Active-site mutants of the VanC2 D-alanyl–D-serine ligase, characteristic of one vancomycin-resistant bacterial phenotype, revert towards wild-type D-alanyl–D-alanine ligases

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Background: The rising number of vancomycin-resistant enterococci (VREs) is a major concern to modern medicine because vancomycin is currently the 'last resort' drug for life-threatening infections. The D-alanyl–D-X ligases (where X is an hydroxy or amino acid) of bacteria catalyze a critical step in bacterial cell-wall peptidoglycan assembly. In bacteria that produce glycopeptide antibiotics and in opportunistic pathogens, including VREs, D-, D-ligases serve as switches that confer antibiotic resistance on the bacteria themselves. Peptidoglycans in vancomycin-sensitive bacteria end in D-alanyl–D-alanine, whereas in vancomycin-resistant bacteria they end in D-alanyl–D-lactate or D-alanyl–D-serine.

Results: We demonstrate that the selective utilization of D-serine by the *Enterococcus casseliflavus* VanC2 ligase can be altered by mutagenesis of one of two residues identified by homology to the X-ray structure of the *Escherichia coli* D-alanyl–D-alanine ligase (DdlB). The Arg322→Met (R322M) and Phe250→Tyr (F250Y) ligase mutants show a 36–44-fold decrease in the use of D-serine, as well as broadened specificity for utilization of other D-amino acids in place of D-serine. The F250Y R322M double mutant is effectively disabled as a D-alanyl–D-serine ligase and retains 10% of the catalytic activity of wild-type D-alanyl–D-alanine ligases, reflecting a 6,000-fold switch to the D-alanyl–D-alanine peptide. Correspondingly, the Leu282→Arg mutant of the wild-type *E. coli* DdlB produced a 560-fold switch towards D-alanyl–D-serine formation.

Conclusions: Single-residue changes in the active-site regions of D-, D-ligases can cause substantial changes in recognition and activation of hydroxy or amino acids that have consequences for glycopeptide antibiotic efficacy. The observations reported here should provide an approach for combatting antibiotic-resistant bacteria.

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Introduction

Vancomycin resistance in the gram-positive pathogenic bacteria is of particular concern among health professionals. Vancomycin-resistant enterococci (VRE) have increased over 20-fold in prevalence since their discovery a decade ago [1], a phenomenon approaching epidemic proportions that could convert the glycopeptide antibiotic vancomycin from its current niche as the 'last resort' antibiotic for life-threatening infections into a very limited single-use therapeutic agent. Clinical phenotypes for VRE isolates have been designated VanA, VanB, or VanC [2,3] based on three criteria: inducibile versus constitutive resistance, susceptibility to vancomycin versus the related molecule teicoplanin, and the minimal inhibitory concentration (MIC) in VRE. Thus the most common clinical phenotype, VanA, is inducible by vancomycin and teicoplanin, has an MIC about three log higher (1 μg/ml to 1000 μg/ml) for antibiotic than that of susceptible bacteria [4], and is rapidly spread through bacterial populations. VanB is very similar to the VanA phenotype except that it is not inducible by teicoplanin [4]. The VanC phenotype has been detected in fewer clinical isolates [5,6]; recent isolates have included the clinically significant human pathogens *Enterococcus faecalis* and *Enterococcus faecium*, however [7]. The VanC phenotype is largely constitutive [8,9], susceptible to teicoplanin, and shows about a one log elevation in MIC [10].

