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Control of peptidoglycan synthesis in vancomycin-resistant enterococci: D,D-peptidases and D,D-carboxypeptidases

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Abstract. Resistance to glycopeptide antibiotics in enterococci results from the synthesis of peptidoglycan precursors with low affinity for these antibiotics. The resistance proteins are encoded on transposons in VanA and VanB type enterococci and are involved in regulation, synthesis of new resistant precursors and elimination of wild-type sensitive precursors by hydrolysis of D-alanyl-D-alanine (D,D-peptidase activity encoded by *vanX*) and removal of D-alanine from UDP-*N*-acetylmuramyl (UDP-MurNAc)-pentapeptide (D,D-carboxypept-idase activity encoded by *vanY*). The substrate specificities of VanX and VanY ensure that essentially only precursors with low affinity for glycopeptide antibiotics are available for peptidoglycan synthesis in strains induced to resistance.

Key words. Peptidoglycan; vancomycin; enterococci; resistance; D,D-carboxypeptidase.

Introduction

Glycopeptide antibiotics, vancomycin and teicoplanin, inhibit peptidoglycan synthesis by forming complexes with the peptidyl-D-alanyl-D-alanine termini of peptidoglycan precursors as they emerge through the cytoplasmic membrane. Glycopeptide resistance in enterococci is phenotypically and genotypically heterogeneous. Two acquired resistance phenotypes, transferable by conjugation, have been characterized: the VanA-type, mediated by Tn*1546* or closely related transposons, confers high-level inducible resistance to both vancomycin and teicoplanin, whereas the VanB-type, containing Tn*1547* as part of a large chromosomal element, displays variable levels of inducible resistance to vancomycin only. VanA- and VanB-type isolates synthesize peptidoglycan precursors ending in D-Ala-D-lactate which bind glycopeptides with greatly reduced affinity and allow cell wall synthesis to proceed in the presence of these antibiotics [1].

The resistance mechanism in VanA enterococci

Tn*1546* carries a gene cluster that includes the *vanR* and *vanS* genes, encoding a two-component regulatory system that activates transcription of at least four other genes, *vanH*, *vanA*, *vanX* and *vanY* [2]. The induction signal has not been identified: vancomycin and teicoplanin are both inducers, but other inhibitors of cell wall synthesis are also active, as are some cell wall hydrolysing enzymes [3–5]. VanH is a dehydrogenase that reduces pyruvate to D-lactate, and VanA is a ligase that catalyses synthesis of the depsipeptide D-alanyl-D-lactate, which replaces the dipeptide D-Ala-D-Ala in the assembly pathway of peptidoglycan precursors [6]. The VanA ligase has limited amino acid sequence identity (approx 30%) to D-Ala:D-Ala ligases (Ddl) of enterococci or *Escherichia coli*, implying a distant relationship; but it has recently been demonstrated that a change in a single residue (from tyrosine to phenylalanine at position 215 of the *E. coli* DdlB) dramatically alters the substrate specificity, and the mutant enzyme acquires D-Ala: D-lactate ligase activity [7]. The affinity of vancomycin for cell wall precursors terminating in acyl-D-Ala-D-lactate is at least 1000-fold lower than for those ending in acyl-D-Ala-D-Ala [6]. All the enzymes of peptidoglycan synthesis that utilize substrates terminating in D-Ala-D-Ala also function with substrates ending in D-Ala-D-lactate. The vancomycin-susceptible and -resis-

