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GLYCOPEPTIDE ANTIBIOTIC RESISTANCE

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■ **Abstract** Glycopeptide antibiotics are integral components of the current antibiotic arsenal that is under strong pressures as a result of the emergence of a variety of resistance mechanisms over the past 15 years. Resistance has manifested itself largely through the expression of genes that encode proteins that reprogram cell wall biosynthesis and thus evade the action of the antibiotic in the enterococci, though recently new mechanisms have appeared that afford resistance and tolerance in the more virulent staphylococci and streptococci. Overcoming glycopeptide resistance will require innovative approaches to generate new antibiotics or otherwise to inhibit the action of resistance elements in various bacteria. The chemical complexity of the glycopeptides, the challenges of discovering and successfully exploiting new targets, and the growing number of distinct resistance types all increase the difficulty of the current problem we face as a result of the emergence of glycopeptide resistance.

INTRODUCTION

Glycopeptide antibiotics are crucial for the treatment of serious infections caused by gram-positive bacteria. Vancomycin is the most clinically relevant member of the glycopeptide family of antibiotics, which also includes one other medically useful member, teicoplanin (Figure 1). These glycopeptide antibiotics find application in the treatment of bacterial infections caused by gram-positive pathogens including *Staphylococci*, *Streptococci*, *Enterococci*, and *Clostridia*, generally in the hospital setting rather than in the community. Discovered in the early 1950s as a secondary metabolite produced by the soil bacterium *Streptomyces orientalis* (now renamed *Amycolatopsis orientalis*), vancomycin rapidly entered clinical use for the treatment of infections caused by gram-positive bacteria that were unresponsive to available antimicrobial agents. The use of vancomycin was eventually limited as a result of toxicity issues originating from nonhomogenous preparations of the antibiotic, and the drug was eventually largely supplanted by the discovery and approval of second and third generation β-lactam antibiotics such as methicillin, which showed activity against even penicillin-resistant bacteria. Thereafter, there was only nominal use of vancomycin until the late 1970s when antibiotic resistant strains of bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA)

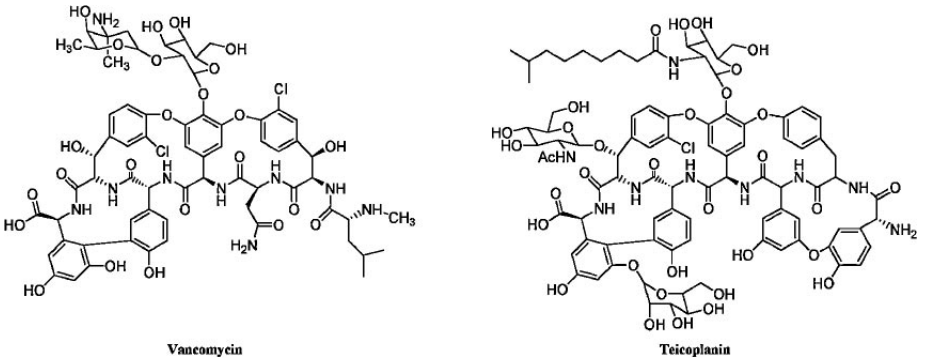

Figure 1 The structures for vancomycin and teicoplanin. Vancomycin (left) and teicoplanin (right) are currently the only clinically used glycopeptide antibiotics.

began to emerge as widespread nosocomial pathogens. Consequently, vancomycin use climbed steadily in the 1980s (1), and resistance eventually emerged and was first reported in 1986. The presence of resistance to this highly important class of antibiotics significantly threatens clinicians' ability to treat serious infections caused by gram-positive bacteria; this change has resulted in great interest in the development of new antibacterial agents and in developing an understanding of the molecular basis of vancomycin resistance.

Glycopeptide Antibiotics: Structure and Mechanism of Action

Vancomycin is a member of the glycopeptide antibiotic family that has several dozen members. These antibiotics consist of one of two core linear heptapeptide structures that in turn are modified by selective glycosylation and amino acid modification to generate distinct compounds. Besides common amino acids found in proteins such as Asn and Leu, glycopeptides also incorporate unusual amino acids including 3,5-dihydroxyphenylglycine (DHPG), β-hydroxytyrosine (β-OHTyr), and *p*-hydroxyphenylglycine (HPG). In addition to linkage through peptide bonds, the aromatic amino acids can also be involved in cross-links via aromatic carbon-carbon or ether linkages between amino acids 2 and 4, 4 and 6, and 5 and 7. The two clinically used glycopeptide antibiotics, vancomycin and teicoplanin, also serve as prototypes for the heptapeptide core structures (Figure 2). Both conserve the C-terminal tetrapeptide consisting of (HOOC)-DHPG-(β-OHTyr)-HPG-HPG-(NH_2). The core structures then diverge in the N-terminal tripeptide with the vancomycin class consisting of (HOOC)-Asn-β-OHTyr-Leu-(NH_2), and the teicoplanin class of (HOOC)-HPG-β-OHTyr-HPG-(NH_2). Diversity in these core structures is achieved through chemical modification of the amino acids, e.g., chlorination of β-OHTyr, N-methylation of Leu, sulfonation of HPG, and through selective glycosylation, most commonly at the central HPG and the penultimate β-OHTyr, but also at the C-terminal DHPG and the N-terminal HPG of antibiotics with the teicoplanin core. Additional glycosylation of the carbohydrate residues

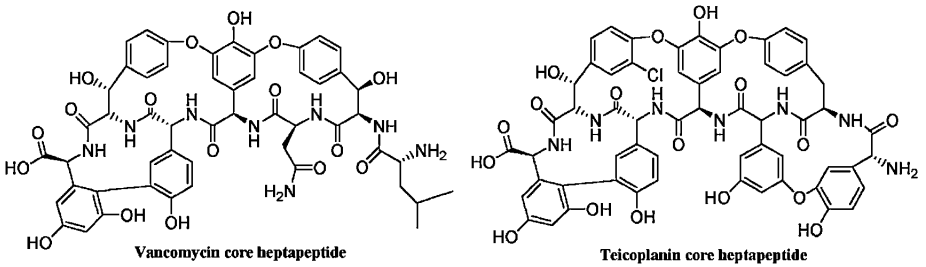

Figure 2 The heptapeptide backbone for vancomycin and teicoplanin. Vancomycin (left) and teicoplanin (right) each contain the same four C-terminal amino acids (3,5-dihydroxyphenylglycine, β-hydroxytyrosine, *p*-hydroxyphenylglycine, and *p*-hydroxyphenylglycine). They differ in the three N-terminal amino acids, which are asparagine—β-hydroxytyrosine—leucine for vancomycin type and *p*-hydroxyphenylglycine—β-hydroxytyrosine—and *p*-hydroxyphenylglycine for teicoplanin-type heptapeptide cores.

can occur, as can substitution of sugars by fatty acyl groups such as in teicoplanin (Figure 1). The teicoplanin class of glycopeptides can also be subdivided into compounds where amino acids 1 (HPG) and 3 (DHPG) are cross-linked, e.g., teicoplanin, and where they are not, e.g., avoparcin.

The generally accepted mechanism of antimicrobial action is through inhibition of extracellular steps in peptidoglycan biosynthesis. Specifically, glycopeptide antibiotics interact with the D-Ala-D-Ala terminus of the uncross-linked peptidoglycan pentapeptide (N-acetyl-muramyl-L-Ala-D-Glu-Lys-D-Ala-D-Ala in most gram-positive bacteria) (*2*). A series of elegant NMR experiments by the group of D. Williams demonstrated that the interaction between N-acyl-D-Ala-D-Ala and glycopeptides consisted of a network of five hydrogen bonds to form a noncovalent complex that effectively sequesters the D-Ala-D-Ala dipeptide, thereby physically inhibiting the action of transpeptidases that generate the cross-linked peptidoglycan necessary for the appropriate tensile strength required by the organisms (Figure 3). Another effect is the inhibition of the transglycosylase (which coincidently is part of a bifunctional extracellular enzyme with transpeptidase activity), which effectively prevents growth of the peptidoglycan chain. This latter activity is the result of direct inhibition of this activity by the vancomycin carbohydrate residues (3). Thus glycopeptides interfere in an intricate fashion with peptidoglycan growth and assembly, in large part by sequestering the substrates of the transpeptidase/transglycosylase with an ultimate bactericidal effect. The requirement for direct access of the large antibiotic (molecular weight >1300 g/mol) with the target peptidoglycan explains the selective inhibition of gram-positive bacteria with surface-exposed peptidoglycan over gram-negative bacteria where the peptidoglycan is protected by the presence of an outer lipopolysaccharide membrane impermeable to large biomolecules.

