Bacterial resistance to vancomycin: five genes and one missing hydrogen bond tell the story

A plasmid-borne transposon encodes enzymes and regulator proteins that confer resistance of enterococcal bacteria to the antibiotic vancomycin. Purification and characterization of individual proteins encoded by this operon has helped to elucidate the molecular basis of vancomycin resistance. This new understanding provides opportunities for intervention to reverse resistance.

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In the general context of the emergence and re-emergence of bacterial resistance to antibiotics, the development of clinically consequential resistance by Gram-positive, opportunistic pathogens such as enterococci to the glycopeptide antibiotic vancomycin (Fig. 1) is an especially compelling cautionary tale of molecular logic that has recently been decoded. Given that vancomycin is often the therapeutic agent of choice in the treatment of methicillin-resistant *Staphylococcus aureus* infections [1,2] and that vancomycin resistance in enterococci had risen dramatically from 0.4 % incidence in hospital intensive care units in 1989 to 13 % by 1993 [3], the specter of transfer of vancomycin resistance to β-lactam-insensitive staphylococci and streptococci has ominous portent.

Mode of action of vancomycin

To deconvolute the molecular logic of the recently detected vancomycin resistance, including underlying chemical alterations in bacterial cell wall structure and the regulation thereof, one must first understand the mechanism of action of the antibiotic. Vancomycin acts just upstream of the penicillin-sensitive, bifunctional transglycosylases/transpeptidases [4] that add disaccharyl pentapeptide units to the growing peptidoglycan (PG) cell wall and then crosslink peptides within and between strands of the PG layer at the external face of the cytoplasmic membrane (Fig. 2a). The target of vancomycin is actually the substrate molecule for the β-lactam-sensitive transglycosylase/transpeptidase, namely lipid-PP-disaccharide-pentapeptide (Fig. 2b). The cup-shaped vancomycin molecule binds with high affinity via its concave pocket to the N-acyl-D-Ala-D-Ala termini of the as yet uncrosslinked pentapeptide by way of five hydrogen bonds between substrate and antibiotic [5].

Binding of vancomycin to the lipid-PP-GlcNAc-pentapeptide chains is thought to physically occlude the approach and prevent subsequent action of the transglycosylase/transpeptidase (Fig. 2c). Failure to attach the disaccharyl pentapeptide and to execute a crosslink lowers the interstrand covalent connectivity, lowers the mechanical strength of the PG layer, and renders the bacteria susceptible to lysis as osmotic pressure changes occur. Vancomycin is effective against Gram-positive bacteria such as enterococci, streptococci, and staphylococci, but not Gram-negatives such as *E. coli* or *Salmonella typhimurium*, because the latter have an intact outer membrane, which serves as a permeability barrier that blocks access of the antibiotic to the PG-D-Ala-D-Ala termini in the periplasm (Fig. 3).

Five *van* genes are necessary and sufficient for resistance

A series of ground-breaking studies by the infectious-disease group at the Pasteur Insititut headed by Patrice Courvalin [6] allowed the molecular logic of enterococcal resistance to vancomycin to be decoded. They isolated a plasmid from bacterial cells in the blood of a cancer patient who had died of a fulminant infection by enterococci that proved resistant to both β-lactam and glycopeptide antibiotics. Courvalin and colleagues determined that the plasmid contained the *van* genes within a transposon. They sequenced seven open reading frames (*vanS,R,H,A,X,Y,Z*) and proved by insertion mutagenesis that five genes, *vanS,R,H,A,X* (Fig. 4), were both necessary and sufficient to induce a high level (minimal inhibitory concentration of 1000 μg ml^{-1} vs 1 μg ml^{-1} for a sensitive strain) of antibiotic resistance. From the DNA sequence of these genes, they inferred putative functions for four of the

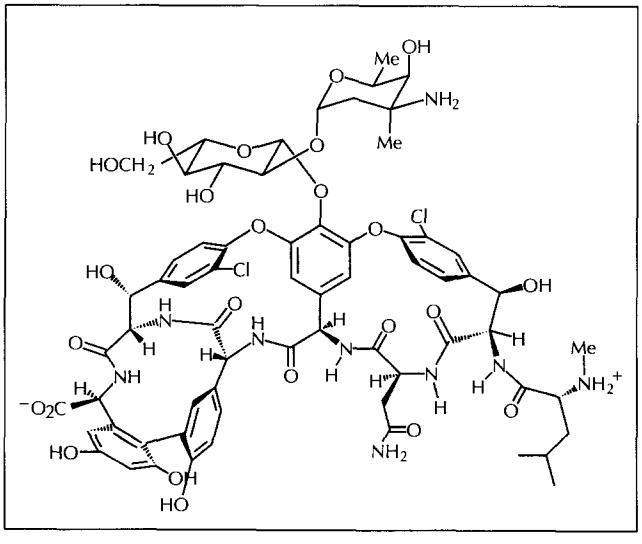

Fig. 1. Structure of vancomycin produced by *Amycolatopsis orientalis*.