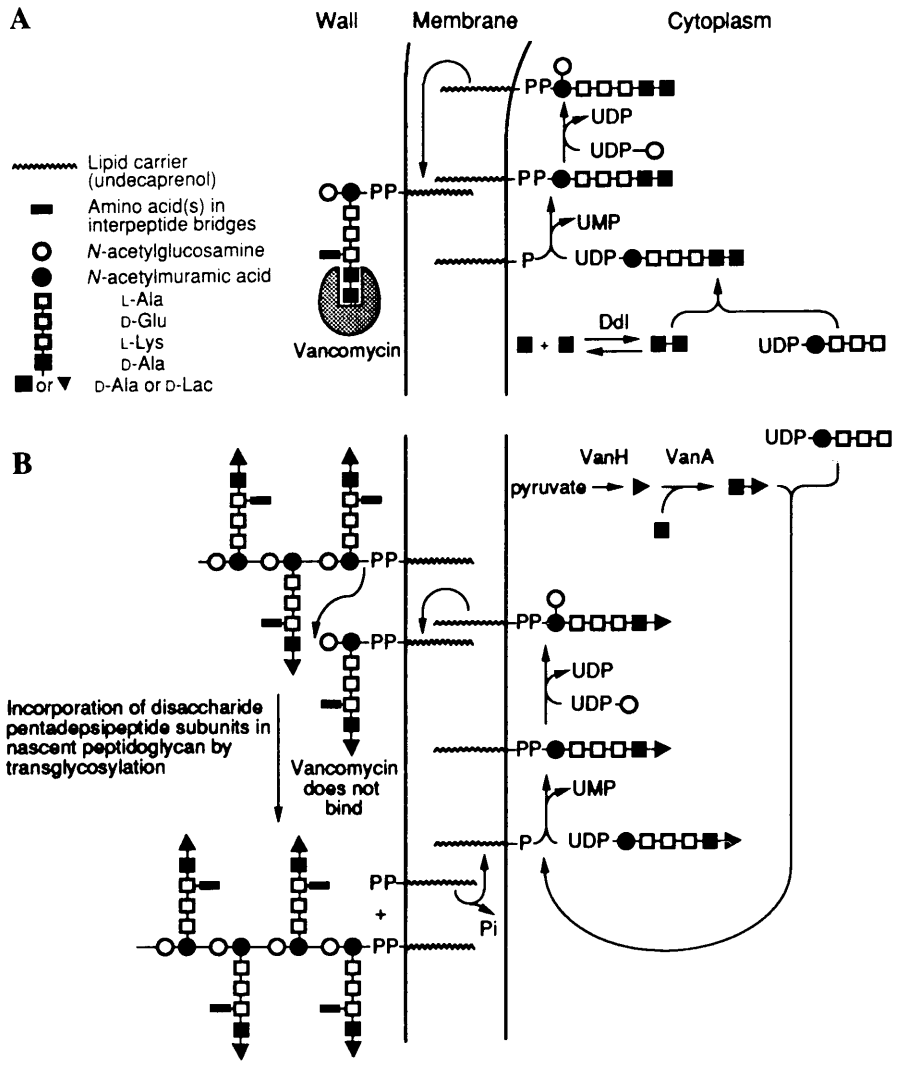

Figure 1. Synthesis of nascent peptidoglycan in vancomycin-susceptible (*A*) and vancomycin-resistant (*B*) enterococci. (Reprinted with permission from: Arthur M., Reynolds P. E. and Courvalin P. (1996) Glycopeptide resistance in enterococci. Trends Microbiol. **4:** 401–407, © 1998 Elsevier Science, Oxford).

tant pathways of peptidoglycan synthesis are depicted in figure 1 [1].

However, the activity of VanA (or VanB, see below) together with VanH is insufficient for glycopeptide resistance to be expressed, because substrates on the glycopeptide-sensitive pathway of synthesis continue to be synthesized. Consequently, two other enzymes are required to eliminate the original precursors. A cytoplasmic D,D-dipeptidase (VanX) and a D,D-carboxypeptidase (VanY) are encoded by genes located in the glycopeptide resistance operon. VanX hydrolyses D-Ala-D-Ala, leading to a large decrease in the rate of UDP-*N*-acetylmuramyl (UDP-MurNAc)-pentapeptide (pentapeptide) synthesis and a rise in the ratio of UDP-MurNAc-pentadepsipeptide (pentadepsipeptide): pentapeptide [8]. VanX is very specific for the hydrolysis of D,D-dipeptides. Unusually for a peptidase, it has no activity against the ester D-Ala-D-lactate, and it does not hydrolyse dipeptides substituted at either the C or N terminus [8]. Consequently, it has the ideal substrate specificity to eliminate D-Ala-D-Ala, which would otherwise compete with D-Ala-D-lactate for addition to UDP-MurNAc-L-Ala-γ-D-Glu-L-Lys. VanY converts pentapeptide to UDP-MurNAc-tetrapeptide (tetrapeptide) by removal of D-Ala, thus reducing the amount of pentapeptide still further and favouring resistance [9]. If the tetrapeptide can be utilised as a substrate in peptidoglycan synthesis, the transglycosylation reaction which is the target of glycopeptides would not be inhibited. VanY catalyses the identical reaction to that carried out by the low molecular mass membrane-bound penicillin-binding proteins (PBPs) of most bacteria, but it is totally penicillin-insensitive. It lacks the classical Ser-X-X-Lys motif found in the active sites of PBPs.