Many glycopeptides have the capacity to form back-to-back dimers (Figure 4), and this property is linked to antibiotic efficacy (4). Glycopeptides such as

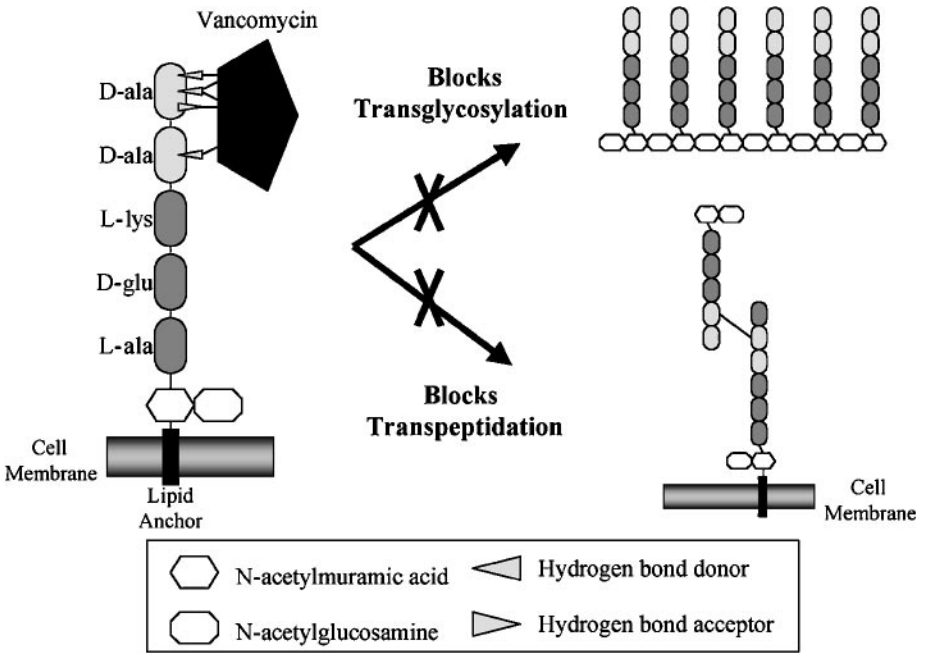

Figure 3 The bactericidal effect of vancomycin on gram-positive bacteria. Vancomycin binds noncovalently via five hydrogen bonds to the D-Ala-D-Ala terminus of the peptidoglycan pentapeptide linker. This tight binding of vancomycin inhibits the cross-linking of the peptidoglycan, lowering the strength of the bacterial cell wall. Transglycosylation is also inhibited, thus stopping peptidoglycan growth by blocking the addition of N-acetylmuramic acid and N-acetylglucosamine.

chloroeremomycin, which readily form asymmetrical back-to-back dimers, show higher affinity for peptidoglycan components and consequently more potent antibiotic activity. A hypothesis that invokes lower entropy barriers for binding of dimeric glycopeptides to peptidoglycan has been proposed to account for the available data (4). A similar explanation has been put forth for the activity of monomeric teicoplanin, which does not form dimers but possesses a fatty acid moiety capable of bacterial cell membrane anchoring that consequently positions the drug adjacent to the peptidoglycan target (5).

GLYCOPEPTIDE RESISTANCE

The extracellular and virtually ubiquitous acyl-D-Ala-D-Ala target of the glycopeptide antibiotics was believed to be a significant impediment to the development of resistance. Indeed, vancomycin was in clinical use for over four decades

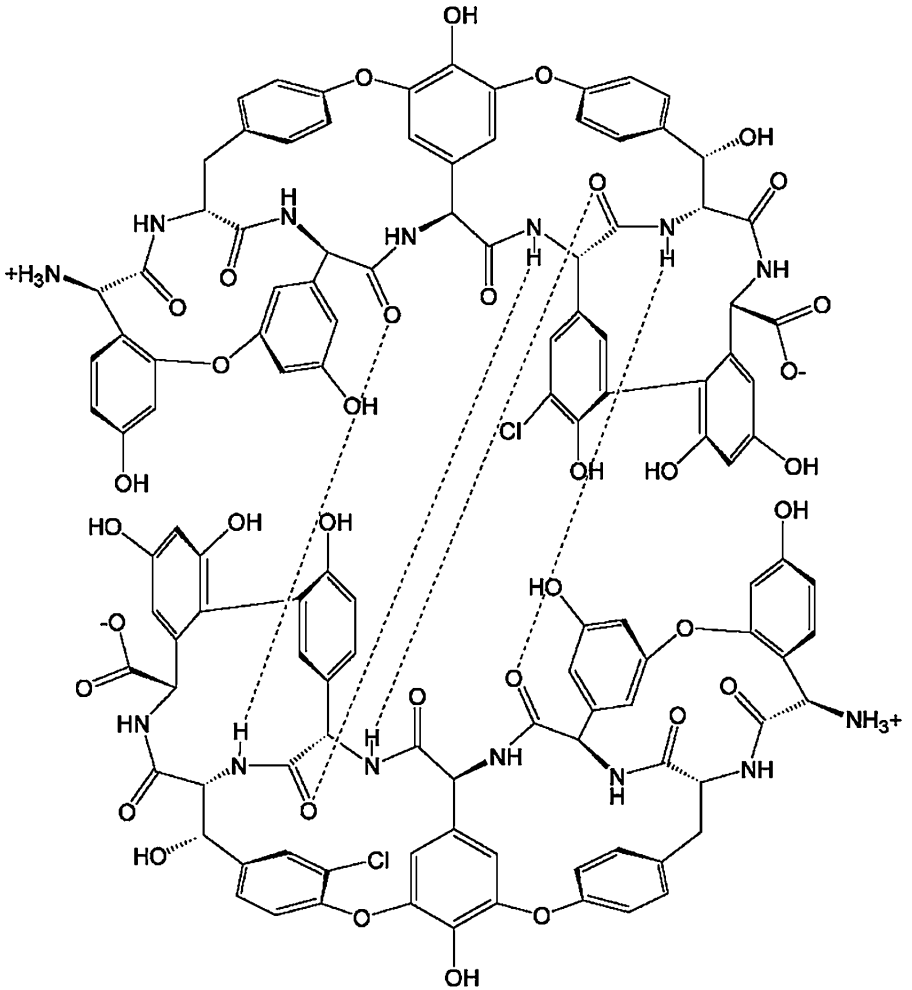

Figure 4 The dimerization of glycopeptide antibiotics. Shown is the back-to-back dimerization of the teicoplanin heptapeptide core structure via four hydrogen bonds. Dimerized glycopeptide antibiotics show a higher affinity for peptidoglycan.

before resistance in bacterial clinical isolates was identified in 1986 (6). The emergence of transferable resistance to glycopeptide antibiotics in species of Enterococci (now collective termed VRE—vancomycin resistant Enterococci) was a grave clinical concern because these bacteria, while generally not highly virulent, are nonetheless major causes of serious hospital-acquired infections. Furthermore, Enterococci are responsive to only a few antibiotic treatment regimens such as penicillin-aminoglycoside combinations, with vancomycin therapy being the method of choice or else the last option (7). Additionally, the concern has been,

and continues to be, that the mechanisms of high-level glycopeptide resistance in VRE will be transferred to the more virulent staphylococci with the potential of generating multi-antibiotic resistant strains of unparalleled impact. Over the past 15 years, knowledge of the molecular mechanism of glycopeptide resistance of the Enterococci has developed considerably, and while resistance has begun to emerge in the more virulent staphylococci and streptococci, the mechanisms appear to be distinct from those in Enterococci.