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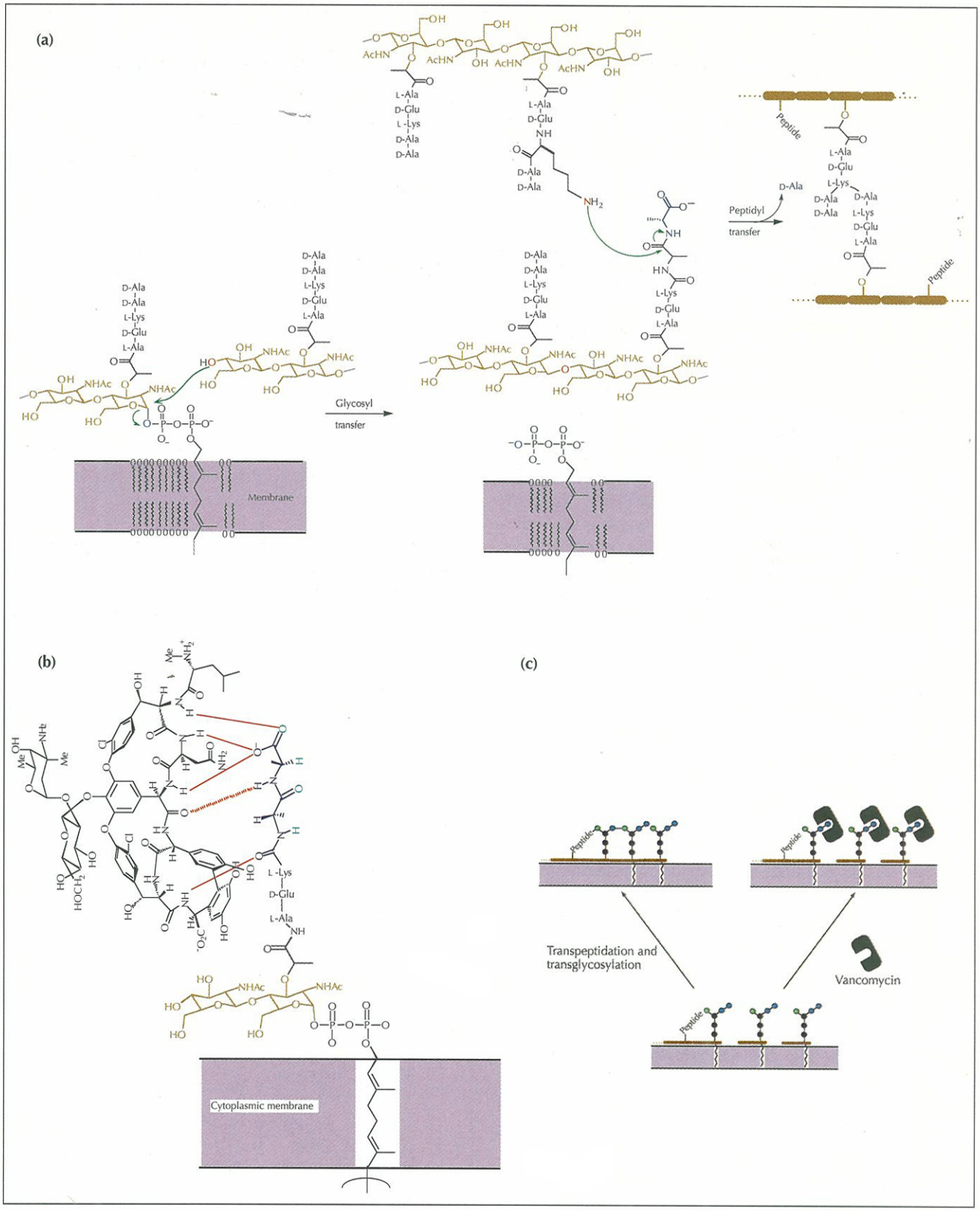

Fig. 2. Steps in the cell wall biosynthetic pathway that are inhibited by vancomycin. **(a)** The lactam-sensitive transglycosylation/transpeptidation steps of cell wall assembly are accomplished by glycosyl transfer via attack of the GlcNAc 4-hydroxyl group on the lipid-PP-disaccharide-pentapeptide intermediate to produce elongated strands of glycopeptide. These strands are further crosslinked through transpeptidase action which proceeds by attack of the Lys ϵ-NH_2 (or diaminopimelic acid in Gram-negative hosts) on the D-Ala-D-Ala termini of neighboring glycan strands to yield the mature, rigid bacterial cell wall. **(b)** Vancomycin (black) binds with high affinity to the D-Ala-D-Ala termini (blue) of the lipid-PP-disaccharide-pentapeptide intermediate through a network of five hydrogen bonds (red lines). **(c)** The vancomycin-PG-D-Ala-D-Ala complex sterically occludes the transglycosylation/ transpeptidation steps of cell-wall assembly, which renders the cell susceptible to lysis through osmotic shock.