Table 1. Relationship between MIC and the ratio of resistant to 'sensitive' peptidoglycan precursors.

Strain	MIC vancomycin (μg/ml)	Induction of resistance	MGT (min)	Tetra	Precursors (%)		Ratio lac/ala
					pentapep (ala)	pentadepsipep (lac)	
JH2-2	2	–	48	2	98	0	–
BM4299	4	+	52	1	45	54	1.2
BM4300	8	+	55	2	31	67	2.2
BM4301	32	+	75	1	6	93	16
BM4302	256	+	130	2	3	95	32

BM4299, BM4300, BM4301 and BM4302 carry 1, 1, 2 and 5 copies, respectively, of the *van*RSHAX gene cluster in a chromosomal location.

VanY contains a hydrophobic amino acid sequence close to the N-terminus that determines its mainly membrane-bound location in VanA strains.

In VanA enterococci, the degree of resistance is dependent on the ratio of resistant precursors (pentadepsipeptide and tetrapeptide) to sensitive precursors (pentapeptide) [10]. In wild-type strains and constructs harbouring the complete operon on multicopy plasmids, the activity of VanX is high: this situation results in almost complete destruction of D-Ala-D-Ala as it is synthesized, and consequently the ratio of pentapeptide to pentadepsipeptide is low. In these strains VanY is inessential for resistance and has been described as having an accessory role. The genes involved in regulation and those essential for resistance were inserted into a chromosomal location in a glycopeptide susceptible *E. faecalis* to study the effect of gene copy number on the degree of resistance, the levels of expression of the resistance genes and the relative proportions of the cytoplasmic peptidoglycan precursors. Derivatives of *E. faecalis* JH2-2 harbouring one, two or five copies of the gene cluster in the chromosome were constructed. The levels of transcription (as indicated by a CAT reporter gene), of resistance and VanX-specific activity all increased with the number of copies of the gene cluster. The relative amounts of pentapeptide and pentadepsipeptide also changed dramatically [10]. The presence of a single copy of the resistance operon resulted in the synthesis of approximately equal amounts of the resistant and sensitive precursors, whereas five copies resulted in a situation in which pentapeptide was almost completely absent (table 1). In one of the constructs containing a single copy of the VanR,S,H,A,X operon, the insertion of a plasmid containing the VanY gene increased the degree of resistance to vancomycin fourfold, implying that VanY may possess an important function when the activities of VanH, A and X are considerably lower than in wild-type clinical isolates of VanA enterococci [10]. The tetrapeptide resulting from the action of VanY can act as a peptidoglycan precursor on the vancomycin-resistant pathway of peptidoglycan synthesis but is unable to function as a donor in transpeptidation reactions. It is evident that in VanA strains, the D,D-peptidase has an essential role in maintaining a high ratio of resistant to sensitive precursors and that VanY, although of more limited significance, can contribute to resistance. The role of the resistance genes in precursor synthesis in VanA strains is summarized in figure. 2.

Induction of resistance in VanB enterococci: early events

Resistance in VanB strains is inducible by vancomycin but not by teicoplanin. In contrast with VanA strains which are slightly leaky in relation to the induction process, the location of the genes is chromosomal in the majority of strains [11], and pentadepsipeptide (synthesized by $VanH_B$ and VanB) and the control enzymes $VanX_B$ and $VanY_B$ are completely absent until the operon is induced with vancomycin. As a result when vancomycin is added to a growing culture of a VanB

