin-resistant enterococci (VRE), which cause high mortality rates, have become a distressingly common reality in hospital settings [10,11]. Three VRE phenotypes have been designated VanA, VanB and VanC on the basis of the minimal inhibitory concentration (MIC) in VRE and susceptibility to teicoplanin (Table 1) [3,11]. The VanA and VanB phenotypes differ in that VanA is resistant to teicoplanin, whereas VanB is not resistant because the VanB resistance pathway is not activated by teicoplanin (MIC ≤ 0.5 μg/ml) [3]. Both phenotypes are the result of the incorporation into cell-wall intermediates of a D-alanyl–D-lactate (D-Ala–D-Lac) depsipeptide synthesized by the VanA or VanB ligase in place of the usual D-Ala–D-Ala dipeptide. Vancomycin binds with a three orders of magnitude lower affinity to D-Ala–D-Lac termini

Figure 1



Structure of glycopeptide antibiotics. **(a)** Vancomycin. **(b)** Chloroeremomycin. **(c)** Teicoplanin.

than to D-Ala–D-Ala termini (Figure 2a), and thus is less able to sterically block the transpeptidation and transglycosylation steps necessary for the formation of the peptidoglycan cell wall [12].

In contrast to the VanA and VanB phenotypes, VanC resistance in *Enterococcus gallinarum* and *E. casseliflavus* is chromosomal, and has only been detected in a few clinical isolates [11,13]. VanC resistance has a VanC D-alanyl–D-serine (D-Ala–D-Ser) ligase in place of the D-Ala–D-Lac ligases (VanA and VanB). The hydroxymethyl sidechain of D-serine is thought to disrupt sterically the optimal geometry for vancomycin binding to the usual D-Ala–D-Ala termini, resulting in about a sixfold decrease in affinity for vancomycin [14] and a corresponding eightfold increase in the MIC for vancomycin (Table 1) [11]. VanD [15] and VanE [16] clinical phenotypes have been described recently, but are not yet completely characterized.

D-Ala–D-X ligase superfamily

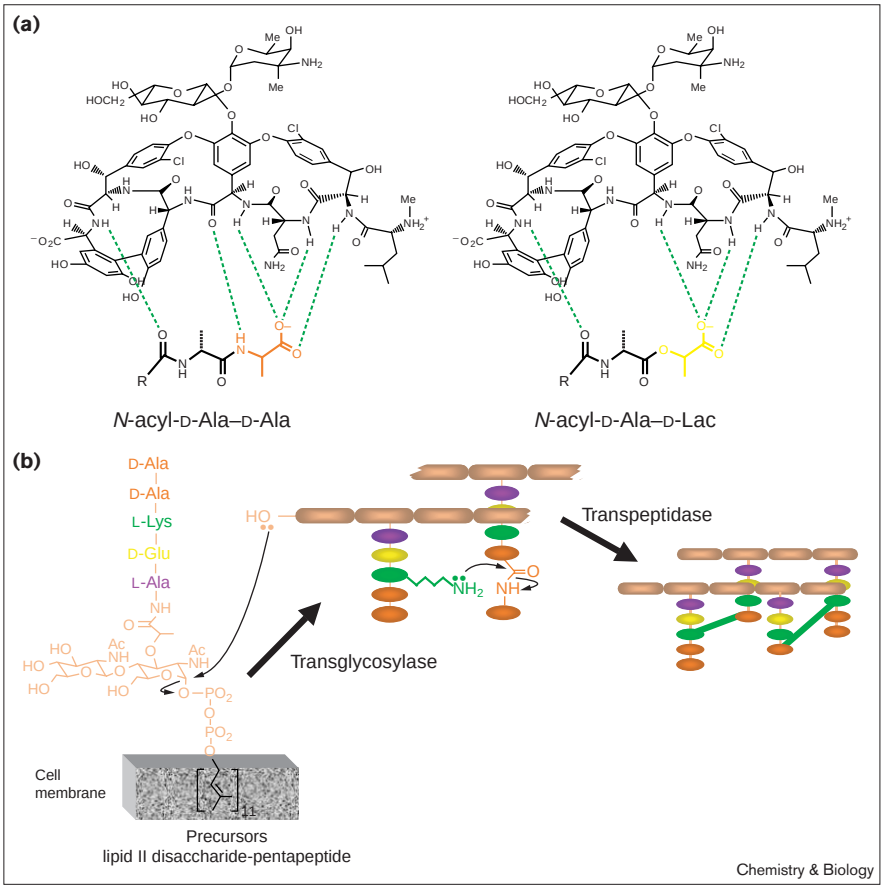
Homology analysis of the D-Ala–D-X (where X = alanine, serine or lactate) superfamily of bacterial ligases revealed five constituent families: two that are D-Ala–D-Ala ligases physiologically, two that are D-Ala–D-Lac ligases and one D-Ala–D-Ser ligase family (Figure 3) [17]. Many of the residues shown by site-directed mutagenesis [18] and structural studies [19] to be important for substrate binding and catalysis by the *Escherichia coli* D-Ala-D-Ala ligase (DdlB) are highly conserved among the entire Ddl superfamily. The D-Ala–D-Ala and D-Ala–D-Ser ligase (VanC) families also have similar sequences for the projected omega loop that closes over the active site of DdlB and the loop contains Lys215 and Tyr216 (DdlB numbering), which are important for catalysis (discussed further in the next section) [17,18,20]. Of the two D-Ala–D-Lac ligase families, the one represented by the *Leuconostoc mesenteroides* ligase (LmDdl2) has a comparable omega loop, whereas the second, represented by the VanA and VanB ligases and the DdlM from the glycopeptide antibiotic producer *Streptomyces toyocaensis*, has a strikingly different sequence of residues for the putative omega loop. The gain of function to make the D-Ala–D-Lac depsipeptide is the key molecular switch that reprograms the VanA and VanB type VRE to produce a vancomycin-resistant cell wall.