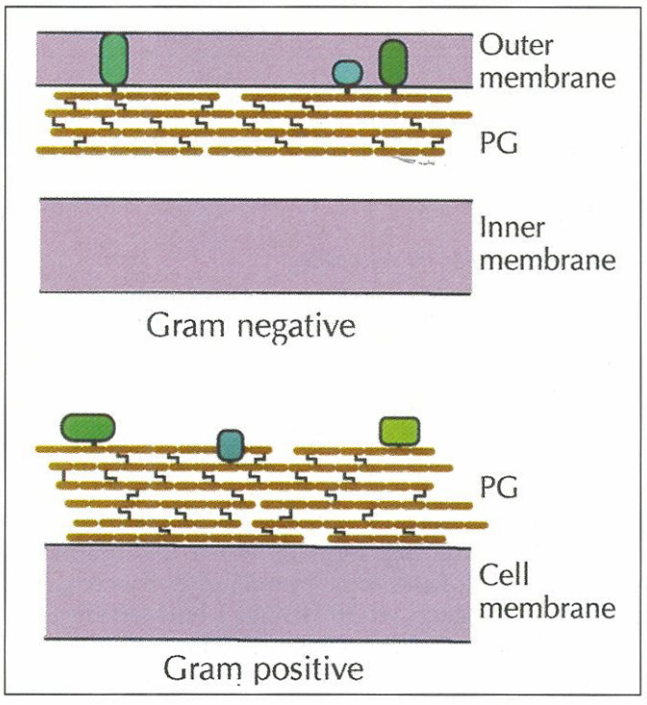

Fig. 3. Comparison of Gram-positive and Gram-negative bacterial cell wall organization. In Gram-negative cells, the peptidoglycan (PG) layer (gold) and the inner membrane are enveloped by an outer membrane which prevents diffusion of glycopeptide antibiotics to the PG layer. In contrast, the PG layer in Gram-positive bacteria is located on the outer surface of the cell membrane and is therefore susceptible to glycopeptide antibiotics.

gene products, VanR,S,H and A. All five encoded proteins have since been overexpressed in *E. coli*, purified, and characterized by our group [7–14]. Their functions, summarized in Table 1, reflect an elegant and ultimately a simple solution to engender resistance. As we shall note below, VanH,A and X are each enzymes that act in collaboration to produce an altered PG biosynthetic intermediate with reduced affinity for vancomycin. VanS is a sensor kinase and VanR is a response-regulator protein; these gene products comprise a two-component transcriptional-activation system that can turn on transcription of *vanH,A,X* to induce the biosynthesis of the altered cell-wall intermediates.

VanA: a D-Ala-D-Ala synthetase homolog that has acquired depsipeptide ligase activity

VanA is a 39-kDa protein that was the first of the five Van proteins whose production was noted to be associated with phenotypically resistant enterococci. [15]. It has 28 % sequence identity to an *E. coli* enzyme, D-Ala-D-Ala ligase (Ddl), a catalyst in the PG biosynthetic pathway that is responsible for ATP-dependent ligation of two D-alanine molecules. The D-, D-dipeptide product of this reaction is subsequently added by the MurF enzyme to the tripeptide intermediate UDP-muramyl-L-Ala-D-γ-Glu-*m*-DAP to yield the UDP-muramyl pentapeptide terminating in the N-acetylated D-Ala-D-Ala dipeptide unit. Once translocated from the cytoplasm to the periplasmic face of the cell membrane (as a C_{55} lipid-PP-intermediate, see Fig. 2b), this pentapeptide is the substrate for vancomycin binding. (Gram-positive bacteria can have lysine in place of the *meso*-diaminopimelate found in Gram-negative organisms.) Ddl homology was thus an intriguing property for the resistance protein VanA, and, upon overproduction in and purification from *E. coli*, the plasmid-encoded VanA had not only a (unphysiologically) weak D-Ala-D-Ala dipeptide ligase activity, but also a new ability to activate the D-hydroxy acids D-lactate and D-hydroxybutyrate as nucleophilic cosubstrates to yield novel ester products (e.g., D-Ala-D-lactate; equation 1) [7]. No Ddl enzymes so far examined have this depsipeptide ligase activity exhibited by VanA, suggesting that this gain of catalytic function is intimately associated with resistance. A VanA homolog, VanB, [6] from an enterococcal plasmid conferring low-level glycopeptide resistance also has depsipeptide ligase activity [16].

$$\text{D-Ala + D-lactate + ATP} \rightarrow \begin{cases} \text{D-Ala}_1\text{-D-Ala}_2 + \text{ADP} + P_i \\ \text{D-Ala}_1\text{-D-lactate} + \text{ADP} + P_i \end{cases} \quad (1)$$

While VanA has yet to be crystallized, the X-ray structure of *E. coli* Ddl in complex with a phosphorylated transition state analog and the product ADP has been solved [17], revealing the molecular architecture of this ligase family (Fig. 5a). As shown in Figure 5b, several protein side chains bind the dialkyl phosphinophosphate inhibitor and in particular, the side chain ϵ-NH_2 of Lys215 coordinates both to ADP and to the PO_3^{2-} group that has just undergone transfer as the γ-PO_3^{2-} of ATP to become the phosphate of the phosphinophosphate inhibitor. Lys215 is on a mobile ω-loop (in yellow in Fig. 5) that has closed over the active site. Tyr216 is also on this loop and forms an H-bond triad with Ser150 and Glu15, which in turn coordinates the α-NH_3 of the bound inhibitor. In VanA, several loop residues have changed including Tyr216, whereas Ser150 and Glu15 and several other active site residues are conserved.