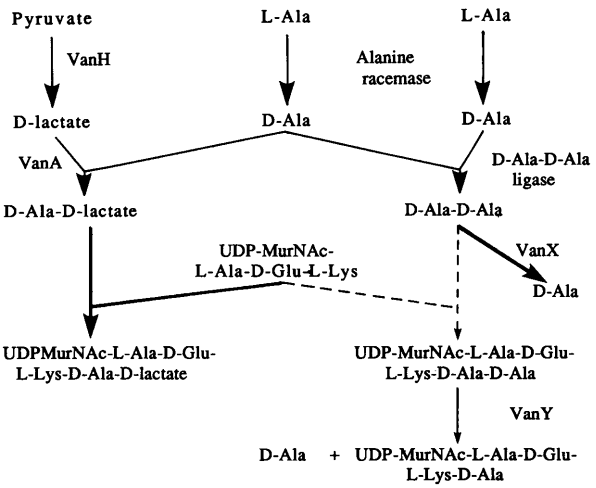

Figure 2. The roles of VanH and VanA in the synthesis of D-Ala-D-lactate and of VanX and VanY in the elimination of D-Ala-D-Ala and UDP-MurNAc-pentapeptide in VanA-type enterococci.

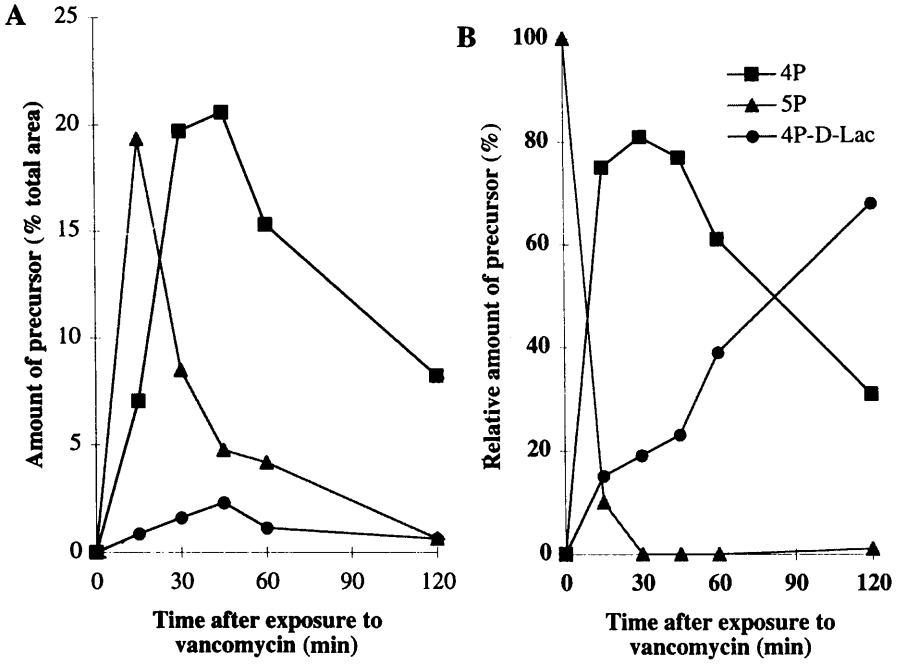

Figure 3. Time course of production of peptidoglycan precursors during induction of vancomycin resistance in a VanB enterococcus. Cultures were induced with vancomycin at time 0: samples were removed at intervals, harvested and the peptidoglycan precursors extracted and analysed by HPLC. (*A*) No ramoplanin added to the culture, (*B*) ramoplanin was added at various times after induction for the equivalent of 0.5 MGT (see text) before harvesting the bacteria.