At one level of molecular analysis, VanA and the related VanB phenotype differ from VanC in that the expression of five genes (*VanR*, *VanS*, *VanH*, *VanA*, and *VanX*) is necessary and sufficient for antibiotic resistance in the first two phenotypes [11–13], whereas the VanC phenotype does not require *VanR*, *VanS*, and *VanH* and expresses the VanC ligase (which is involved in cell-wall peptidoglycan assembly) in place of the VanA (or VanB) ligase. At another level, however, the three clinical phenotypes of vancomycin resistance stem from a fundamentally similar switch in the specificity of a key enzyme in peptidoglycan assembly—a D-alanyl–D-alanine (D-Ala–D-Ala) ligase is

Figure 1

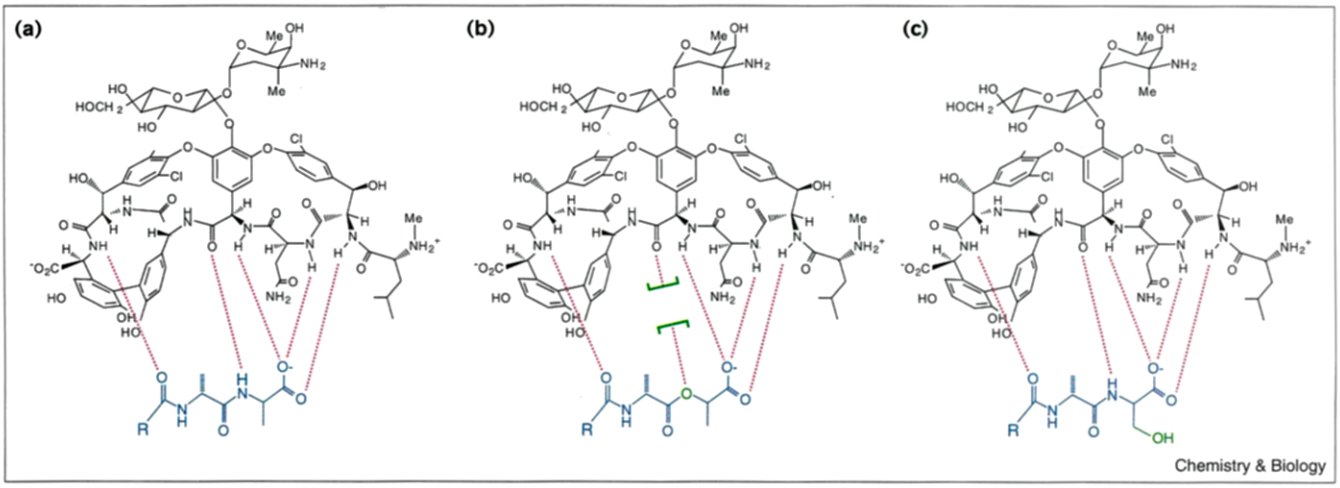


Illustration of vancomycin interacting with the peptidoglycan termini of **(a)** vancomycin-sensitive bacteria, *N*-acyl–D-Ala–D-Ala; **(b)** vancomycin-resistant bacteria of the VanA, VanB or *Leuconostoc mesenteroides* type (lactate-specific residues shown in green); **(c)** mildly vancomycin-resistant bacteria of the VanC group, *N*-acyl–D-Ala–D-Ser (with serine-specific residues shown in green) interacting with vancomycin.