Mutagenesis studies indicated that Tyr216→Phe, Ser150→Ala, and Glu15→Gln mutants of *E. coli* Ddl retain substantial dipeptide ligase activity [18]; close analysis of the Tyr216→Phe and Ser150→Ala mutants, however, reveals that they have gained the depsipeptide ligase activity of VanA, catalyzing both amide (dipeptide) and ester (depsipeptide) synthesis (eq. 1) with acid and

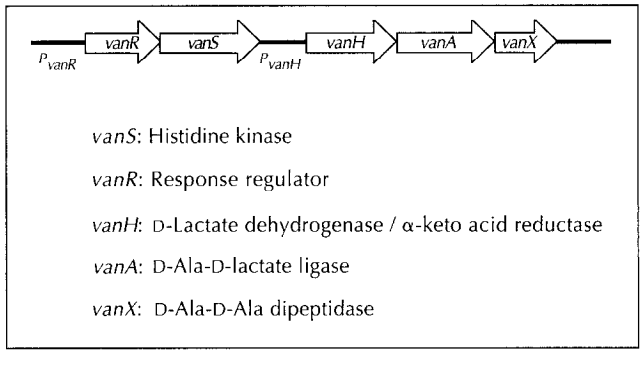

Fig. 4. Organization of the *van* gene cluster from the *Enterococcus faecium* BM4147 transposable element Tn*1545*.

Table 1. Activities and functions of the products of the five *van* genes that are necessary and sufficient for vancomycin resistance.

Protein	Activity	Function
VanS	Transmembrane histidine kinase	Sensor protein that initiates signal transduction pathway
VanR	Two-domain response regulator	Accepts PO_3^{2-} group from phospho-VanS, activates *vanH,A,X* transcription
VanH	D-Specific α-keto acid reductase	Generates D-lactate required for VanA action
VanA	Depsipeptide ligase for D-Ala-D-lactate	Generates an ester D-Ala-D-lactate in competition with normal amide D-Ala-D-Ala
VanX	Zn^{2+}-dependent D-Ala-D-Ala dipeptidase	Selective removal of D-Ala-D-Ala allows accumulation of D-Ala-D-lactate for addition to growing UDP-muramyl-tripeptide

alkaline pH optima, respectively (I.-S.P. & C.T.W., unpublished observations). These studies may reveal how an enzyme with depsipeptide ligase activity evolved from a dipeptide ligase.

VanH is a D-hydroxy acid dehydrogenase

The deduced amino-acid sequence of *vanH* has homology to α-keto acid reductases; it was thus possible that the reaction catalyzed by VanH produces substrates for the novel D-,D-depsipeptide ligase activity of VanA. To investigate the function of the *vanH* gene product, we expressed VanH in *E. coli*, purified it on a resin that is often used for NADPH binding proteins, and showed that the homogeneous VanH is an α-keto acid reductase, producing D-lactate or D-hydroxybutyrate from the readily available pyruvate or α-ketobutyrate substrates, respectively (Fig. 6a) [8]. In an enterococcal cell expressing VanH and VanA (both necessary for resistance) the tandem action of the two enzymes would produce D-Ala-D-lactate from pyruvate and D-Ala in competition with D-Ala-D-Ala (see Fig. 6a). When UDP-muramyl peptide intermediates were examined in vancomycin-resistant enterococci, UDP-muramyl tetrapeptide ester species, terminating in D-Ala-D-lactate, were detected [19–22], proving that the D-, D-depsipeptide produced in the reaction catalyzed by VanH,A could be used as a substrate by MurF (Fig. 6b).

VanX: A Zn^{2+}-dependent D-, D-dipeptidase

The *vanX* gene is co-transcribed with *vanH,A*, and VanX is also required to confer vancomycin resistance. The deduced amino-acid sequence of VanX gave no clue to its probable function, and its role remained obscure until Reynolds *et al.* [23] found evidence that it could hydrolyze D-Ala-D-Ala. We purified VanX from *E. coli* harboring an overproduction vector, confirmed the dipeptidase activity, showed a Zn-dependence and quantitated the selectivity for hydrolysis of D-Ala-D-Ala compared to