strain, wall synthesis is rapidly inhibited, and lipid II will complex with vancomycin when it is exposed to the antibiotic on the external face of the membrane. Lipid intermediates I and II on the cytoplasmic side of the membrane are saturated with substrate in a few seconds. Peptidoglycan synthesis is effectively frozen until new C_{55} (undecaprenol) molecules are synthesized and until most precursors terminating in acyl-D-Ala-D-Ala have been eliminated. If these events are prolonged and wall lytic enzymes continue to function, irreparable damage to the cell wall may occur. In order to test this possible scenario, we studied the early events that occurred in a VanB strain (J. Stigter and P. E. Reynolds, unpublished observations). Cell wall precursors in the cytoplasm were analysed by high performance liquid chromatography (HPLC) in cultures induced for various periods of time and in which wall synthesis was then inhibited for half the mean generation time (MGT) of an uninduced culture by ramoplanin, which blocks the utilization of cytoplasmic wall precursors. Parallel cultures were induced but without the addition of ramoplanin. Precursors that increased in amount in the absence of ramoplanin were an indication of the primary effect of vancomycin on that culture: accumulation is indicative of a reaction that is inhibited, coupled with an absence of sufficient undecaprenol carrier molecules on which the complete wall subunit can be assembled. Hence this picture represents the dynamic sequence of events that occurs on addition of the inducer. The alternative experimental strategy employed, in which peptidoglycan synthesis was inhibited, at a different site, for a fixed time after varying periods of induction, provided information on the total pool of wall precursors that could be synthesized: some of these would already have been present before ramoplanin was added, but others that appeared would have been synthesized and utilized until wall synthesis was inhibited.

In the high-level resistant *E. faecium* L1836, pentapeptide accumulated rapidly after addition of vancomycin, followed by a rapid increase in the amount of tetrapeptide and an equally rapid diminution of pentapeptide (fig. 3A). The most likely explanation of this result is that pentapeptide was converted rapidly to tetrapeptide by the activity of $VanY_B$. The subsequent slow disappearance of tetrapeptide from the cytoplasmic pool of precursors suggests that it could function as a substrate for peptidoglycan synthesis. The small rise and subsequent fall in the amount of the pentadepsipeptide indicated that it was able to be utilized in peptidoglycan synthesis as rapidly as it was synthesized. Treatment with ramoplanin for short periods provided an indication of the rate at which induction of $VanH_B$ and VanB occurred, as shown by the gradually increased rate of

synthesis of pentadepsipeptide (fig. 3B). In the case of the pentadepsipeptide, each point represents the amount of the precursor that was synthesized by the bacteria in a 22-min period (0.5 MGT of the uninduced resistant strain). Induction was a relatively slow process, and the amounts of VanB and $VanH_B$ (as indicated by the amount of pentadepsipeptide synthesized) were still increasing 2 h after the start of induction.

The results of enzyme activity assays supported the suggestions based on precursor analysis. $VanY_B$ was induced rapidly and reached a maximum within 45 min. This activity was likely to be primarily responsible for the conversion of pentapeptide to tetrapeptide and its consequent 'detoxification'. $VanX_B$ activity increased more slowly than that of $VanY_B$, and the final activities determined were approximately the same under the conditions of the assays. This situation is unlike that in VanA strains in which vancomycin resistance is encoded on multicopy plasmids: in these strains VanX activity is high, much greater than that of VanY, and it results in rapid elimination of the capacity of the bacteria to synthesize pentapeptide. However, VanX does not affect the amount of pentapeptide already present in the bacteria, which, if incorporated into lipid intermediates, would prevent the rapid onset of resistance. In VanB strains in which the location of the resistance operon is chromosomal, the single copy resistance genes result in a low rate of elimination of D-Ala-D-Ala, so the early presence of VanY activity is important in controlling the amount of pentapeptide present.

Vancomycin resistance in VanC enterococci

The strategy employed by the intrinsically resistant VanC-type enterococci (*E. gallinarum* and *E. casseliflavus*) is different from that of VanA and VanB enterococci: the organisms possess two D,D-dipeptide ligases, the normal wild-type enzyme catalysing the synthesis of D-Ala-D-Ala and a second ligase designated VanC that is presumed to synthesize D-Ala-D-Ser that is present at the C-terminus of peptidoglycan precursors [12, 13]. Insertional inactivation of VanC leads to loss of resistance [14]. The binding site of vancomycin is more restrictive for acyl-D-Ala-D-Ser than for acyl-D-Ala-D-Ala, hence there is a decrease in binding affinity of approximately fivefold [15]. There is a much smaller change in affinity for teicoplanin; consequently, the susceptibility of VanC strains for teicoplanin is virtually unchanged. The small change in structure of the dipeptide in which a methyl is replaced by a hydroxymethyl side chain on the C-terminal residue poses increased difficulty in relation to the evolution of a VanX-type enzyme. Extracts containing VanA and VanB D,D-peptidases also hydrolyse D-Ala-D-Ser, though at a reduced rate compared with D-Ala-D-Ala; and if the VanX enzyme of a VanC strain had a similar substrate specificity this type of resistance may not have evolved. However, studies of the substrate specificity of $VanX_C$ in extracts indicated that the enzyme had little or no activity against D-Ala-D-Ser or D-Ala-Gly (table 2), whereas in other respects it resembled VanX (no activity against D-Ala-D-Ala-D-Ala or the amide of D-Ala-D-Ala; table 2). VanY has low activity against wall precursors terminating in acyl-D-Ala-D-Ser in comparison with its activity against acyl-D-Ala-D-Ala, and a similar but more rigorous specificity was observed with $VanY_C$ (table 3). It differs from VanY in that virtually all activity is located in the cytoplasm, whereas VanY and $VanY_B$ have membrane anchors and the majority of activity is membrane-bound. Furthermore, the ratio of VanY/VanX activity in VanC strains is greater than in VanA strains. A summary of precursor synthesis in VanC strains is given in figure 4.