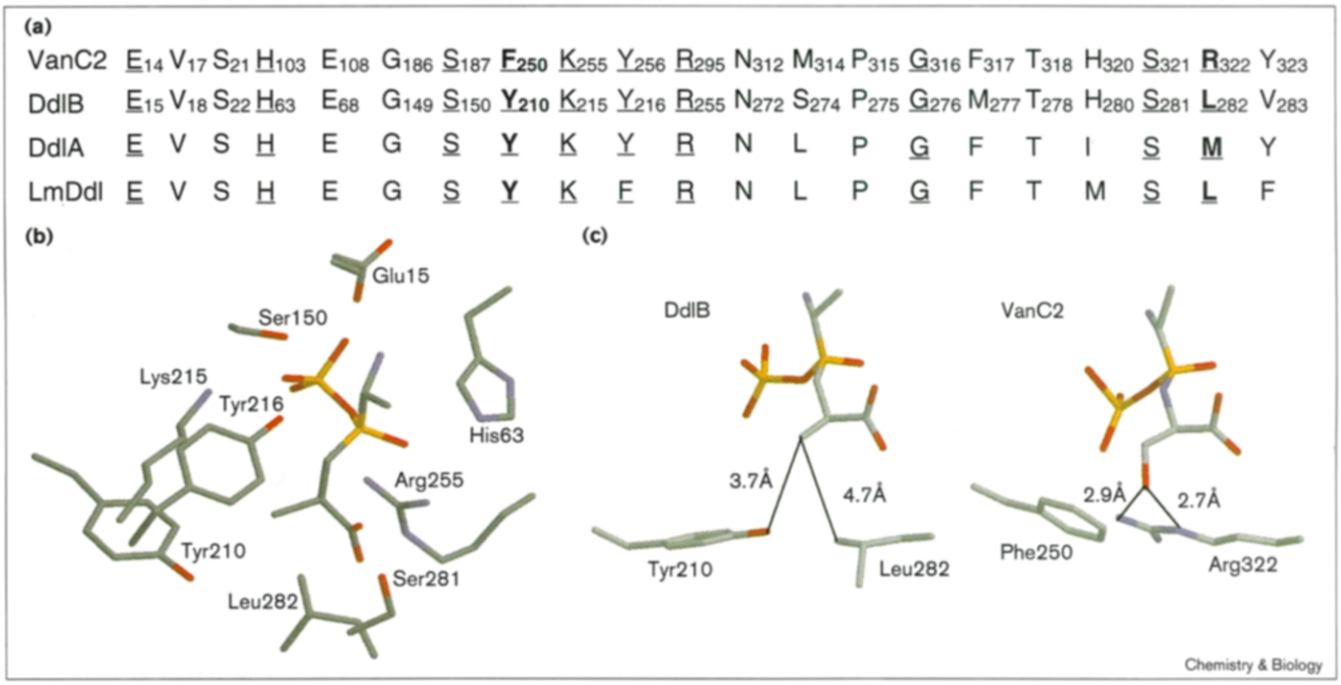
replaced or competed by a D-Ala–D-X ligase (encoded by *VanA*, *VanB*, or *VanC* genes). Bacteria that have peptidoglycan termini with the D-Ala–D-Ala motif bind vancomycin with high affinity, resulting in the blockage of enzymatic cross-bridge formation between adjacent peptide strands at the penultimate D-Ala residue, which leads to a concomitant decrease in wall tensile strength, which in turn leads to osmotic lysis of the bacterium.

Peptidoglycan termini ending in D-alanyl–D-lactate (D-Ala–D-Lac) or D-alanyl-D-serine (D-Ala–D-Ser) have 1000-fold [14] or sixfold [15] lower affinity, respectively, for vancomycin, mirroring the MIC values observed, and quantitatively accounting for resistance. The altered D-Ala–D-X peptidoglycan termini arise through the action of VanA, VanB, or VanC D-Ala–D-X ligases acting instead of, or in competition with, the normal D-Ala–D-Ala ligase in the bacterial cytoplasm.

In this sense, vancomycin resistance is a molecular disorder of D-Ala–D-X ligases where X is Ala in sensitive strains and Lac or Ser in resistant gram-positive bacteria. To understand the switch in specificity and to permit rational design of D-, D-ligase inhibitors, with the intent of reversing vancomycin resistance, we have been carrying out structure–function studies on the D-, D-ligase (Ddl) family, including the *Escherichia coli* DdlA and DdlB isoforms [16,17], the *VanA* D-Ala–D-Lac ligase [14,18], *E. coli* Ddl mutants with a VanA-type gain-of-function [19], the depsipeptide-forming ligase from *Leuconostoc mesenteroides* (Lm) [20] and, most recently, the VanC-type D-Ala–D-Ser ligase [21]. The VanA and VanB phenotypes differ from the VanC phenotype in that in the first two, a D-, D-depsipeptide is made, in which the amide NH to oxoester switch is the molecular determinant of the elevated K_d for the antibiotic, whereas the VanC-type continues to make a D-, D-dipeptide product in which the methyl sidechain of the carboxy-terminal D-Ala is replaced by the larger, polar CH_2OH substituent of D-Ser that is the source of molecular dislocation when complexed with vancomycin (Figure 1).