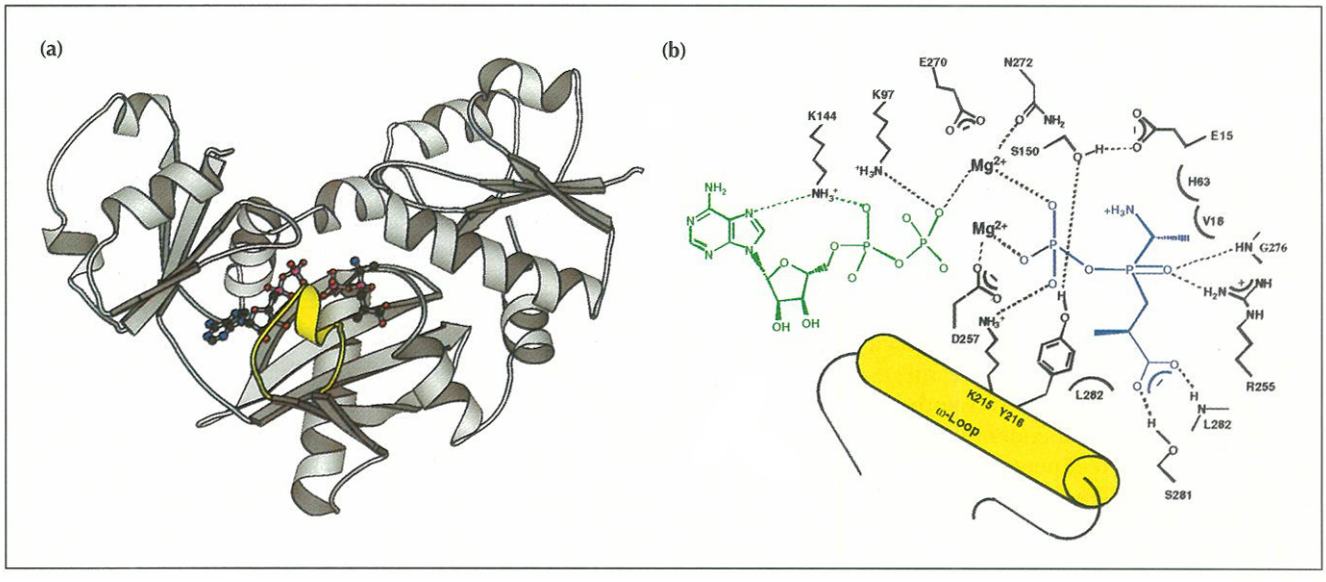

Fig. 5. Structural studies on the VanA-related enzyme, D-Ala-D-Ala ligase (Ddl). **(a)** MOLSCRIPT [32] representation of the enzyme complexed with ADP and a phosphinophosphate transition state analog inhibitor (both are represented in ball-and-stick form). The ω-loop (residues 205–220), believed to be involved in substrate binding and catalysis, is shown in yellow. **(b)** Schematic representation of the active site residues with hydrogen bonds (dashed lines) to ADP (green) and a phosphinophosphate transition state inhibitor (blue). The ω-loop is shown in yellow. Reprinted with permission from [18].

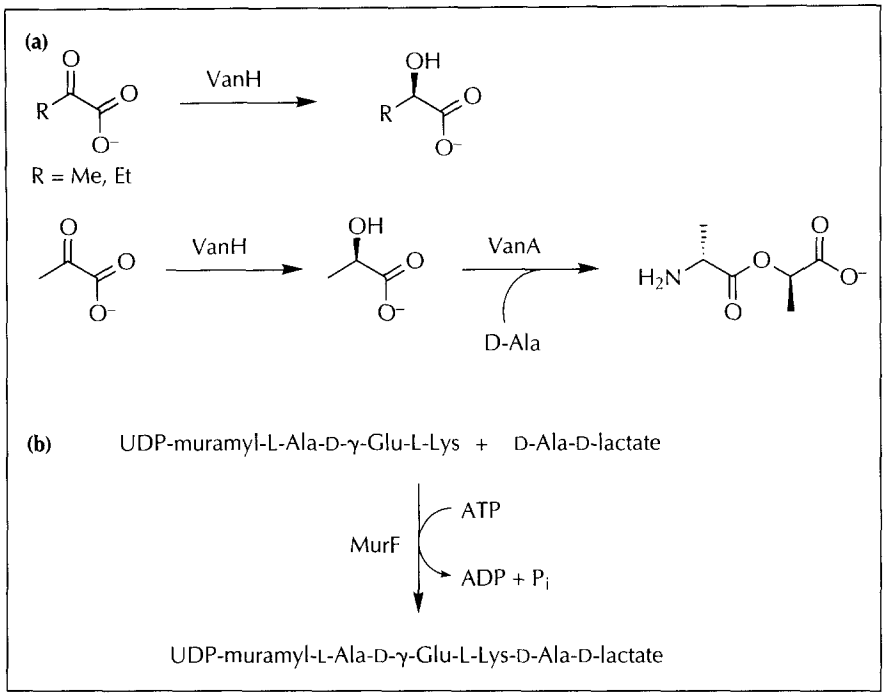

Fig. 6. Incorporation of D-lactate produced by VanH into cell wall precursors. **(a)** VanH functions as an α-ketoreductase to reduce pyruvate (R = Me) or α-ketobutyrate (R = Et), producing D-lactate or D-hydroxybutyrate, respectively. D-Lactate can serve as a substrate for VanA to produce D-Ala-D-lactate. **(b)** D-Ala-D-lactate can be used as a substrate by MurF to produce a UDP-muramyl tetrapeptide ester species terminating in D-lactate.