Glycopeptide Resistance in Enterococci

Glycopeptide resistance in enterococci manifests itself in at least six distinct phenotypes classified based on inducibility, breadth of resistance to individual compounds, and level of resistance (Table 1).

VanA The first vancomycin resistance type to be studied in depth was VanA from the Enterococcus transposon *Tn*1546 (8) (Figure 5). The seven genes found on this transposon include three genes that are essential for vancomycin resistance: *vanH*, *vanA*, and *vanX*. The *vanH* gene encodes an α-keto acid reductase that generates exclusively the D-isomer of lactate (9). A thioredoxin fusion of the enzyme has been prepared and crystallized, generating crystals that diffract to 3.0 Å resolution, but the complete structure of the protein has not yet been solved (10). The structure of a homologous D-lactate dehydrogenase from *Lactobacillus pentosus* (11) is known however, and based on this structure, site-directed mutagenesis and mechanistic analysis of the VanH orthologue VanHst from the glycopeptide producing bacterium *Streptomyces toyocaensis* confirmed the roles of active site residues Arg231, Glu260, and His292 (VanH from *Tn*1546 numbering) as important in substrate orientation and catalysis (Figure 6) (12).

TABLE 1 Glycopeptide antibiotic resistance in Enterococci

Phenotype	Peptidoglycan terminus	Resistance (MIC in μg/mL)	Source	Induction	Organisms
VanA	D-Ala-D-Lact	Vanc ($\geq$64) Teic ($\geq$16)	Acquired e.g., Tn 1546	Inducible	*E. faecium* *E. faecalis*
VanB	D-Ala-D-Lact	Vanc ($\geq$4)	Acquired e.g., Tn 1547	Inducible	*E. faecalis* *E. faecium*
VanC	D-Ala-D-Ser	Vanc ($\geq$2)	Intrinsic	Constitutive and Inducible	*E. gallinarum* *E. casseliflavus*
VanD	D-Ala-D-Lact	Vanc ($\geq$16) Teic ($\geq$2)	Intrinsic	Constitutive	*E. faecium*
VanE	D-Ala-D-Ser	Vanc (16)	Acquired	Inducible	*E. faecalis*
VanG	D-Ala-D-Ser?	Vanc (16)	?	?	*E. faecalis*

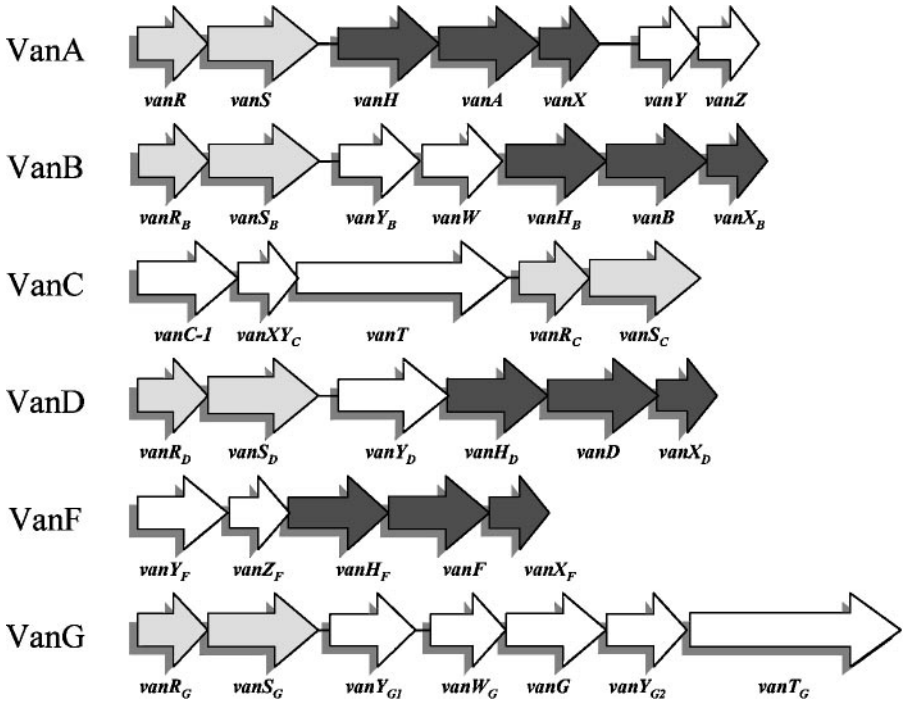

Figure 5 The *van* gene clusters that confer resistance to glycopeptide antibiotics. Each cluster shows the genes associated with that particular resistance phenotype. Arrows in dark grey represent the *vanHAX*- or *vanHAX*-like genes that confer resistance in an anologous manner. Arrows in light grey represent the similar *vanR-vanS* two-component regulatory system. The cluster for VanE type resistance has yet to be sequenced.

The *vanA* gene in this cluster encodes a D-Ala-D-Ala ligase of altered specificity. The D-Ala-D-Ala dipeptide that caps the pentapeptide portion of the peptidoglycan is synthesized inside the cell through the aegis of an ATP-dependent peptide synthetase termed D-Ala-D-Ala ligase. The VanA amino acid sequence shows 20%–40% homology to these ligases and has weak but demonstrable D-Ala-D-Ala synthesis activity (13), but it has robust ester synthesizing activity preferentially generating the despipeptide D-Ala-D-Lactate (D-Ala-D-Lact) (14). The crystal structure of VanA in complex with ADP and a phosphorylated phosphinate inhibitor has recently been determined (15), and as expected the structure shows significant homology in folding to the previously determined structure of *E. coli* D-Ala-D-Ala ligase DdlB (16). The enzymes do differ in a flexible ω-loop that provides important catalytic residues and closes over the active site upon binding of the ATP and two D-Ala substrates. The ω-loop is the key to the differential peptide vs. depsipeptide synthesis activity. Careful enzyme mechanism studies have demonstrated that VanA shows weak but significant D-Ala-D-Ala synthesis

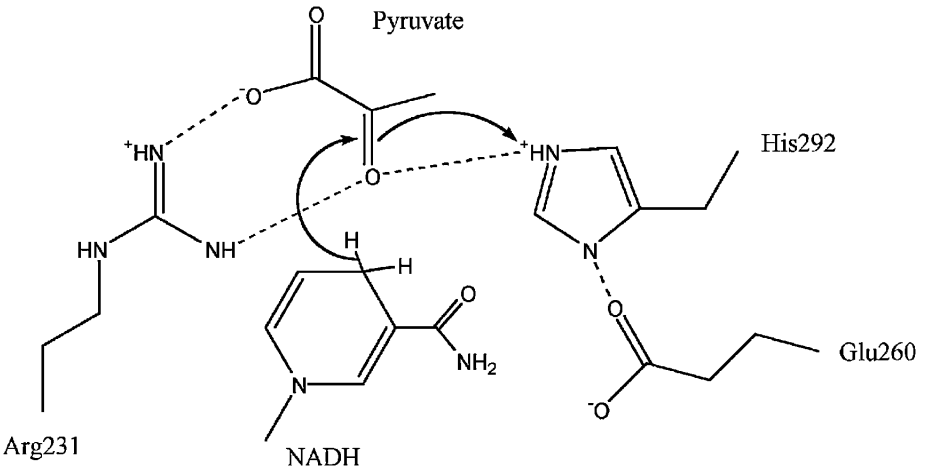

Figure 6 Orientation of vanH active site. Production of D-lactate occurs by hydride transfer from NADH to pyruvate through the action of His292. The correct orientation of pyruvate is maintained by Arg231. Numbering is from VanH in *Tn*1546.

capacity at neutral pH, but that activity rivaling bone fide D-Ala-D-Ala ligases can be measured at higher pH (17). One interpretation of these results is that VanA's specificity toward D-Lact at physiologic pH is the result of preferential capture of neutral hydroxyl at position 2 over the ammonium cation of D-Ala [although this has been disputed based on calculations of electrostatics (18)]. The molecular basis for this discrimination is likely the replacement of an ω-loop Tyr (Tyr216 in DdlB) of D-Ala-D-Ala ligases with a His (His244 in VanA) in D-Ala-D-Lact synthesizing ligases such as VanA. The presence of a protonated His would repel the zwiterinonic D-Ala at neutral pH (Figure 7). The critical role of Tyr216 in D-Ala-D-Ala vs. D-Ala-D-Lact synthesis has been demonstrated by several site-directed mutagenesis studies (17, 19, 20).