In previous studies we have utilized the crystal structure of *E. coli* DdlB [22] to guide mutagenesis studies that led to some understanding of the switch from dipeptide D-Ala–D-Ala to depsipeptide D-Ala–D-Lac synthesis. The *E. coli* DdlB Tyr216→Phe (Y216F) mutant gains D-Ala–D-Lac activity [19] whereas a corresponding phenylalanine to tyrosine mutation in the D-, D-ligase from the vancomycin-resistant *L. mesenteroides* loses depsipeptide ligase activity but retains D-, D-dipeptide ligase function [20]. In this study, we have begun to address specificity determinants between the Ddl and VanC proteins that specify D-Ser versus a second D-Ala (D-Ala_2) in a D-, D-dipeptide product. Whereas the wild-type VanC2 ligase from *Enterococcus casseliflavus* has a 240-fold preference for using D-Ser over D-Ala_2 (400-fold reported by Park *et al.* [21]), mutation of two VanC2 residues, Phe250 and Arg322, which are predicted to interact with D-X, leads to a D-Ala–D-X ligase that now activates D-threonine, D-allothreonine, and D-homoserine at least 10-fold more efficiently than does the wild-type VanC2 ligase. The double mutant Phe250→Tyr, Arg322→Met (F250Y R322M) VanC2 ligase has been reverted almost exclusively back to a D-Ala-D-Ala ligase. Analogously, mutation of Leu282 in *E. coli* DdlB (which corresponds to residue 322 in VanC) leads to discrimination in favor of D-Ser over D-Ala_2.

Figure 2



Choice of mutations predicted to affect D-Ala–D-X recognition in the VanC2 ligase by comparison to D-Ala–D-Ala ligases. **(a)** Alignment of residues (using single-letter amino-acid code) in D-, D-ligases from *E. coli* (DdlA and DdlB), *Leuconostoc mesenteroides* (Lm Ddl) and *Enterococcus casseliflavus* (VanC2) based on X-ray analysis of *E. coli* DdlB [22] that lie within a 6 Å sphere of any atom of the phosphinate transition-state analog. Those residues within 4 Å are underlined. The subscripts refer to the postions of the amino acids in the primary sequence of the corresponding enzymes. **(b)** *E. coli* DdlB residues within 4 Å of any atom of the phosphinate transition-state analog, which defines the active site. **(c)** A comparison of the distance between the sidechain methyl of the bound phosphinate in *E. coli* DdlB to Tyr210 and Leu282 sidechains and a model for VanC2 with putative distances to Phe250 and Arg322M, assuming equivalent orientations.