E. coli DdlB as D-Ala–D-Ala ligase prototype

Until recently, the only available X-ray structure of a D-Ala–D-X ligase superfamily member has been that of the D-Ala–D-Ala ligase from *E. coli* (DdlB) with bound ADP and a phosphorylated D-Ala–D-Ala phosphinate transition-state inhibitor (Figure 4a,b) [19]. DdlB belongs to the ATP-grasp superfamily of enzymes that have an unusual nucleotide-binding fold [21–24]. All of these ATP-grasp enzymes hydrolyze ATP to ADP and inorganic phosphate and ligate one substrate with a carboxylate to a second substrate with a primary amine, secondary amine, hydroxyl or thiol [25]. The catalytic mechanism of these enzymes is also thought to include formation of an

Figure 2

Mode of action of vancomycin. **(a)** Binding of vancomycin to D-Ala–D-Ala and D-Ala–D-Lac peptidoglycan termini. **(b)** Inhibition of transglycosylation and transpeptidation steps in cell-wall biosynthesis by vancomycin.



activated acyl phosphate intermediate that is nucleophilically attacked by the second substrate [26–29] (Figure 5).

Structural alignment of DdlB with two other ATP-grasp proteins, *E. coli* glutathione synthetase and biotin carboxylase, revealed a similar binding site for ATP in a cleft formed by two structural elements, each containing two antiparallel β strands and a loop [22]. Despite only a 12% sequence identity between biotin carboxylase and glutathione synthetase, 127 Cα atoms superimpose with a root mean square deviation (rmsd) of only 1.64 Å [22]. Similarly, 101 Cα atoms of biotin carboxylase and DdlB superimpose with a rmsd of 1.91 Å. In addition, most of the amino acid residues that interact with ATP in DdlB are conserved in all ATP-grasp proteins. For example, Lys97 and Lys144 (DdlB numbering) are conserved in all sequences except for substitution by arginine in carbamoyl phosphate synthetases [25]. This sequence conservation of all ATP-grasp proteins and the conserved tertiary structure of three crystallized members support a common evolutionary ancestor for these enzymes.

On the basis of the crystal structure of DdlB, two loops close over the catalytic cavity, as observed in other ATP-grasp enzymes (Figure 4b) [19]. The first loop connects two β strands and contains Glu148–Gly–Ser–Ser–Val–Gly. This loop is part of a X–Gly–Ser–Ser–X–Gly motif, where position X is variable, that is conserved in all D-Ala–D-X ligases [17]. The second loop, called the omega loop, also connects β strands and forms a short helix consisting of amino acids 206–220 [19].

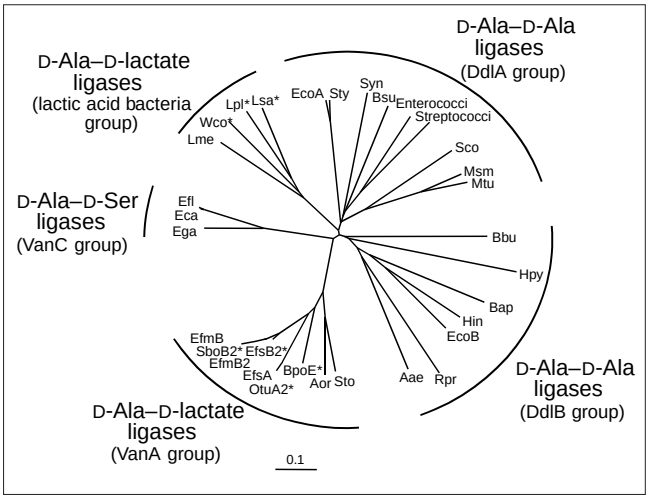
The omega loop is believed to be flexible and exposed in the uncomplexed enzyme [30]. The hydroxyl groups of Ser150 and Ser151 of the first loop form three hydrogen bonds to the omega loop and help hold it in place (Figure 4b). Closing of the omega loop over the active site

Table 1

Resistance to glycopeptide antibiotics in the three classes of vancomycin-resistant enterococci [3,11].

Clinical phenotype	Vancomycin MIC (μg/ml)	Teicoplanin MIC (μg/ml)
VanA	>512	64
VanB	>512	0.5
VanC	8	0.5

Figure 3



Phylogenetic tree of D-Ala–D-X ligase superfamily. From [17] with permission. This alignment was obtained using the program Clustal W [60]. Syn, *Synechocystis* sp.; Bsu, *Bacillus subtilis*; Enterococci, *Enterococcus* sp. (*Enterococcus faecalis*); Streptococci, *Streptococcus* sp. (*Streptococcus pneumonia*); Sco, *Streptomyces coelicolor*; Msm, *Mycobacterium smegmatis*; Mtu, *Mycobacterium tuberculosis*; Bbu, *Borrelia burgdoferi*; Hpy, *Helicobacter pylori*; Bap, *Buchnera aphidicola*; Hin, *Haemophilus influenzae*; EcoB, *Escherichia coli* (*ddlB*); Rpr, *Rickettsia prowazekii*; Aae, *Aquifex aeolicus*; Sto, *Streptomyces toyocaensis* (*ddlM*); Aor, *Amycolatopsis orientalis* (*ddlN*); BpoE, *Bacillus popilliae* (*vanE*); OtuA2, *Oerskovia turbata* (*vanA2*); EfsA, *Enterococcus faecium* (*vanA*); EfmB2, *Enterococcus faecium* (*vanB2*); EfsB2, *Enterococcus faecalis* (*vanB2*); SboB2, *Streptococcus bovis* (*vanB2*); EfmB, *Enterococcus faecium* (*vanB*); Ega, *Enterococcus gallinerum* (*vanC*); Eca, *Enterococcus casseliflavus* (*vanC2*); Efl, *Enterococcus flavescens* (*vanC3*); Lme, *Leuconostoc mesenteroides*; Wco, *Weassella confusa*; Lpl, *Lactobacillus plantarum*; Lsa, *Lactobacillus salivarius*; EcoA, *E. coli* (*ddlA*); Sty, *Salmonella typhimurium*. Stars refer to alignments based on partial sequences.