D-Ala-D-lactate [11]. Remarkably, the k_{cat}/K_M catalytic efficiency ratio indicated a preference for D-Ala-D-Ala hydrolysis of $>10^6$-fold; k_{cat} for D-Ala-D-lactate was $<10^{-4}$, and this substrate has 250-fold lower binding affinity than D-Ala-D-Ala. This comparison explains the role of VanX in the antibiotic-resistant cell. A mix of D-Ala-D-Ala and D-Ala-D-lactate are produced by the enterococcal Ddl (dipeptide) and by VanA (dipeptide and depsipeptide) ligases, but VanX spares the depsipeptide while hydrolyzing the dipeptide. Thus, in a VanH,A,X-producing bacterium, only D-Ala-D-lactate accumulates as a substrate for MurF, and thus the PG advanced intermediates in these bacteria contain only D-Ala-D-lactate termini (Fig. 7).

Vancomycin binds poorly to N-acyl-D-Ala-D-lactate

VanH,A,X-expressing enterococci are 1000-fold more resistant to vancomycin than sensitive enterococci [6]. To evaluate the contribution of the N-acyl-D-Ala-D-lactate moiety in the PG termini to this decreased sensitivity, we assayed binding constants of vancomycin for NAc-D-Ala-D-Ala or for the corresponding NAc-D-Ala-D-lactate and measured a 1000-fold reduction in K_d for the depsipeptide [8]. Thus the loss of the hydrogen bond from the NH of the D-Ala-D-Ala dipeptide moiety to the vancomycin backbone carbonyl (Fig. 8) quantitatively accounts for the resistance phenotype. The loss of one H-bond is the elegant and simple solution that can spell life or death to the bacterium and, perhaps, to an infected patient.

Signal transduction by VanS and VanR

In the absence of vancomycin, the VanH,A,X genes are transcriptionally inactive on a plasmid carrying the *vanRSHAX* operon (pIP816) [24]. Vancomycin may be the direct inducing ligand or may act indirectly, for example, by altering the synthesis/degradation balance of the cell wall. The DNA sequence of *vanS* and *vanR* indicated that this pair of genes was clearly related to the two-component regulatory systems that bacteria use to sense some environmental signal, transduce it across the membrane, and activate selective gene transcription to

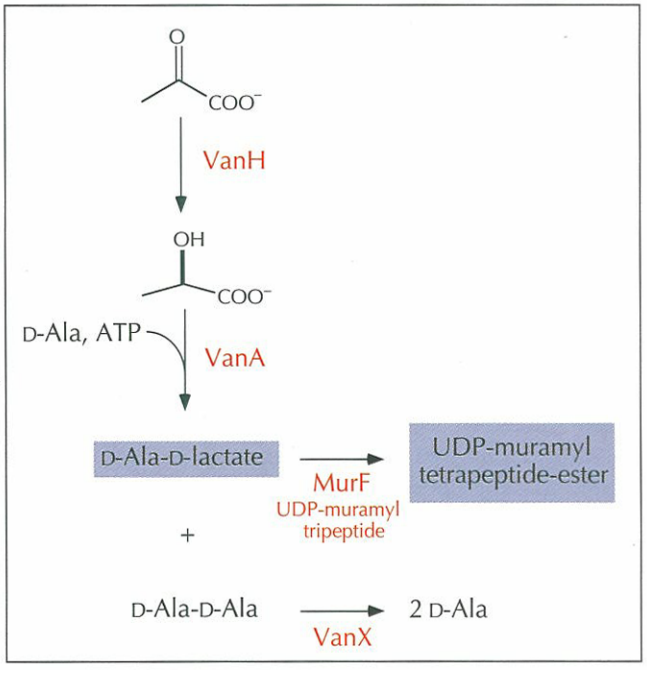

Fig. 7. Alternative cell wall biosynthetic pathway of the VanH,A,X proteins to produce peptidoglycan intermediates with D-Ala-D-lactate termini. Reduction of pyruvate by by the NADP-dependent dehydrogenase VanH leads to the production of D-lactate, which is an effective substrate for the ATP-dependent D-Ala-D-lactate depsipeptide synthase VanA. The D-Ala-D-lactate depsipeptide formed by VanA is then taken forward by the adding enzyme MurF to produce the muramyl-peptide-ester intermediate. VanX, a Zn^{2+}-dependent dipeptidase that acts to specifically hydrolyze the D-Ala-D-Ala dipeptide pool produced by the native, vancomycin-sensitive biosynthetic pathway, effectively shunts the flux of the cell wall biosynthesis to the VanH,A pathway.