Results

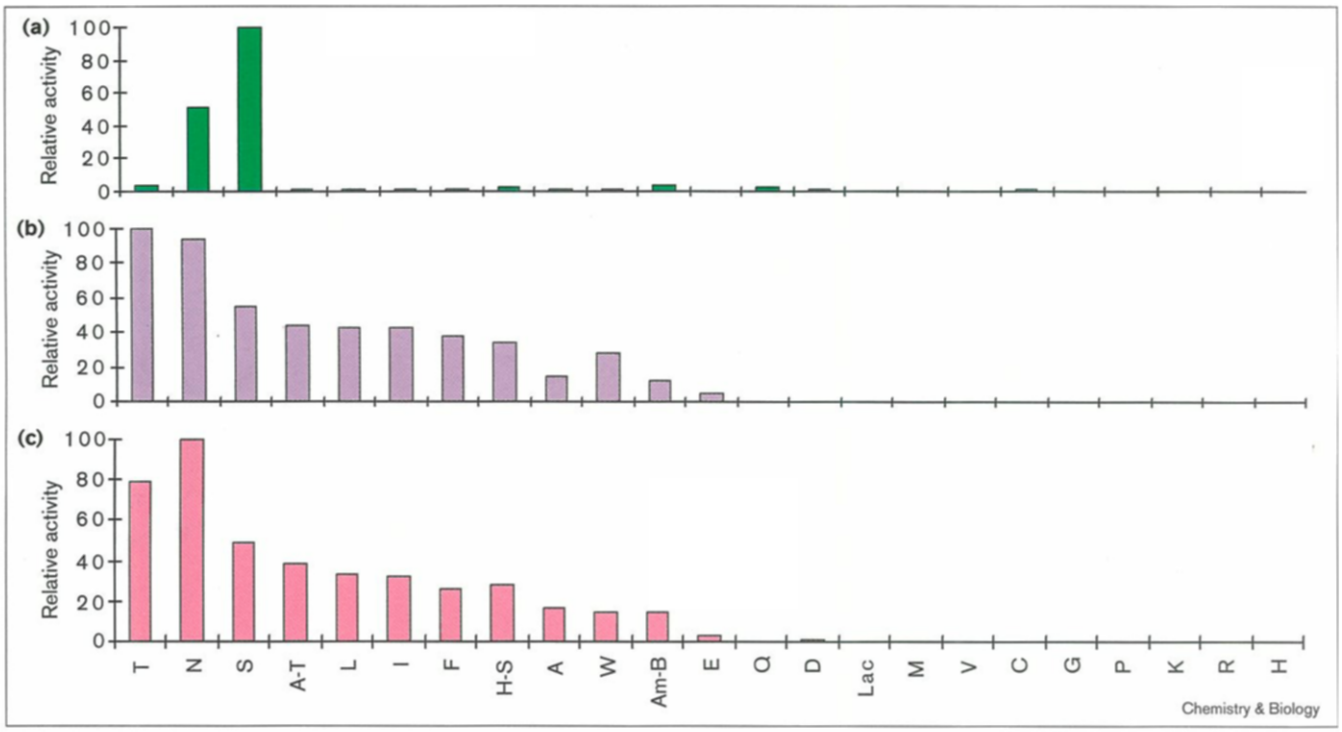
Rationale for F250Y and R322M mutations

Based on sequence homology (Figure 2a), the amino acids shown by X-ray analysis to be within 4 Å of the phosphinate transition-state inhibitor crystallized with *E. coli* DdlB [22] are identical to those of VanC2 except for Tyr210 and Leu282, whose locations are noted in Figure 2b. The Tyr210 and Leu282 sidechains are 3.7 and 4.7 Å, respectively, from the methyl group in the transition-state analog that corresponds to the methyl sidechain in D-Ala_2 (Figure 2c). A model for VanC2 with equivalent orientation would place Phe250 and Arg322 in van der Waals contact with the CH_2OH sidechain of a D-Ser variant of a phosphinate transition-state analog. Thus the corresponding VanC2 residues, Phe250 and Arg322, might be responsible for the 240-fold preferred K_m of the VanC2 ligase for serine compared to alanine in the second site. To test this hypothesis, we constructed the F250Y and R322M VanC2 single mutants, as well as the double mutant. The R322M mutation was made instead of the Arg322→Leu (R322L) mutation because the arginine to methionine mutation yields a less bulky sidechain, and *E. coli* DdlA has methionine in this position (Figure 2a). We expected these mutations to selectively disable D-Ala–D-Ser ligase activity and augment D-Ala–D-Ala ligase activity. The single mutants were purified in a manner analogous to wild-type VanC2, to > 95% homogeniety, as assayed by SDS–PAGE (data not shown). The F250Y R322M double mutant was, however, substantially more problematic to obtain in quantity, as it is much less soluble than either single mutant or the wild-type VanC2 ligase.