of DdlB is thought to be required for most of the interactions with ADP. The hydrogen-bond network of Glu15, Ser150 and Tyr216 also orients Lys215 for stabilization of γ-PO_3 transfer to D-Ala in subsite 1 (D-Ala_1; Figure 5b). A solvent accessibility calculation shows that only 2% of the surface area of the enzyme bound ADP and D-Ala–D-Ala phosphinophosphate transition-state inhibitor is exposed, so the loops are expected to be effective in preventing hydrolysis of reactive intermediates (e.g. D-alanyl phosphate) by water [19].

Kinetic analysis of purified DdlB revealed a k_{cat} value of 1870 min^{-1} at pH 7.8 for D-Ala–D-Ala synthesis but no detectable D-Ala–D-Lac activity [18]. At pH 6.0, there is very low D-Ala–D-Lac ligase activity that is less than 10% of the dipeptide ligase activity and probably not physiologically significant, but this intrinsic activity could contribute to the possible evolution of D-Ala–D-Lac ligases from DdlB [31]. Electrostatic calculations [32] and the slight decrease in D-Ala K_{m2} values as pH increases [31] suggests that DdlB can utilize both the protonated and deprotonated forms (NH_3^+ or NH_2) of D-Ala at subsite *2* (D-Ala_2) as substrates but may bind the deprotonated form, which is more abundant at high pH, with higher affinity. For D-Ala–D-Ala synthesis, DdlB binds Ala_1 in subsite 1 with high affinity (1 μM) but binds Ala_2 in subsite *2* with a 1000-fold weaker K_{m2} of 1.1 mM [18]. On the basis of the *in vivo* concentration of 0.5 mM D-Ala in exponentially growing *E. coli*, the rate of D-Ala–D-Ala formation *in vivo* could be limited by the possible failure to saturate subsite *2* of DdlB [33].

VanA/B family

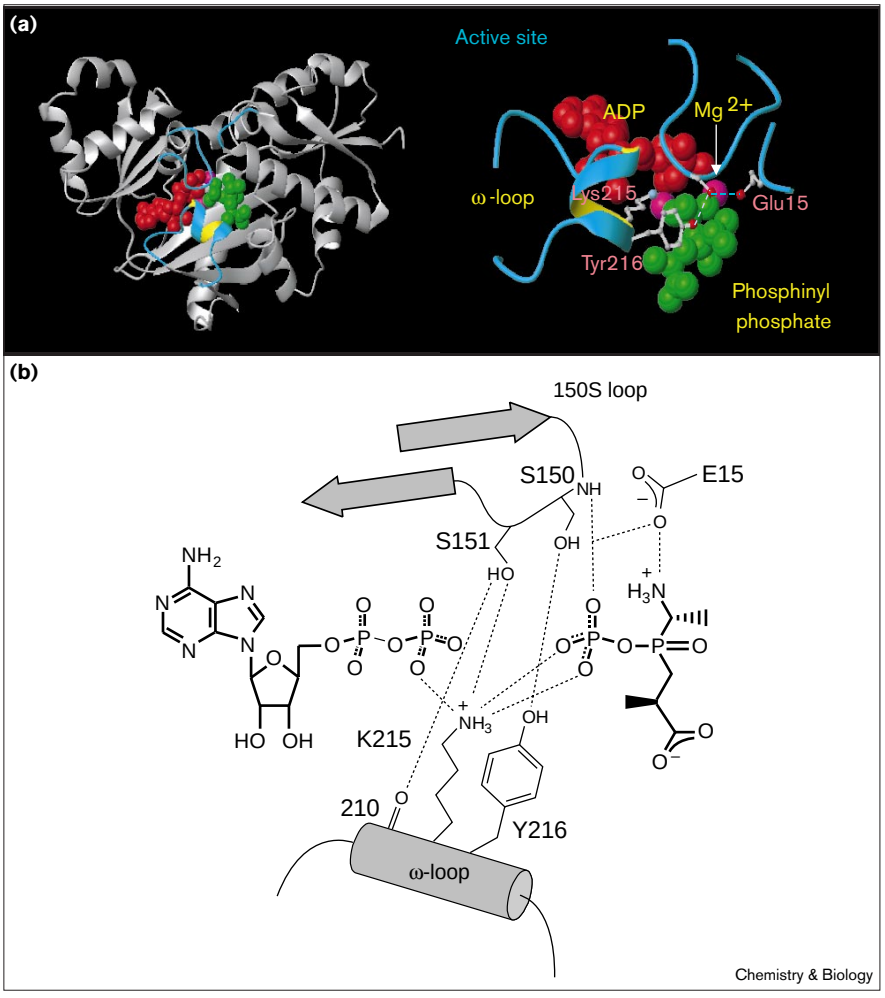
In contrast to the D-Ala–D-Ala ligase families, the VanA family synthesizes predominantly D-Ala–D-Lac by selectively activating D-lactate while discriminating against D-Ala_2 (Table *2*) [17,31]. Based on the pH profile of D-Ala–D-Ala synthesis, VanA only utilizes the deprotonated (NH_2) form of D-Ala_2 as a substrate [17,31]. Thus, one mechanism that VanA uses to minimize the amount of D-Ala–D-Ala produced at physiological pH is to reject the protonated (NH_3^+) form of D-Ala_2 that is predominantly present in solution (99.9% at pH 7.0). This result is in contrast to the electrostatic calculations [32] and weaker pH dependence of dipeptide synthesis by DdlB [31] and VanC2 (V.L.H., unpublished observations) that imply that DdlB and VanC2 can utilize both the protonated and deprotonated forms of amino acid substrates at subsite *2*.