In addition to the VanH D-Lact dehydrogenase and the VanA D-Ala-D-Lact ligase, high-level glycopeptide resistance requires the presence of VanX, a Zn-binding metallodipeptidase (21, 22). This dipeptidase cleaves D-Ala-D-Ala virtually exclusively with no reactivity toward the thermodynamically more labile D-Ala-D-Lact ester (1010-fold discrimination). The structure of the VanX enzyme has been determined at the Abbott Laboratories to 2.2 Å resolution (23). The structure reveals the predicted active site Zn^{2+} ion and a small active site, tailored for dipeptide recognition. Structure-guided, site-directed mutagenesis of VanX has confirmed the roles of active site residues in catalysis and substrate recognition (Figure 8), however the stringent specificity favoring dipeptidase vs. esterase activity is not obvious. The biochemical role of VanX in glycopeptide resistance is therefore to selectively clear intracellular pools of D-Ala-D-Ala, thereby increasing the effective concentration of VanH/VanA-produced D-Ala-D-Lact and ensuring that the

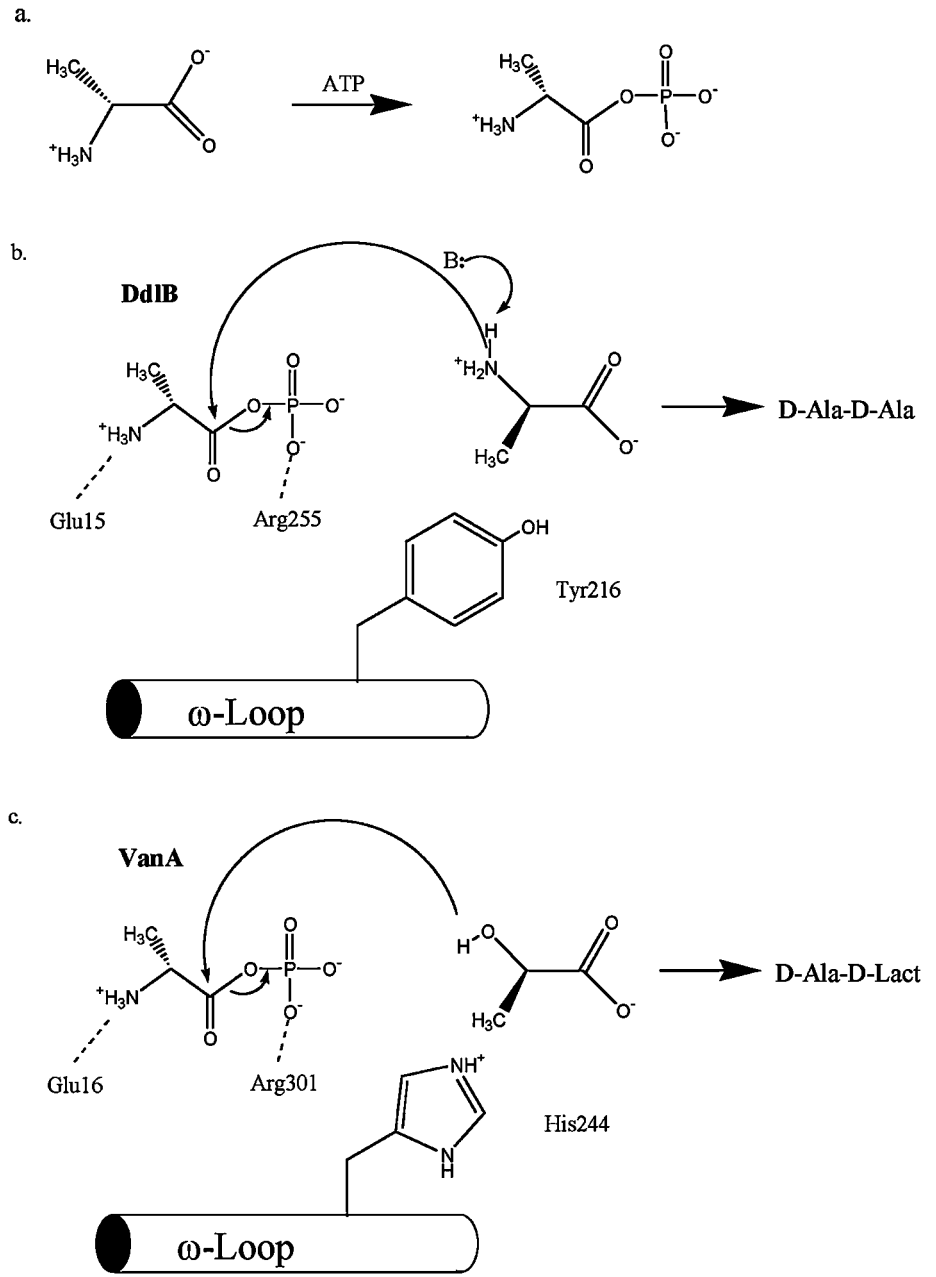

Figure 7 Production of D-ala-D-X dipeptides by bacterial ligases. (*a*) Activation of D-alanine with high energy phosphate from ATP. (*b*) Comparison of the catalytic mechanism for *E. coli* DdlB (top) and *E. facaelis* VanA (bottom). In VanA, substrate selectivity is controlled by the presence of His244, which replaces Tyr216 in DdlB.

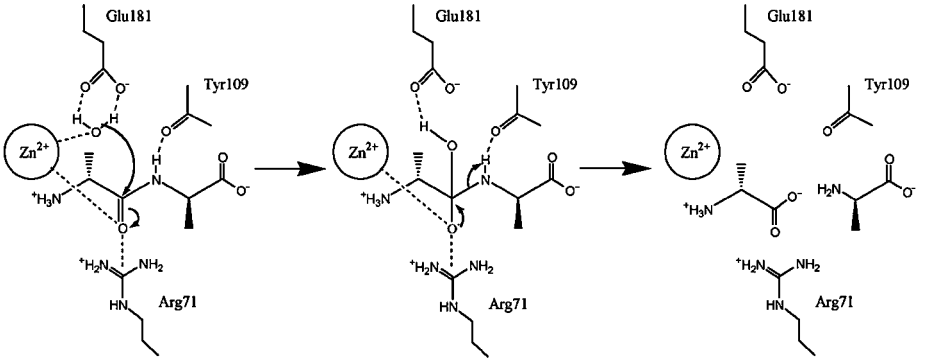

Figure 8 Hydrolysis of D-Ala-D-Ala dipeptide by VanX. The addition of water across peptide bond is coordinated by a single Zn^{2+}.

peptidoglycan terminates in this depsipeptide rather than the nearly ubiquitous D-Ala-D-Ala dipeptide (Figure 9).

This form of peptidoglycan terminating in D-Ala-D-Lact therefore lacks a critical hydrogen bond donor in the D-Ala-D-Ala amide hydrogen that plays a role in glycopeptide binding (Figure 9). The net loss of this bond results in a weakening of the interaction by approximately ~4 kcal/mol and commensurate 1000-fold drop in *Kd*. Cells expressing the three essential enzymes VanH, VanA, and VanX are therefore glycopeptide resistant by virtue of a change in antibiotic target structure to one that is no longer effectively recognized by the drug and simultaneously by VanX-mediated biochemical suppression of the constitutive metabolic pathway generating D-Ala-D-Ala. This strategy of altered peptidoglycan biosynthesis and structure is common to the acquired and intrinsic glycopeptide resistance mechanisms in enterococci, lactic acid bacteria, and glycopeptide producing organisms.

Two regulatory proteins, VanS and VanR, are also found in the VanA cluster. These comprise a canonical two-component regulatory system that responds to the presence of glycopeptides by upregulating the expression of the *vanH*, *vanA*, *vanX* genes. The regulation of resistance is detailed in the section below.