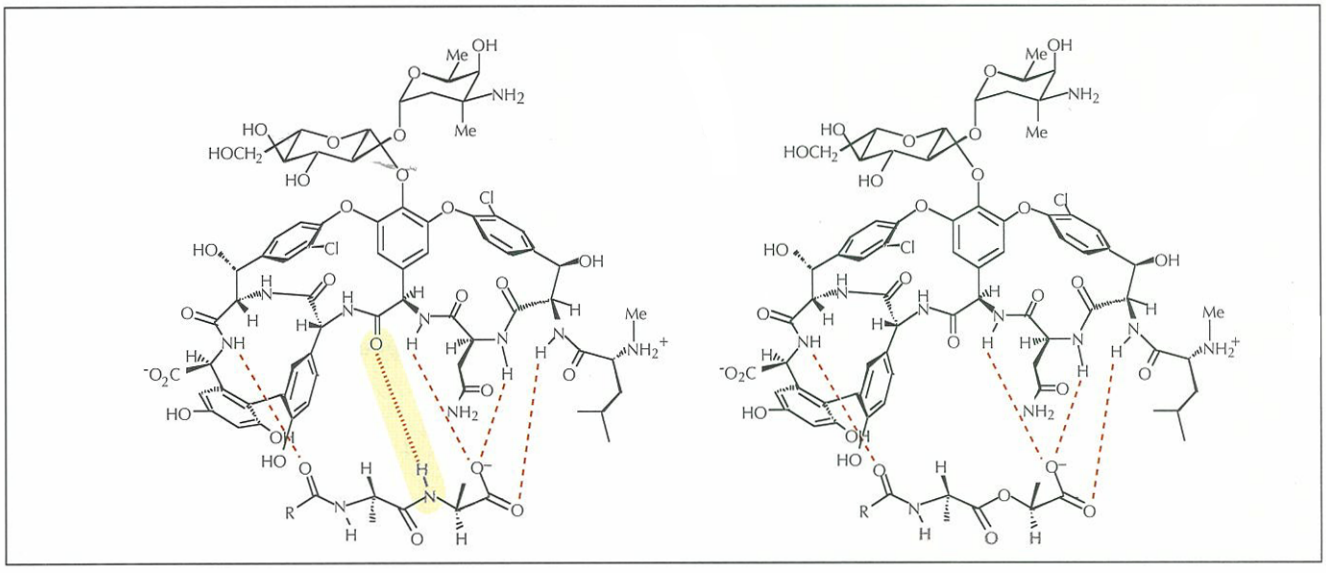

Fig. 8. Structures of the vancomycin complexes with N-Acyl-D-Ala-D-Ala (left) and N-Acyl-D-Ala-D-lactate (right). The hydrogen bond network responsible for the tight binding affinity to the drug is shown in red. The key hydrogen bond between the D-Ala amide NH and the vancomycin backbone, which is lost in the D-Ala-D-lactate complex, is highlighted in yellow.

effect a desired response. Such systems, comprising a transmembrane sensor kinase (such as VanS) and cytoplasmic response regulator (such as VanR), are found in bacterial responses to chemical gradients (chemotaxis), osmotic changes (EnvZ, OmpR in osmoregulation), nitrogen starvation, inorganic phosphate limitation, and dozens of other responses [25,26].

To test whether VanS and VanR followed this precedent, we expressed the predicted cytoplasmic fragment of VanS [12] fused to maltose binding protein (MBP) in *E. coli*, purified it, and demonstrated autokinase activity, yielding a phospho-His164 form of the VanS protein. When homogeneous VanR was obtained [12] and incubated with phospho-His-MBP-VanS, the PO_3^{2-} group was transferred to a specific conserved aspartyl residue (Asp53) in the amino-terminal domain of VanR (Fig. 9). The carboxy-terminal half of the 27-kDa VanR protein was predicted to contain a DNA-binding domain. Using gel-shift assays and footprinting analysis, we demonstrated that the phospho-Asp form of VanR bound more tightly than the dephospho form to symmetry elements in the promoter regions for the the *vanH,A,X* triad (P_{vanH}) and for *vanS,R* [13]. Since Courvalin and coworkers [24] have shown *in vivo* that VanR acts as a transcriptional activator, it is highly likely that the 500-fold increase in K_d for P_{vanH} binding by phospho-VanR compared to VanR correlates with increased transcription of *vanH,A,X* (Fig. 10). The signal-transduction pathway leading to vancomycin resistance clearly proceeds via the receptor histidine kinase/phosphoaspartyl transcriptional activator molecular logic in which external ligand binding is translated into interprotein phosphoryl group movement as the cytoplasmic information transfer currency. Selective transcription of *vanH,A,X* yields the three catalysts that remodel the biosynthetic termini of PG intermediates. The ester linkage in the PG-D-Ala-D-lactate strands is an effective substrate for crosslinking by the penicillin-sensitive transpeptidase, so there is no osmotic liability to the *Enterococcus* in this strategy, even though vancomycin affinity has been drastically reduced.