Because the pH profiles for His244→Ala and Arg242→Ala VanA mutants were less pH dependent and consisted of K_{m2} values that were 2–3-fold lower than those of wild-type VanA, His244 and Arg242 appeared partially responsible for the rejection of the protonated (NH_3^+) form of D-Ala at subsite *2* by VanA [17]. In the recently solved X-ray structure of VanA from *Enterococcus faecium*, the sidechain of His244 is 3.5 Å from the position in the D-Ala–D-Ala phosphinate inhibitor corresponding to the amino group D-Ala_2 (Figure 6; D.I.R., T. Huyton, A. Veguine and G. Dodson, unpublished observations). His244 is therefore positioned in the active site such that the protonated imidazole ring could help reject the protonated form of D-Ala_2. His244 is also involved in D-Ala–D-Lac formation: mutation of His244→Ala resulted in a 100-fold higher K_{m2} but only a twofold lower k_{cat} [17]. Thus, the imidazole ring of His244 probably participates in binding D-Lac. In contrast, Arg242 is exposed to solvent, and thus, is less likely to play a role in repelling D-Ala_2 or binding D-Lac (D.I.R., T. Huyton, A. Veguine and G. Dodson, unpublished observations).

Inclusion of the D-Ala–D-Lac ligase from the antibiotic producer *S. toyocaensis* (DdlM) in the VanA ligase family suggests that the VanA and VanB ligases in VRE may have evolved from the resistance cassette in glycopeptide producers (Figure 7) [34]. The presence of DdlM in addition

Figure 4

Tertiary structure of the *E. coli* D-Ala–D-Ala ligase DdlB [19]. **(a)** The overall folding of DdlB showing the protected location of the binding sites. ADP is shown in red; the phosphinophosphate transition-state inhibitor, in green. **(b)** The flexible omega loop and Ser–Ser loop close over the DdlB active site and orient residues for substrate binding and catalysis.



to a D-Ala–D-Ala ligase in *S. toyocaensis* allows the bacteria to switch from production of D-Ala–D-Ala to D-Ala–D-Lac PG termini to generate self-immunity to the antibiotic it produces. *ddlM*, with a high GC content of 65%, may have

Figure 5

Formation of D-alanyl phosphate intermediate and its capture by the nucleophilic substrate in the reactions catalyzed by D-Ala–D-X ligases.

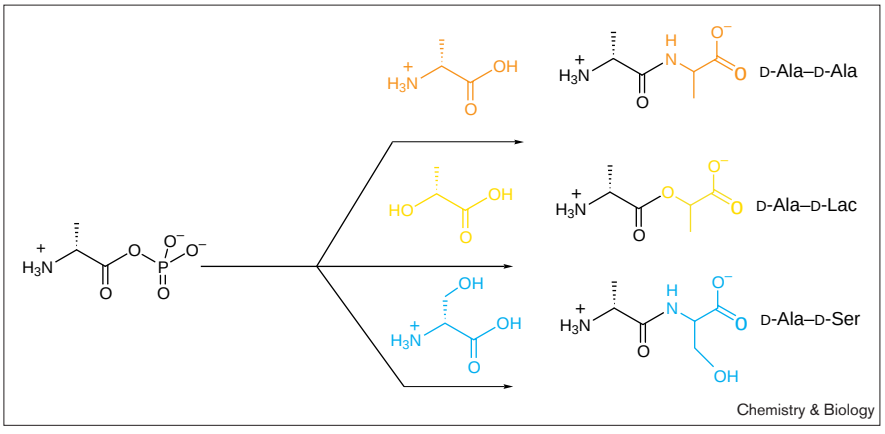


Table 2

Gain of D-Ala–D-Lac ligase function [31,63].

Ligase	D-Ala–D-lactate D-Ala–D-Ala	Ligase
E. coli DdlB	<0.001	1
E. coli DdlB Y216F	0.04	>40-fold
L. mesenteroides	3	>3000-fold
S. toyocansis DdlM	4	>4000-fold
Enterococcal VanA	30	>30,000-fold

From [39].

been transferred via one or more bacterial intermediates with a lower GC content to VRE, resulting in *vanA* and *vanB* with GC contents (45% and 48%, respectively) that are high for enterococcal genes. In addition the *vanHAX* gene cluster in VRE has the same orientation and gene alignment as that in *S. toyocaensis* [35,36]. Alternatively, VanA and VanB may have evolved independently from other D-Ala–D-X family members.