Two additional genes are also associated with the VanA cluster, *vanY and vanZ*. VanY is a membrane-associated Zn-dependent D,D-carboxypeptidase with both UDP-pentapeptide-D-Ala-D-Ala and pentapeptide-D-Ala-D-Lact hydrolysis activity (24, 25). The presence of VanY contributes to high-level vancomycin resistance in the presence of the *vanH-vanA-vanX* cluster (26, 27), by removing D-Ala-D-Ala and D-Ala-D-Lact termini on the exterior of the cell. The presence of VanY is not essential, however, because clinical isolates of VRE in which *vanY* has been disrupted by insertion elements are nonetheless vancomycin resistant (28). The biochemical role of VanZ, a protein of predicted mass of 18.5 kDa, basic pI and membrane association, is unknown but is coupled with teicoplanin resistance (29).

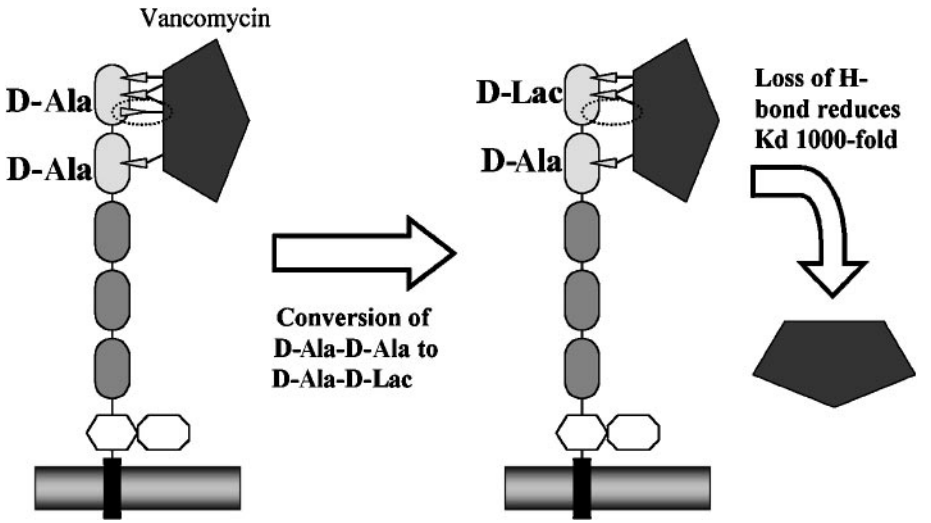

Figure 9 Resistance mechanism to vancomycin by *vanHAX* type resistance. Conversion of the terminal D-Ala-D-Alanine in the pentapeptide linker to D-Ala-D-Lactate occurs through the action of VanH, VanA, and VanX. Conversion to D-lactate results in the replacement of an amide bond by an ester bond that now lacks one hydrogen bond donor. The result is vancomycin binds with only four hydrogen bonds to D-Ala-D-lact compared to five for D-ala-D-ala and a lowering of the Kd by 1000-fold.

VANB The VanB phenotype is also linked with the production of D-Ala-D-Lact terminating peptidoglycan and consequently requires expression of the appropriate $VanH_B$, $VanA_B$, and $VanX_B$ enzymes with identical roles to their VanA counterparts and significant primary sequence homology (>70%) (30–32). In addition, a $VanY_B$ is generally present as is a gene of unknown function, *vanW* (32, 33). There is also a two-component system associated with this phenotype $VanR_B$-$VanS_B$, but the sequence homology with the VanA two-component system is lower (∼30%) than between the VanH,A,X enzymes (32). The VanB phenotype is characterized both by its sensitivity to teicoplanin and by vancomycin-inducible glycopeptide resistance to vancomycin (Table 1). This phenotype also differs from VanA in that it is more often (but not exclusively) found localized to the bacterial chromosome (34) rather than on highly mobile genetic elements such as plasmids. However, this may reflect the mobilization of very large genetic elements, such as the 34 kb transposon Tn*1549* (33), that are more difficult to identify and characterize.

VANC The VanC phenotype provides intrinsic, low-level resistance (MIC <32 μg/mL) to vancomycin only in *E. gallinarum*, *E. casseliflavus*, and *E. flavescens* (Table 1). These organisms also produce a D-Ala-D-Ala ligase of altered specificity (35, 36), not with ester synthesis capacity but with preferential

D-Ala-D-Ser generation (37–39). The production of peptidoglycan terminating in D-Ala-D-Ser impairs glycopeptide antibiotic binding, likely by steric means, lowering the association constant by sixfold (40). Sequencing of the regions upstream and downstream of the *vanC-1* gene in *E. gallinarum* has revealed four additional genes *vanXYc*, *vanT*, *vanRc*, and *vanSc* (41). VanXYc is a predicted Zn^{2+}-dependent peptidase with VanX-like D-Ala-D-Ala hydrolysis and VanY-like UDP-MurNAc-pentapeptide-D-Ala-D-Ala carboxypeptidase activity, but with no ability to cleave the C-terminal D-Ser from UDP-MurNAc-pentapeptide-D-Ala-D-Ser (42). The requisite D-isomer of Ser is generated by the membrane-bound pyridoxal phosphate-dependent racemase VanT (43, 44). The racemase domain is located in the cytosolic C-terminus of the protein, and it also has sufficient Ala racemase activity to complement a temperature-sensitive *Escherichia coli* alanine racemase mutant (43, 44). Thus, in a fashion analogous to the VanA/B clusters, the VanC cluster generates the requisite substrate, D-Ser via VanT, for a D-Ala-D-Ala ligase of altered specificity, VanC with D-Ala-D-Ser synthesis capacity, to generate peptidoglycan terminating in a DD-dipeptide that is refractory to glycopeptide antibiotic binding. The presence of VanXYc ensures a homogenous D-Ala-D-Ser incorporation and clearance of D-Ala-D-Ala termini. The presence of a two-component couple, *vanRc*/*vanSc*, downstream from the *vanT* gene, suggests that the VanC system may be inducible, despite being generally thought to be constitutively expressed and despite the determination that, in strain BM4174, that *van* gene expression was not vancomycin-dependent (41). The characterization of some strains of *E. gallinarum* that show vancomycin-dependent *vanc-1* expression (45) suggests a) that there is likely a bone fide D-Ala-D-Ala ligase in the genome and b) that VanRc and VanSc are likely candidates for regulation of inducible resistance in these strains.

VanD The VanD phenotype is characterized by constitutive vancomycin and teicoplanin resistance (Table 1) (46). Cloning of the glycopeptide resistance cluster revealed the canonical *vanR$_D$*, *vanS$_D$*, *vanY$_D$*, *vanH$_D$*, *vanD*, *vanX$_D$* gene assembly predicting D-Ala-D-Lact synthesis in this strain (47). The sequence of the chromosomal D-Ala-D-Ala ligase gene, *ddl*, revealed a 5-bp insert that caused a catastrophic frameshift resulting in inactive enzyme (47). This is reminiscent of enterococcal *ddl* mutants that can grow only in the presence of vancomycin, as the antibiotic is required to induce the *vanA* or *vanB* genes so that peptidoglycan biosynthesis and assembly can continue (48, 49). Since VanD strains are constitutively expressing D-Ala-D-Lact, they are no longer responsive to the $VanR_D/VanS_D$ two-component system, and accordingly strains in which VanSD has been inactivated by frameshift mutation have been described (50).

VanE and VanG Recently, two novel glycopeptide resistance types were identified in *E. faecalis*, VanE and VanG (51, 52). Both confer low level resistance to vancomycin (MIC 16 mg/mL) and show no resistance to teicoplanin. Several lines of evidence suggest that the VanE phenotype is associated with VanC-like

D-Ala-D-Ser incorporation in the peptidoglycan. First, VanE D-Ala-D-X ligase shows 55% identity with the VanC ligase and only 43%–45% identity with VanA, VanB, or VanD; second, vancomycin-inducible Ser racemase activity was detected in the membrane fractions; and third, the peptidoglycan precursor UDP-MurNAc-pentapeptide-D-Ala-D-Ser was identified in the presence of vancomycin only (51).