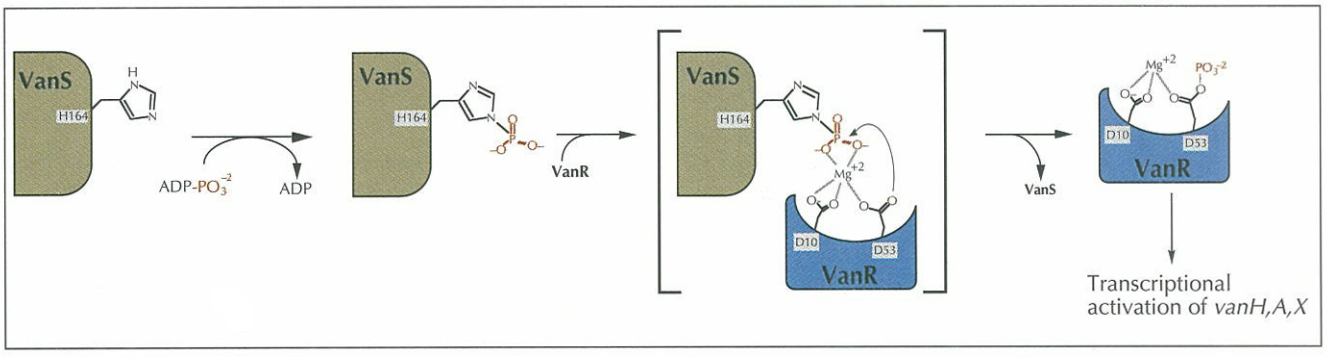

Fig. 9. Phosphotransfer relay in the VanS–VanR two-component signal-transduction system. VanS undergoes autophosphorylation with ATP on a histidine residue (His164) to yield the acid-labile, phosphorylated kinase. The phosphate group is then transferred to an aspartate residue on VanR (Asp53) through an Mg^{2+}-activated intermediate.

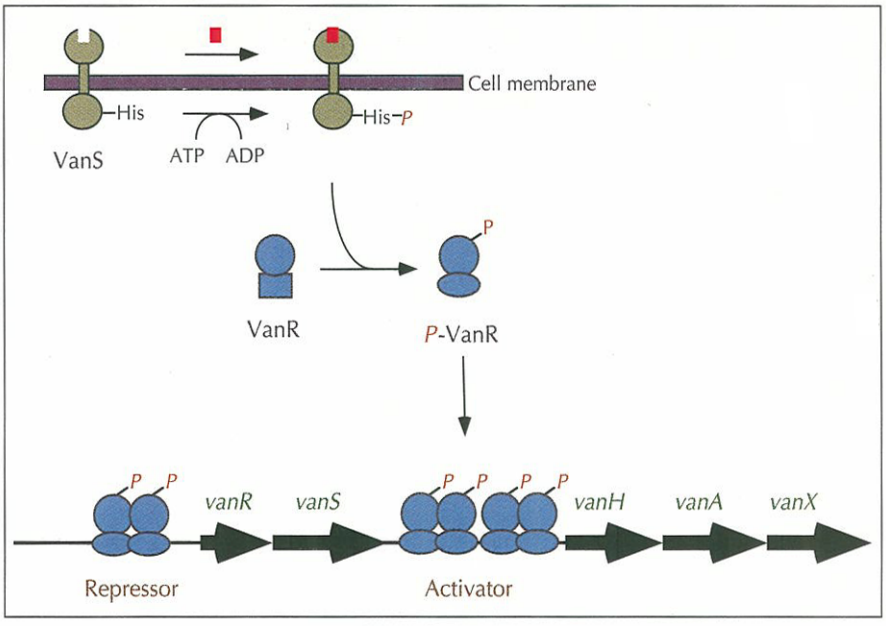

Fig. 10. Activation of transcription of the *vanH,A,X* multicistronic RNA by the VanS–VanR two-component system. Upon activation of VanS by a sensory ligand (magenta), VanS undergoes autophosphorylation at a histidine residue (His164). Subsequent transfer of the phosphate group to an aspartate group on VanR (Asp53) results in a conformational change on VanR to promote dimerization and DNA binding. Binding of phospho-VanR to the *vanH,A,X* promoter (P_{VanH}) results in transcriptional activation, while phospho-VanR binding to the *vanR,S* (P_{VanR})promoter results in repression of transcription.

Opportunities for intervention to reverse vancomycin resistance

With the molecular logic of each of the five necessary and sufficient vancomycin-resistance proteins revealed, the opportunities increase to intervene and convert vancomycin resistance back to sensitivity. On the one hand, rational approaches may be used for inhibition of the novel VanA depsipeptide ligase and for the unique Zn^{2+}-dependent VanX D-, D-dipeptidase, and slow-binding inhibitors have been described for each of these proteins [7–11]. On the other hand, one can establish screens against any of the five Van proteins. *Inter alia*, a screen that blocks phosphoryl transfer from sensor histidine autokinases to their cognate response regulators should be of general antibacterial utility [27]. Recent screening efforts against enterococci have yielded effective glycopeptide-antibiotic variants with increased hydrophobicity [28], supporting the hypothesis that the equilibria of glycopeptide dimerization [29] (which results in bidentate ligation to peptidoglycan termini) and the membrane avidity of the glycopeptide are important factors in the antibacterial potency of this class of antibiotics.

Finally a note on lessons from the past that may be relevant to future study of vancomycin/antibacterial resistance. In speculating on the molecular origin of the five *van* genes collected for high level resistance, we note that certain Gram-positive bacteria such as *Leuconostoc*, lactobacilli, and pediococci are intrinsically resistant to the vancomycin class of glycopeptides. These bacteria have now been examined and found to contain PG-D-Ala-D-lactate termini in their cell wall intermediates [30,31]. It is likely that this will also be the molecular mechanism for immunity by the vancomycin-producing *Amycolatopsis orientalis*. The ability to collect five genes to fashion the elegant, but simple, amide to ester switch in the vancomycin-resistance strategy, reinforces the concentrated ability of large microbial populations to go to great lengths to combat antibiotic action.

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