Leuconostoc D-Ala–D-Lac ligase family

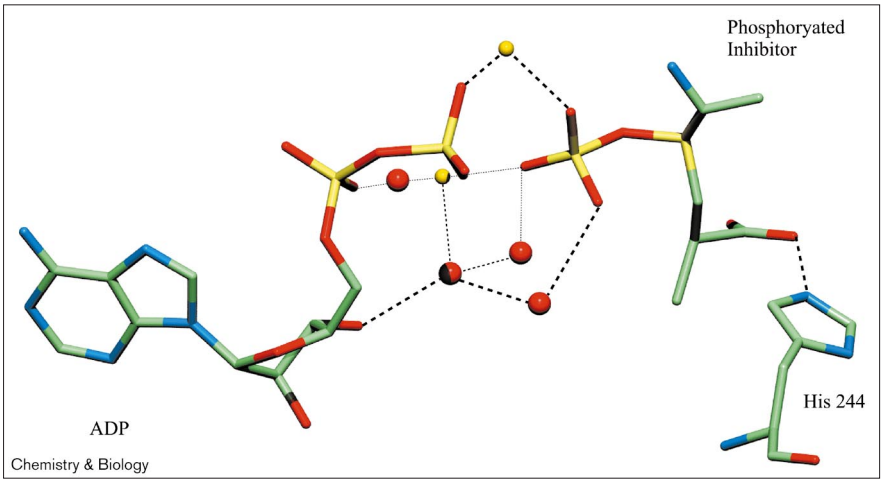
In addition to VRE, other gram-positive bacteria, such as the Leuconostoc ligase family, are intrinsically resistant to vancomycin because D-Ala–D-Lac is incorporated into their cell wall [37,38]. The omega loop sequence for LmDdl2 is similar to that of DdlB, except for the presence of Phe261, which is required for D-Ala–D-Lac synthesis and corresponds to the Tyr216→Phe (Y216F) DdlB mutant with a gain of depsipeptide synthesis [20]. The recent X-ray structure of LmDdl2 suggests that the smaller size and greater hydrophobicity of the active site than that of DdlB contribute to the 75-fold greater selectivity for depsipeptide over dipeptide synthesis compared to Y216F DdlB (Table 2; Figure 8) [20,39]. For example, the replacement of the solvent-exposed His280 in DdlB by the buried Met326 in van der Waals contact with the phosphinate inhibitor in LmDdl2 would fill 55–60 Å^3 of the binding cavity, favoring binding of the smaller D-Lac over D-Ala$_2$ [39].

VanC family

In contrast to VanA and VanB classes of VRE, VanC-mediated resistance is predominantly chromosomal and constitutive [11,40]. VanC2 from *E. casseliflavus* is a D-Ala–Ser ligase with no detectable D-Ala–D-Lac activity [41]. VanC2 has a 240-fold preference for D-serine over D-Ala$_2$ in subsite 2 (Table 3) [41,42]. Modeling the structure of VanC2 on the basis of the X-ray structure of the D-Ala-D-Ala ligase DdlB [19] predicted that Arg322 and Phe250 could be responsible for the greater affinity of D-Ser in the second binding site of VanC2 [42]. This hypothesis was supported by mutating these residues in VanC2 to the corresponding residues in DdlB, resulting in a Phe250→Tyr/Arg322→Met VanC2 double mutant with reversed substrate specificity: the mutant retained D-Ala–D-Ala ligase activity but had negligible D-Ala–D-Ser activity (Table 3) [42]. The guanidino group of Arg322 probably hydrogen bonds to the hydroxyl sidechain of D-Ser; however, the role of Phe250 in D-Ser binding by VanC2 has not been determined.

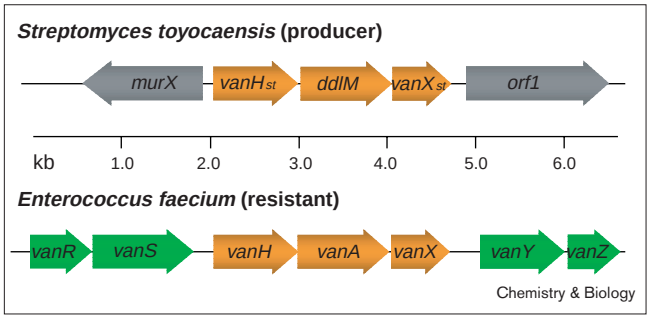
The role of the putative VanC2 omega loop in substrate specificity was further explored by generating VanA-based chimeric proteins with portions of the omega loop of VanC2 replacing that of VanA. These chimeras had a 5–6-fold improvement in K_{m2} for D-Ala and D-Ser, possibly resulting from a lessened discrimination against the zwitterionic form of substrate 2 [17]. This result suggests the possibility that VanA could have evolved from a VanC2-type framework. In addition, the chimeras failed to produce detectable D-Ala–D-Lac, suggesting that the VanA omega loop is necessary for utilization of D-Lac.

Figure 6



Active site of the VanA D-Ala–D-Lac ligase from *E. faecium* (D.I.R., T. Huyton, A. Veguine and G. Dodson, unpublished observations). The hydrogen-bond network involving four water molecules shown in red and two magenesium ions shown in yellow is thought to help stabilize γ-phosphate transfer to the phosphinate inhibitor or to D-Ala$_1$ in the normal reaction mechanism.

Figure 7



Comparison of vancomycin-resistance cassette in *E. faecium* to the orientation of the corresponding genes in the antibiotic producer *S. toyocaensis* [34].