The *vanG* gene cluster has been sequenced and contains a two-component system *vanRG*, *vanSG*, and also *vanYG1*, *vanWG*, *vanG*, *vanYG2*, and *vanTG*. The absence of VanH and VanX homologues and the *vanD*, *vanYG2*, *vanTG* gene arrangement suggest a D-Ala-D-Ser peptidoglycan; however, this awaits confirmation by biochemical methods, especially in view of the fact that the VanG ligase is slightly more similar to the VanA and VanB ligases (44% and 47% identity) than are the VanC and VanE ligases (42% and 39% identity).

Glycopeptide Resistance in Staphylococci

Emergence of vancomycin resistance in the Staphylococci has lagged behind resistance in the Enterococci but has materialized as a growing problem over the past decade. *S. aureus* in particular is a major cause of both hospital-acquired and community-acquired infections; and it is significantly more virulent than the Enterococci (53). While as early as 1992 it was demonstrated that vancomycin resistance could be conjugatively transferred from *E. faecalis* to *S. aureus* under laboratory conditions (54), clinical emergence of van-gene dependent glycopeptide resistance in Staphylococci has not yet been reported. Nonetheless, since the mid-1990s vancomycin resistant *S. aureus* strains have been isolated on several occasions from clinical specimens around the world (55–57). These strains are characterized by intermediate level resistance to vancomycin (MIC ≈ 8 μg/mL) and teicoplanin (MIC ≈ 8 μg/mL); these have therefore been termed VISA (vancomycin intermediate *S. aureus*) or GISA (glycopeptide intermediate *S. aureus*) (58).

The molecular mechanism of intermediate resistance in *S. aureus* is not well established. Resistance is independent of the enterococcal van genes and can be mimicked in vitro by serial passage under selection of increasing concentrations of vancomycin (59) or teicoplanin (60); however, it is generally unstable in the absence of antibiotic (61). Vancomycin is not chemically altered by these strains, and whereas the antibiotic concentration decreases in cell culture, the active drug can be recovered from the washed cells. VISA strains are generally characterized by a thickened abnormal cell wall, a decreased sensitivity to the lytic enzyme lysostaphin, a slow doubling time, increased peptidoglycan synthesis, altered penicillin-binding protein expression, and an increase in peptidoglycan autolytic activity (59, 61–64). Resistance is likely multifactorial with several mutations necessary to achieve the full effect (65). In *S. aureus* strains resistant to the β-lactam antibiotic methicillin (MRSA), vancomycin resistance parallels increased sensitivity to β-lactam antibiotics, altered penicillin-binding protein activity, and

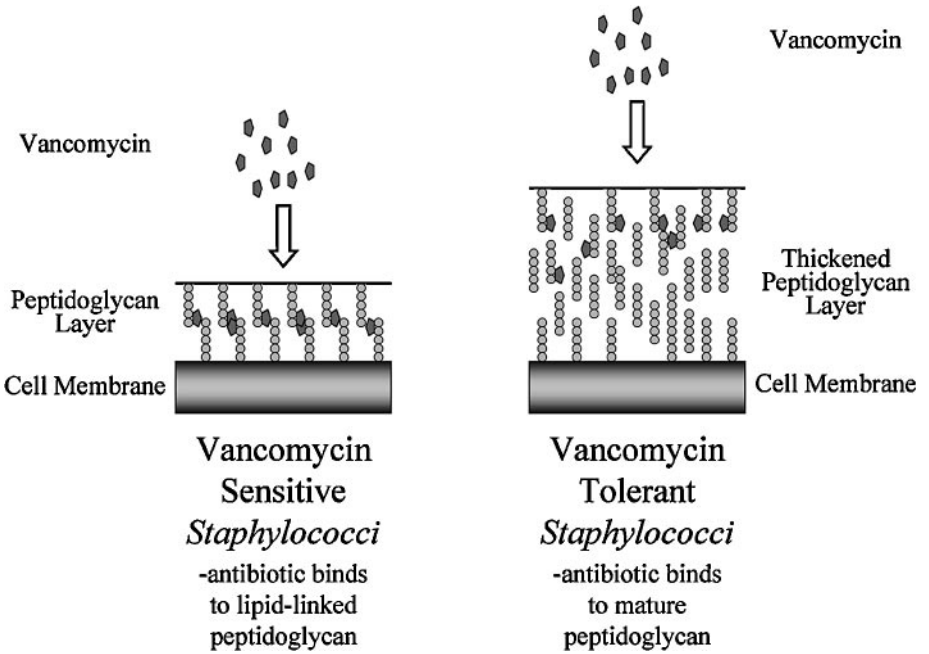

Figure 10 Mechanism of vancomycin resistance in Staphylococci. Vancomycin resistance occurs in Staphylococci without the presence of a *van* resistance cluster. Instead, resistant Staphylococci have a thickened cell wall. The thickened cell wall now has more D-Ala-D-Alanine available to bind to the vancomycin. This has the effect of tying up all the vancomycin, enabling the cell wall to continue peptidoglycan growth.

consequent alteration of peptidoglycan structure including decreased cross-linking and increased glycan chain length (59, 65, 66). Taken together, these results suggest a mechanism (66) where VISA strains express 'molecular flak' in the form of an altered peptidoglycan with exposed D-Ala-D-Ala termini in sufficient quantity to soak up vancomycin and prevent it from reaching the site of peptidoglycan growth (Figure 10).

Glycopeptide Resistance in Streptococci

A few vancomycin-resistant streptococcal isolates bearing the enterococcal *van* genes have been characterized. For example, a *vanB* containing *Streptococcus bovis* strain was isolated from an AIDS patient (67), and several strains of *vanA* and *vanB* containing *Streptococcus gallolyticus* were described from veal fecal samples (68). Vancomycin resistance in *Streptococcus pneumoniae*, a common cause of bacterial meningitis and pneumonia, has not been reported; however, vancomycin tolerance in this species has been described in several clinical isolates

(69, 70). Tolerance differs from resistance in that tolerant bacteria fail to grow in the presence of vancomycin but will continue to grow if the antibiotic is removed. On the other hand, resistant organisms can grow unimpeded in the presence of antibiotic unless a threshold level of drug is present, at which point they are killed. Vancomycin tolerance in *S. pneumoniae* is associated with mutations in VncS, a sensor His kinase that is part of a two-component regulatory system that triggers autolysis in response to various antibiotics including vancomycin (71). Like most two-component His kinases, VncS has both kinase and phosphatase activity toward its cognate response regulator partner, VncR in this case. Phosphorylated VncR represses autolysin gene expression and under normal conditions is maintained in its phosphorylated state by the action of the VncS kinase. In the presence of antibiotics, VncS normally acts in phosphatase mode, dephosphorylating phospho-VncR, which results in autolysin production and cell lysis. Inactivation of VncS results in loss of the capacity to dephosphorylate VncR and consequently autolysin gene expression remains repressed in the presence of antibiotic signals resulting in tolerance. The normal signal that is sensed by VncS is a secreted peptide, Pep27, that triggers the autolysis cascade (72).

Glycopeptide Resistance in Other Organisms

Glycopeptide resistance occurs in other gram-positive bacteria including the lactic acid bacteria such as Leuconostocs, Pedicococci, and Lactobacilli. Here resistance is the result of D-Ala-D-Lact terminating cell wall structure (73, 74); however, there does not appear to be an associated *van* cluster of the type diagramed in Figure 5. The D-Ala-D-Lact ligase associated with resistance in *Leuconostoc mesenteroides* has been cloned and characterized, and its 3-dimensional structure determined (19, 75, 76). This enzyme resembles more closely the *E. coli* D-Ala-D-Ala ligase DdlB rather than the VanA D-Ala-D-Lact ligase in ω-loop structure with the critical Tyr216 now replaced by Phe (77).