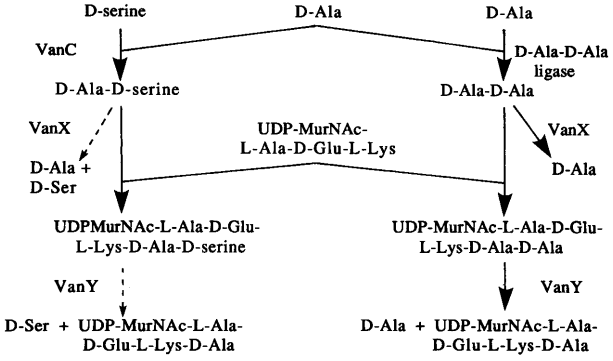

Figure 4. Synthesis of peptidoglycan precursors in intrinsically resistant *E. gallinarum*.

Table 2. Specificity of D,D-peptidase (Van X-type) of *Enterococcus faecium* and *E. gallinarum*.

Substrate	Relative activity (%)	
	E. faecium (VanA)	*E. gallinarum* (VanC)
D-Ala-D-Ala	100	100
D-Ala-Gly	26	2
D-Ala-D-Ser	49	3
D-Ala-L-Ser	ND	1
D-Ala-D-Ala-D-Ala	0	2
D-Ala-D-Ala-NH_2	0	0

Substrates were present at 10 mM.

Table 3. Specificity of D,D-carboxypeptidases (VanY-type) of *Enterococcus faecium* and *E. gallinarum*.

Substrate	Relative activity (%)	
	E. faecium (VanA)	*E. gallinarum* (VanC)
X-L-Lys-D-Ala-D-Ala	100	100
X-L-Lys-D-Ala-D-lactate	8	5
X-L-Lys-D-Ala-D-Ser	9	0
X-meso-DAP-D-Ala-D-Ala	17	ND

X, UDP-MurNAc-L-Ala-γ-D-Glu; DAP, diaminopimelic acid. Substrates were present at 10 mM; ND, not determined.

Comparison of D,D-peptidases and D,D-carboxypeptidases in VanA, VanB and VanC enterococci

There is a high level of amino acid sequence identity in VanX and $VanX_B$ (71%), similar to that in the D-lactate dehydrogenases (VanH, $VanH_B$) and D-Ala:D-lactate ligases (VanA, VanB). The D,D-peptidases of both phenotypes contain 202 amino acids. On the other hand, the D,D-carboxypeptidases (VanY, $VanY_B$) are not closely related, with only 30% amino acid identity, and the genes encoding them are in different relative positions within the resistance operons [16]. VanX requires Zn^{2+} or a related cation for activity, and two short sequences of amino acids containing residues believed to be involved in binding Zn^{2+} and in catalysis have been identified in both D,D-peptidases and D,D-carboxypeptidases [17]. These consensus sequences are present in the D,D-peptidase/D,D-carboxypeptidase of *E. gallinarum* (P. E. Reynolds, C. A. Arias and P. Courvalin, unpublished observations) and are also found in the D,D-peptidase of *Streptomyces albus* G (table 4) [18].