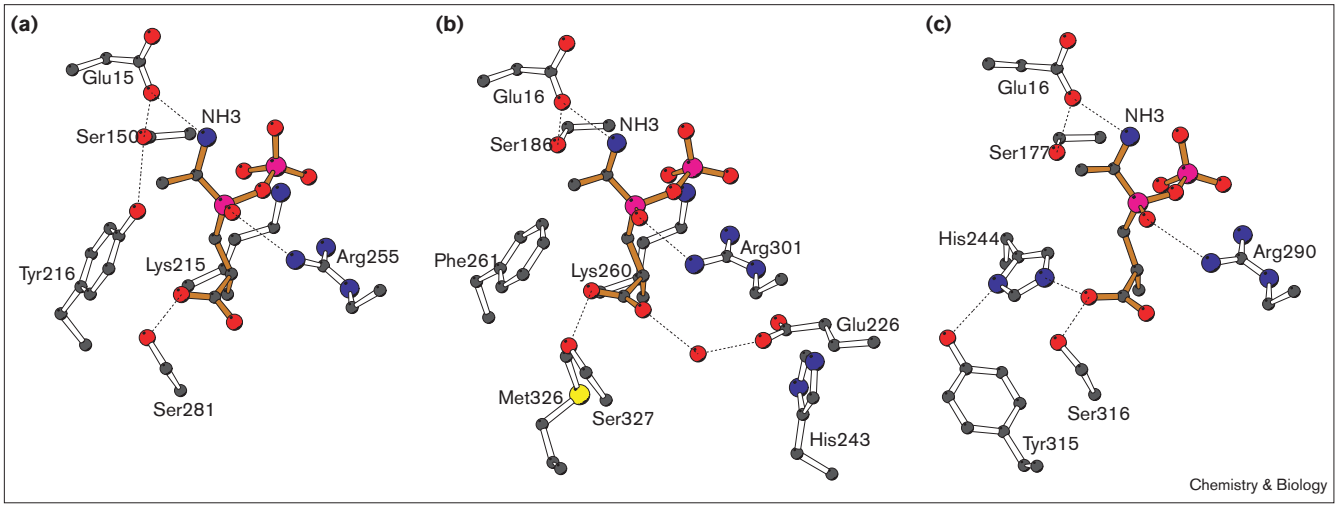
Chemical mechanism of D-Ala–D-X ligases

The first step in the chemical mechanism of D-Ala–D-X ligases is transfer of the γ-phosphate from ATP to D-Ala forming the putative D-alanyl phosphate intermediate (D-Ala$_1$-P; Figure 5). In the second step, D-Ala$_1$-P is captured by the nucleophilic substrate to generate the dipeptide or depsipeptide product. The reversible formation of D-Ala$_1$-P in the active sites of VanA, VanC2 and Ddl (*Salmonella typhimurium* Ddl or *E. coli* DdlB) is supported by the D-Ala-dependent positional isotope [β,γ]-bridge to nonbridge oxygen-18 scrambling of [γ-$^{18}O_4$]-ATP, molecular isotope exchange, and rapid quench studies [26] (and V.L.H., unpublished observations). Although the common intermediate D-Ala$_1$-P is formed in each active site, the VanA ligase differs from the others in not having the expected Lys215 (DdlB numbering) cationic sidechain to stabilize the PO_3 group transferring from ATP to the carboxylate of D-Ala$_1$, but instead has evolved an ordered array of water molecules and metal ions to provide transition state stabilization, as shown in Figure 6 (D.I.R., T. Huyton, A. Veguine and G. Dodson, unpublished observations).

The X-ray structures for the DdlB [19], LmDdl2 [39] and VanA ligases (D.I.R., T. Huyton, A. Veguine and G. Dodson, unpublished observations), complexed with the identical transition-state analog phosphinophosphate (Figure 8a–c), reveal full conservation of the Glu15, Ser150, Arg255 and Ser281 (DdlB numbering) that position and orient the D-Ala$_1$ and D-X substrates. The D-Ala–D-Ala ligase (DdlB; Figure 8a) has Tyr216, whereas the D-Ala-D-Lac ligase LmDdl2 (Figure 8b) has a change to phenylalanine, disrupting a hydrogen-bond network. This Tyr/Phe switch is one architectural element for dipeptide to depsipeptide switching because the *E. coli* DdlB F216Y mutant has a noticeable gain of D-Ala–D-Lac synthesis (Table 2) and the LmDdl2 F261Y loses the depsipeptide ligation activity [20]. VanA (Figure 8c) uses His244 in this locale, oriented by Tyr315, suggesting a different mechanism from LmDdl2, which is a distinct family of D-Ala–D-X ligases (Figure 3).

The second step in the reaction mechanism of these ligases is the capture of D-Ala$_1$-P by the nucleophilic cosubstrate, D-Ala$_2$, D-Ser or D-Lac, which must be deprotonated (from zwitterion to free amine form for D-Ala$_2$ and D-Ser; from alcohol to alkoxide for D-Lac) to engage in peptide or depsipeptide bond formation. Despite the X-ray structures, the identity of the catalytic base in these

Figure 8



Comparison of environments of the phosphinophosphate analog in **(a)** the DdlB ligase (Protein Data Bank 2DLN), **(b)** the LmDdl2 ligase (Protein Data Bank 1EHI), and **(c)** the VanA ligase [19,39] (D.I.R., T. Huyton, A. Veguine and G. Dodson, unpublished observations). Hydrogen bonds are indicated by dashed lines. Hydrogen atoms are not drawn. Figure generated using MOLSCRIPT [61].

Table 3

Point mutants in VanC2 active site: reversion from D-Ala–D-Ser to D-Ala–D-Ala ligase.

Mutants	k_{cat}/K_{M2} ratio $\frac{\text{D-Ala}_2}{\text{D-Ser}}$	Fold switch in specificity
VanC2	0.0042	1
F250Y	0.19	44
R322M	0.15	36
F250Y and R322M	26	6200

From [42].

steps is not resolved, and could, for example, be bound water, Asp257 or even the phosphate group of D-Ala_1-P for DdlB [18]. For VanA, where only the minor, free base form of D-Ala_2 binds productively, a catalytic base may not be provided by the protein when it makes D-Ala–D-Ala [17]. When the protein makes D-Ala–D-Lac, His244 could be involved, as indicated by the selective loss of two logs of activity for D-Ala–D-Lac but not D-Ala–D-Ala formation rates in the His244→Ala mutant [17] (D.I.R., T. Huyton, A. Veguine and G Dodson, unpublished observations).