A *van* gene cluster (termed VanF in Figure 5) has been found in *Paenibacillus popillae*, an organism commonly used as a biopesticide in the control of insect populations (78). Glycopeptide producing bacteria also have been found to harbor a *van* gene cluster consisting of at least the VanH, VanA, VanX triad (79, 80). The proteins from the *Streptomyces toyocaensis*, which produces the teicoplanin-like antibiotic A47934, have been best studied at the molecular level with heterologously expressed proteins showing the predicted D-Lact and D-Ala-D-Lact synthesizing (VanHst, DdlM) (12, 81), and D-Ala-D-Ala hydrolyzing (VanX) activities (82). Given the identical roles of these proteins in glycopeptide resistance, and their conserved gene arrangement, it is likely that these environmental organisms are the source of the van gene clusters that have emerged on mobile genetic elements in the Enterococci. While it is evident from the vast C+G differences between glycopeptide producers and VRE ($>$70% vs. $\sim$45%) that direct transfer of the *van* genes from these soil bacteria to Enterococci is highly unlikely, the

producers are the logical font from which VRE have emerged following a chain of genetic exchanges that likely have occurred over many years, first occurring in soil organisms as a defense against glycopeptide producers, and the eventually to enterococci.

Regulation of Van Gene Expression

As noted above and in Table 1, the majority of the glycopeptide resistance types are characterized by induction by the antibiotics. The regulatory proteins responsible for sensing glycopeptides and responding to them by expression of the *van* genes are the two-component system consisting of the membrane bound sensor His-kinase, VanS, and the response regulator, VanR. Molecular studies have been pursued primarily on the VanR/S couple associated with the VanA transposon Tn*1546* (83). Autophosphorylation of VanS on His194 was demonstrated on a C-terminal truncated enzyme fused to maltose-binding protein, and this precedes phosphoryl transfer to VanR on Asp53 (84). Phospho-VanR binds as a tetramer to the promoter region upstream of the *vanH* gene to promote transcription of the resistance genes as a result of the presence of vancomycin (85, 86). Phospho-VanR also binds with lower affinity to the promoter region upstream of the *vanR* gene but does not activate expression of the downstream *vanR*/*vans* (85, 86). Thus in response to the presence of glycopeptide antibiotics, VanS undergoes autophosphorylation, transfers the phosphate to VanR, which then activates expression of the vancomycin resistance genes *vanH*, *vanA*, *vanX*. In the absence of induction, VanS activity dephosphorylates phospho-VanR, thereby providing strict control of expression (Figure 11) (87). The VanB system presumably functions in a manner similar to the VanA type discussed above as expression of $vanH_B$, *vanB*, $vanX_B$ is dependent on the $VanS_B$-$VanR_B$ couple, but teicoplanin is not an inducer (32, 88, 89).

The identity of the signal that initiates the signal transduction mechanism that activates *van* gene expression is not known but is not the result of direct binding of glycopeptides to the VanS sensor kinase. Initial work showed that in addition to glycopeptides, antibiotics that target the transglycosylase step in peptidoglycan biosynthesis such as moenomycin could induce the van genes, but that other antibiotics that target either post-or pre-glycosylation steps were not inducers of the system (90). Various reporter gene fusions to the promoter upstream of $vanH_A$ have been prepared including chloramphenicol acetyltransferase (91), LacZ (92), and firefly luciferase (93). A green fluorescent protein construct under the control of the promoter upstream of $vanY_B$ in the VanB type system has also been reported (94). The consensus of these studies is that antibiotics that block later stage peptidoglycan synthesis at the cell membrane, e.g., transglycosylation and carrier lipid metabolism, can induce *van* gene expression. Accumulation of lipid-linked peptidoglycan precursors or metabolites on the outer leaflet of the cell membrane is therefore the unifying element in triggering transcription of the *van* genes. The mechanism of this induction and the precise role of VanS as the likely first step in the cascade remain a mystery.

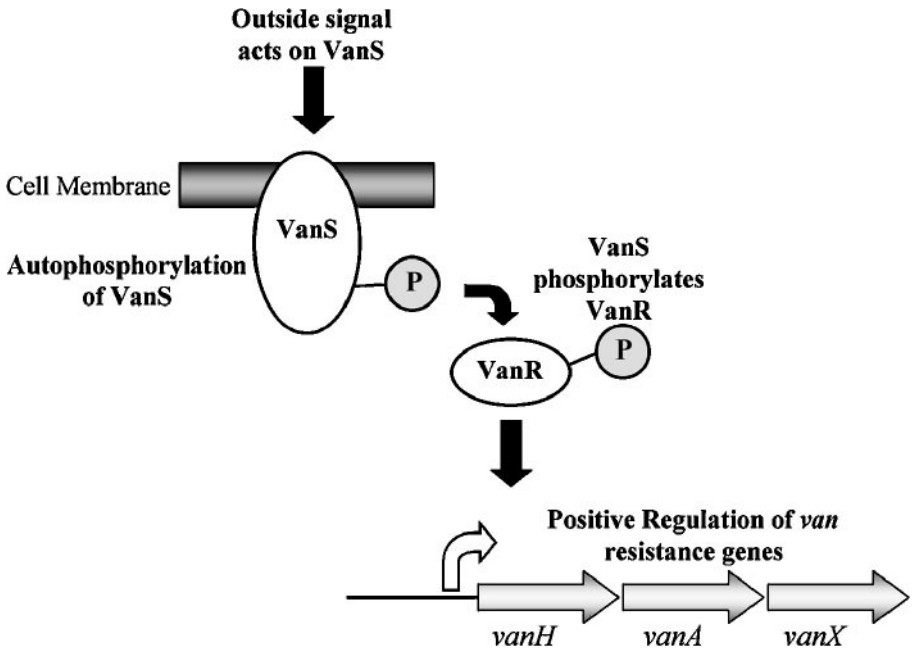

Figure 11 Mechanism of action of the *vanR-vanS* two-component regulatory system. A known outside signal acts on VanS, stimulating autophosphorylation on a conserved histidine residue. The phosphorylated VanS then transfers the phosphate to a conserved aspartic acid on VanR. Once phosphorylated, the active VanR is a transcriptional activator of the *vanHAX* genes.

STRATEGIES FOR OVERCOMING VANCOMYCIN RESISTANCE

Ever since vancomycin resistance was first described, approaches to circumvent this problem have been sought. These have included the use of new antibiotics of different classes, the development of glycopeptide antibiotics with activity toward resistant strains, and the inhibition of vancomycin resistance mechanisms to rescue the activity of the antibiotic. The first strategy has proven to be successful with a few new compounds now in clinical use for treatment of vancomycin-resistant Enterococci, including the streptogramin mixture Synercid (95), and the oxazolidinone linezolid (96). Additionally, several other nonglycopeptides are in clinical or preclinical development, including the natural products daptomycin (97) and everninomicin (98), and the semi-synthetic glycylcycline derivatives of tetracycline (99).

Given the intricate nature of the glycopeptide structures, de novo total synthesis of glycopeptides is not economically viable for the generation of new antibiotics. The preferred route for the expansion of chemical diversity of these antibiotics

has been semi-synthesis where the natural products isolated from producing bacteria are derivatized using traditional chemical methods (100). A number of semi-synthetic glycopeptides have been produced by reaction at the C-terminal carboxyl, the N-terminal amine, and by alkylation of various amines on the sugar residues. For example, BI 397, a C-terminal alkyl amide derivative of teicoplanin shows good antibiotic activity against staphylococci, streptococci, and enterococci, but no improved activity against VanA-type VRE (101). Other C-terminal amides have been prepared with the capacity to cleave N-acyl D-Ala-D-Lact, but with unreported antibiotic activity (102). On the other hand, several derivatives of vancomycin and chloroeremomycin with hydrophobic alkyl groups linked to the amine of the vancosamine sugar have excellent antibiotic activity even against VRE (103). One of these, LY333328 (oritavancin) is a chlorobiphenyl derivative of the vancomycin-like glycopeptide A82846B that shows antibiotic activity against VanA and VanB VRE (MIC ≤ 1 μg/ml) (104, 105) (Figure 12). The mode of action of these derivatives is not fully understood. The hydrophobic chlorobiphenyl group was introduced in part to mimic similarly placed lipid chain on teicoplainin. This chain has been suggested to act as a lipid anchor that assists in localizing teicoplanin to the site of active peptidoglycan assembly (4, 106). Like other glycopeptides, LY333328 has low affinity for N-acyl-D-Ala-D-Lact but does bind to membranes, and unlike teicoplanin, it does form dimers; this membrane localization is likely important for activity (107). Resistance to LY333328 can be achieved by decreasing the relative concentration of D-Ala-D-Ala terminating peptidoglycan either by increasing copies of the *van* gene cluster or by exogenous addition of D-2-hydroxyacids, and by the expression of vanZ (108). These results suggest that, unlike teicoplanin, the hydrophobic group linked to the vancosamine sugar is not primarily involved in membrane targeting; rather, the requirement for a fraction of the peptidoglycan terminating in D-Ala-D-Ala for full LY333328 activity may reflect fixing of the antibiotic in the vicinity of the transglycosylase where the chlorobiphenyl sugar can directly inhibit this enzyme as has shown in an in vitro transglycosylase assay (3).