Important events during the early stages of induction

The main features of acquired glycopeptide resistance include the synthesis of new cell wall precursors and the elimination of the wild-type precursors (see above). An equally important aspect is the presence of a pool of undecaprenylphosphate lipid carrier molecules that can accept the new resistant precursors. The lipid cycle of reactions in peptidoglycan synthesis plays an important role during the process of induction of the vancomycin resistance proteins. Immediately following the addition of a glycopeptide to an uninduced culture, the existing 'sensitive' pathway of peptidoglycan synthesis continues to function, and the membrane-bound stages of synthesis which involve recycling of the lipid portion of the lipid intermediates are blocked. In VanA strains, after an initial rapid rise in UDP-MurNAc-pentapeptide, the cytoplasmic concentration is kept low by the rapid elimination of D-Ala-D-Ala by VanX. In VanB strains, VanY activity converts the pentapeptide precursor to tetrapeptide. However, in both instances at very early time points, pentapeptide is the only precursor present in large amounts. Therefore, the existing lipid molecules become immobilized as lipid II intermediates complexed with vancomycin on the external face of the protoplast membrane or as lipid I intermediates (containing peptides terminating in D-Ala-D-Ala). Incorporation of the new 'resistant' intermediates terminating in acyl-D-Ala-D-lactate is dependent on synthesis of new lipid molecules or on the recovery of undecaprenyl phosphate from the existing complexes by a variety of mechanisms, including the reversal of synthesis of lipid I, the action of VanY on lipid I and/or lipid II or the dissociation or breakdown of externally oriented lipid II:vancomycin complexes. The recycling or resynthesis of C_{55}-undecaprenylphosphate will only be effective if the ratio of sensitive/resistant precursors is low. The action of D,D-peptidases and D,D-carboxypeptidases is vital in this respect in VanA, VanB and VanC strains.

Table 4. Conservation of motifs in D,D-dipeptidases and D,D-carboxypeptidases and comparison with related hydrolases.

VanX	113	K	**S**	S	**H**	S	R	**G**	S	A	I	**D**	L	. . 53 aa . .	A	Y	S	L	**E**	W	**W**	**H**	Y	V . . .
$VanX_B$	113	Q	**S**	S	**H**	S	R	**G**	S	A	I	**D**	L	. . 53 aa . .	S	Y	R	F	**E**	W	**W**	**H**	Y	K . . .
$VanX_C$	91	C	**S**	E	**H**	Q	I	**G**	L	A	I	**D**	V	. . 46 aa . .	G	I	S	Y	**E**	P	**W**	**H**	F	R . . .
VanY	160	Y	**S**	E	**H**	N	S	**G**	S	S	L	**D**	V	. . 35 aa . .	G	I	Q	Y	**E**	P	**W**	**H**	I	R . . .
$VanY_B$	185	T	**S**	E	**H**	Q	L	**G**	L	A	V	**D**	N	. . 35 aa . .	G	V	S	N	**E**	P	**W**	**H**	Y	R . . .
$VanY_C$	91	C	**S**	E	**H**	Q	I	**G**	L	A	I	**D**	V	. . 46 aa . .	G	I	S	Y	**E**	P	**W**	**H**	F	R . . .
Ddp	151	N	**S**	R	**H**	M	Y	**G**	H	A	A	**D**	L	. . 31 aa . .	P	G	H	N	**D**	H	T	H	V	A . . .
PLY118	77	R	**S**	Y	**H**	L	V	**G**	Q	A	L	**D**	F	. . 37 aa . .	S	G	F	V	**D**	N	P	**H**	L	Q . . .
PLY500	77	Q	**S**	N	**H**	N	Y	**G**	V	A	V	**D**	L	. . 37 aa . .	K	S	F	K	**D**	Y	P	**H**	F	E . . .
CPL2438	77	Q	**S**	N	**H**	N	F	**G**	V	A	V	**D**	L	. . 37 aa . .	K	S	F	K	**D**	Y	P	**H**	F	E . . .
Consensus	. . .	x	**S**	x	**H**	x	x	**G**	x	x	x	**D**	x	. . aa . .	x	x	x	x	**D** E	x	x	**H**	x	x . . .

Ddp is a D,D-carboxypeptidase from *S. albus* G; PLY118, PLY500 and CPL2438 are peptidoglycan hydrolysing enzymes from bacteriophages [17].

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