In all the D-Ala–D-X ligases, it is likely that the loops bearing Tyr216 (DdlB), Phe261 (LmDdl2) or His244 (VanA) open and close in each catalytic cycle to let products out and then when new substrates, D-Ala, D-Lac or ATP, are bound they close down, orient substrates, and sequester labile intermediates from hydrolysis and prevent their release from the active site [19].

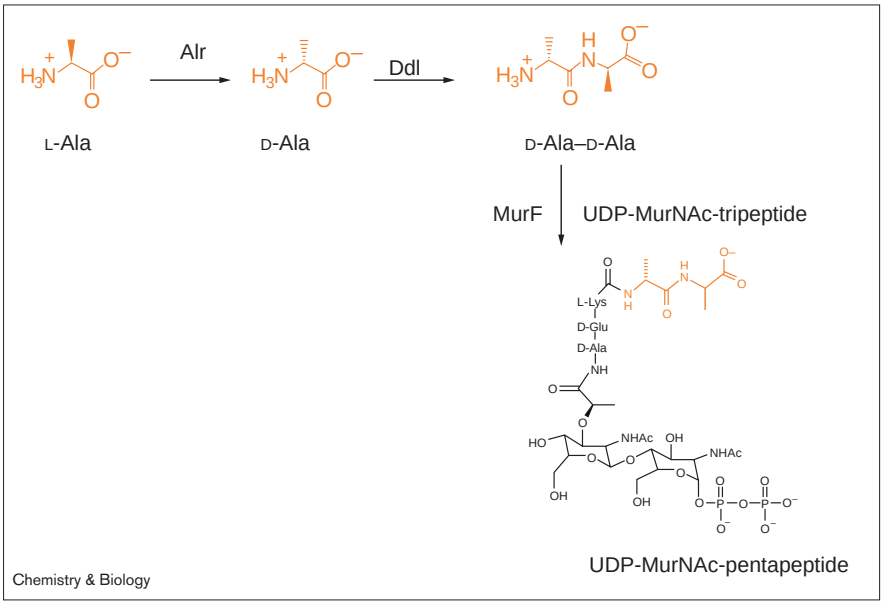
Companion enzymes

The Ddl, VanA/B and VanC pathways all require two additional enzymes to make PG: an alanine racemase for generation of D-Ala as a D-Ala–D-X ligase substrate and an enzyme for adding the D-Ala–D-X dipeptide/depsipeptide product to a cytoplasmic PG precursor (Figure 9). D-Ala is formed from conversion of L-Ala by alanine racemase. *E. coli* has two genes encoding alanine racemases: *alr* encodes a constitutive biosynthetic enzyme, while *dadX* encodes a catabolic enzyme that is inducible by D/L-Ala.

After synthesis, D-Ala–D-X is added to an UDP-*N*-acetylmuramic acid (UDP-MurNAc) tripeptide moiety by the enzyme MurF in an ATP-dependent mechanism [43]. Similar to the reaction catalyzed by the D-Ala–D-X ligases, this reaction probably involves γ-phosphate transfer to the UDP-MurNAc-tripeptide to form an acyl phosphate that is then attacked by the D-Ala–D-X peptide/depsipeptide, generating the UDP-MurNAc pentapeptide and inorganic phosphate. The UDP-MurNAc pentapeptide is subsequently modified by addition of a lipid carrier and *N*-acetylglucosamine to form the lipid-PP-disaccharide-pentapeptide, which is translocated to the extracellular side of the cytoplasm for incorporation into the cell wall.

For the VanA and VanB classes of vancomycin resistance, the genes *vanS*, *R*, *H* and *X* are also required for resistance [5,44]. VanH catalyzes the formation of D-Lac from pyruvate, which is then used by VanA or B to synthesize a D-Ala–D-Lac depsipeptide [12]. VanX acts selectively as a D-Ala–D-Ala dipeptidase, allowing only D-Ala–D-Lac to accumulate and become incorporated into bacterial cell wall intermediates in the presence of vancomycin [45,46].

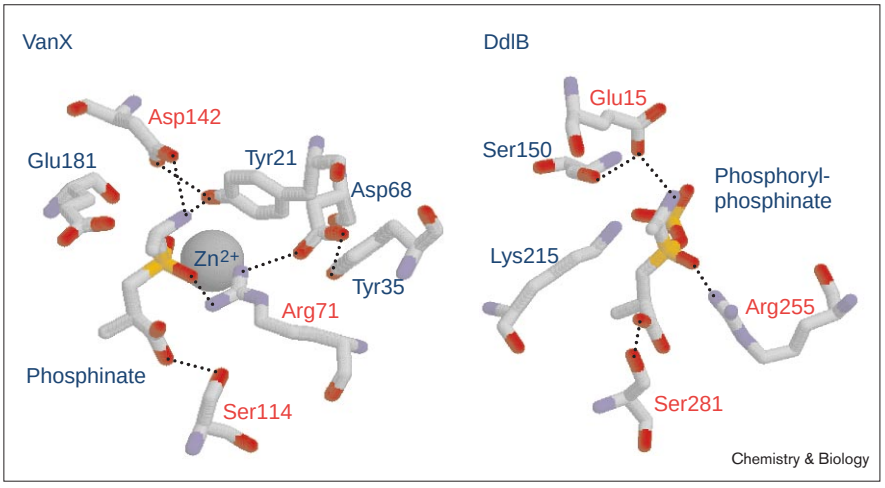
Figure 9



Reactions catalyzed by alanine racemase and MurF in cell-wall biosynthesis [43,62].