The success of the N-alkylvancosamine derivatives prompted efforts to introduce an amine in place of the C6 hydroxyl of the glucose residue that is directly linked to the HPG residue (109). The success of this chemistry has permitted the synthesis of additional hydrophobic vancomycin derivatives with alkyl groups linked to the glucose residue. These compounds also show excellent activity against Enterococci and some increased activity against VanA-Type VRE; however the activity is not as potent as derivatization of the vancosamine sugar.

An alternative means of blocking vancomycin resistance is direct inhibition of the resistance mechanisms. Small molecule inhibitors of VanX have been described, including various nonhydrolysable phosphonate, phosphinate, and phosphonamidate (but not isosteric phosphothioate) D-Ala-D-Ala analogues that mimic the predicted tetrahedral transition state (22, 110, 111). These show both reversible and slow binding behavior, with K_i values in the nM to μM range.

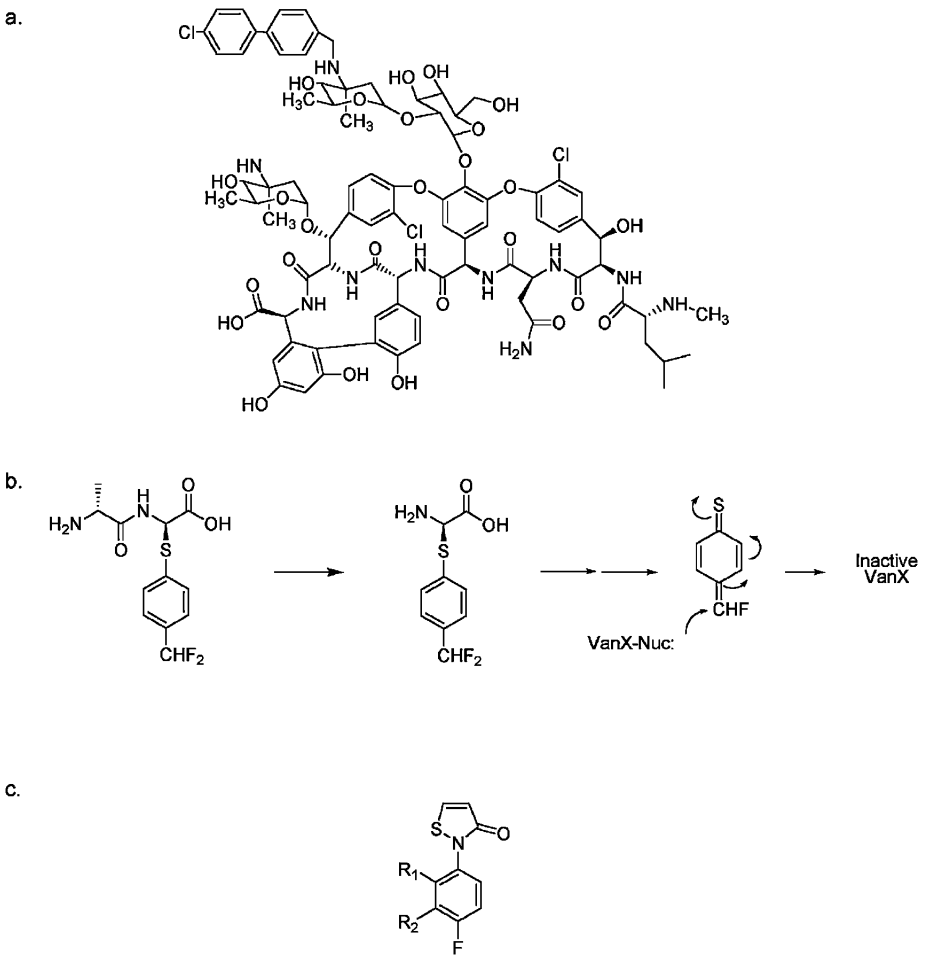

Figure 12 Methods to overcome vancomycin resistance. Shown are three different approaches in overcoming the problem of vancomycin resistance. (*a*) LY333328 (oritavancin) is a semisynthetic vancomycin-like glycopeptide antbiotic. By chemically modifying an existing glycopeptide antibiotic, one can hope to increase its effectiveness. (*b*) Another route to overcoming resistance is to create an inhibitor for the enzymes that confer resistance. The VanX inhibitor shown is a modified D-Ala-D-Ala in which the carboxy-terminal D-Alanine has been modified with the replacement of the methyl group by a difluromethylthiophenol group. The result is a compound that covalently modifies the VanX active site, rendering it inactive. (*c*) Halophenyl isothiazolone compounds have been shown to inhibit the transfer of the phosphate from VanS to VanR in the two-component regulatory system, interfering with induction of the van^R phenotype.

Mechanism-based inhibitors of VanX have recently been reported that consist of the D-Ala-D-Ala dipeptide modified by replacement of the C-terminal D-Ala with a D-Ala analogue where the CH3 has been replaced by a difluromethylthiophenol group (D-Gly(SΦp-CHF_2) (Figure 10) (112). VanX-dependent cleavage of the peptide bond releases the D-Gly(SΦp-CHF_2), which can undergo spontaneous rearrangement in the active site generating reactive 4-thioquinone fluoromethide that covalently modifies the enzyme (Figure 12). These results indicate the possibility of generating inhibitory compounds with the capacity to inhibit VanX with sufficient potency in vivo despite the small size and highly selective nature of the enzyme active site (23). The description of a robust continuous colorimetric assay for VanX activity should be amenable to high throughput screening methods (113).

The central role of the Van ligases and the related D-Ala-D-Ala ligases make these enzymes highly attractive drug discovery targets. Various phosphinate and phosphonate dipeptide inhibitors are also good inhibitors of VanA (13, 114), and the VanA crystal structure was determined in the presence of one of these phosphinate inhibitors (15). The availability of this structure will now provide important insights into the development of new inhibitors.

The VanR/VanS two-component system is also a viable target for compounds with the potential to block vancomycin resistance. A membrane-reconstituted system that monitored phosphoryl transfer from membrane-bound phospho-VanS to VanR was established, and inhibition of this phosophorelay was observed in the presence of halophenyl isothiazolone compounds (Figure 12). Other compounds may also have the potential to inhibit signal transduction throughVanR/VanS. In VRE, for example, a recent report indicated that flavanoid compounds delay growth of VRE in the presence of vancomycin and potentiate the action of the antibiotic. The flavanoid genistein, which has a structure similar to the compounds tested in this report, is an inhibitor of protein kinases and a mammalian His kinase (115). Therefore, it is possible the action of flavanoids on vancomycin activity in VRE may reflect inhibition of induction of D-Ala-D-Lact synthesis through the VanR/VanS couple, though this is an untested hypothesis and genistein itself did not inhibit autophosphorylation of a soluble C-terminal VanS fragment (84).

An alternative to small molecule inhibition of vancomycin resistance mechanisms is a gene-based approach. Reversal of vancomycin resistance has been demonstrated with the introduction of a *vanA* antisense gene under the control of the *vanH* promoter (116). This creative approach shows proof of principle for the use of gene therapy methods to intervene in antibiotic resistance at a new level.

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