Figure 10

Comparison of *E. faecium* and *E. coli* DdlB active sites with bound phosphinate inhibitor [19,49]. From [50].



An accessory enzyme not required for resistance, VanY, which has D-carboxypeptidase activity, removes D-Ala from peptidoglycan precursors ending in D-Ala–D-Ala [47]. In VanC-mediated resistance, a $VanXY_c$ protein has both D-,D-carboxypeptidase and D-,D-dipeptidase activity [48]. The recently solved X-ray structure of VanX from *E. faecium* with a bound D-Ala–D-Ala phosphinate inhibitor reveals an active site with residues that serve a similar function as DdlB residues Glu15, Ser218 and Arg255 that are involved in electrostatic interactions with the ammonium group of $D\text{-}Ala_1$, the carboxylate of $D\text{-}Ala_2$ and the anionic transition state, respectively (Figure 10) [19,49,50]. The structure of VanX in the presence of a weakly bound phosphonate inhibitor explains the poor binding of D-Ala–D-Lac compared to D-Ala–D-Ala by VanX. The partial negative charge of the phosphonate bridging oxygen is repelled by the negatively charged Glu181 sidechain and a mainchain carbonyl group of Tyr109; additionally, the bridging oxygen could form an intraligand hydrogen bond with the terminal amino group of the inhibitor, resulting in the loss of a hydrogen bond between the amino group of the inhibitor and the enzyme [49].

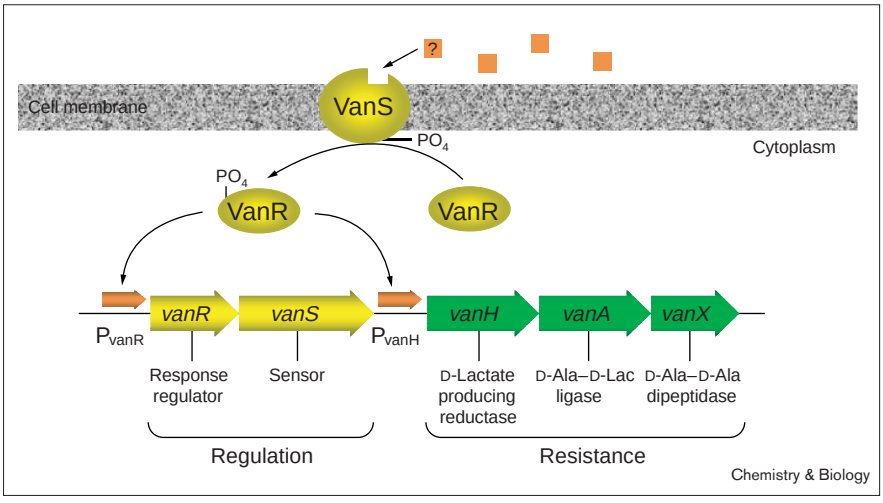
VanS and VanR form a two-component regulatory system that senses vancomycin and regulates expression of the *vanH*, *A* and *X* genes (Figure 11) [51]. VanS is a sensor kinase that undergoes autophosphorylation on a conserved histidine residue in its cytoplasmic domain and then phosphoryl transfer to an aspartate residue on the response regulator VanR, increasing the affinity of VanR for the promoter region of the *vanH, A,* and *X* triad by 500-fold [52]. VanS autophosphorylation is induced by vancomycin and teicoplanin in the VanA VRE phenotype but only by vancomycin in the VanB phenotype. VanC resistance is constitutive and thus does not seem to require VanS or VanR.

The VanC class of resistance also requires a membrane-bound serine racemase that has been recently isolated from *E. gallinarum* [53]. This pyridoxal 5′-phosphate-dependent enzyme, VanT, racemizes L-Ser to the D-enantiomer for use by the VanC ligase to generate D-Ala–D-Ser.

Strategies and progress in combating VRE

Given the rapid spread of VRE in hospitals and the high mortality from such infections in immunocompromised patients, there has been great interest in finding antibiotics that will kill VRE. One approach has been to use the knowledge gained about the molecular logic of the cell wall reprogramming from vancomycin-sensitive to vancomycin-resistant bacteria to identify new targets. Clearly VanA is one, along with the associated enzymes VanH (the D-Lac dehydrogenase/pyruvate reductase) and VanX (the D-Ala–D-Ala dipeptidase). The *vanHAX* structural genes are controlled by a two-component sensor kinase-response regulator pair, VanS and VanR, so all five of these proteins are targets for inhibition of function and reversal of VRE. The phosphinates that cocrystallize are potent inhibitors of the D-Ala–D-X ligases *in vitro* [54] and there are reports of inhibitors of two-component sensor/response regulators [55], but to date no inhibitors of these five proteins have been reported that can gain access to the bacterial cytoplasm and have activity *in vivo*. A second approach has been to screen natural product and other compound libraries for efficacy against VRE. This strategy has revealed that hydrophobic derivatives of vancomycin family members, such as the N-(chlorobiphenyl)-chloroeremomycin LY333328, is about two logs more active than vancomycin against VRE [56]. Other peptide-based drugs that have efficacy against VRE are the quinuprustin/dalfoprustin combination Synercid [57] and the lipodepsipeptide ramiplanin [58].

Figure 11



VanRS two-component system that activates transcription of *vanHAX* genes [51].

The D-Ala–D-X ligases remain an attractive target and as ATP-grasp proteins they share common structural elements with members of the protein kinase family fold [24]. There are large libraries of compounds that target ATP sites of protein kinases [59] that have yet to be evaluated systematically against the D-Ala–D-X ligases.

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