R322M and F250Y VanC2 D-Ala–D-X ligases

Initial analysis of the regioselectivity of the wild-type VanC2 ligase for substrates in the carboxy-terminal position demonstrated recognition of only two D-amino acids at appreciable rates: D-Ser (k_{cat}/K_m = 7.0×10^{-3} $M^{-1}s^{-1}$) and D-Asn (2.3×10^{-3} $M^{-1}s^{-1}$). In contrast, recognition of D-Thr was very low (9.3×10^{-5} $M^{-1}s^{-1}$) as was D-Ala (2.9×10^{-5} $M^{-1}s^{-1}$); values of 4.5×10^{-3}, 1.2×10^{-3}, 2.8×10^{-5}, and 1.1×10^{-5} $M^{-1}s^{-1}$, respectively were reported by Park *et al.* [21]. Because VanC2 is believed to have little uncoupled ATPase activity, each of the two single-mutant VanC2 ligases was assayed for recognition of various D-amino acids, using a spectrophotometric assay in which the production of ADP is coupled to the oxidation of NADH through pyruvate kinase (PK) and lactate dehydrogenase (LDH). From these assays, it became

Figure 3



The ability to activate D-amino acids (D-X) in D-Ala–D-X ligase assays using **(a)** wild-type VanC2 ligase compared to **(b)** the R322M and **(c)** the F250Y single mutants. Catalytic efficiency assays measuring k_{cat}/K_m(D-X) for the F250Y and R322M single mutant VanC2 ligases were measured using 16 µg or 17 µg enzyme, 10 mM D-Ala, and 21 mM D-isomers or 42 mM DL-isomers of the indicated amino acids, as described in the Materials and methods section. For wild-type VanC2, 34 µg enzyme, 10 mM D-Ala, and 10 mM D-amino acids or 20 mM DL-amino acids were used, as previously reported [21]. A-T, allothreonine; H-S, homoserine; Am-B, α-amino-*n*-butyric acid; Lac, lactate.

clear that, rather than just a specific reversal of selectivity from a D-Ala–D-Ser back towards a D-Ala–D-Ala ligase, the ligase's recognition selectivity was 'loosened'. From Figure 3b and 3c it is evident that both single mutants demonstrate a substantial gain in ability to activate and incorporate several D-amino acids, including D-Thr, D-Allothr, D-Leu, D-Ile, D-Phe, and D-Trp at mM levels. The nonproteinogenic D-Homoser is also recognized by the modified ligases. There is no detectable depsipeptide ligase activity with D-Lac, as is the case for wild-type VanC2.

Steady-state kinetic data were acquired using the F250Y and R322M single-mutant enzymes and compared to wild-type VanC2 for D-Ser, D-Ala, D-Thr, D-Allothr, D-Homoser, D-Asn and D-Leu (shown in Table 1). Confirmation that the D-X and ATP-dependent coupled ADP-production assay was indeed measuring D-Ala–D-X dipeptide formation was validated by thin-layer chromatography (TLC) analysis with ^{14}C-D-Ala or ^{14}C-D-Ser and comparison with R_f values of authentic L-, L-dipeptides for D-Ala–D-Ala, D-Ala–D-Asn, D-Ala–D-ser, and D-Ala–D-Thr activities (data not shown).

Two trends are apparent from the kinetic data. First, both the R322M and F250Y mutants showed decreased catalytic efficiency as D-Ala–D-Ser ligases by about 10–12-fold ($K_{cat}/K_m = 7.0 \times 10^{-3}$ to 5.7×10^{-4} or 6.7×10^{-4} $M^{-1}s^{-1}$) compared to VanC2. Most of the effect, ninefold and sevenfold, respectively, was a change to the K_m for D-Ser, not a decrease of the k_{cat} for dipeptide bond formation. Second, there was a modest improvement in catalytic efficiency of each mutant as a D-Ala-D-Ala ligase, of threefold to fourfold. Although not necessarily interpretable alone, the change in catalytic efficiency ratios k_{cat}/K_m(D-Ala_2): k_{cat}/K_m(D-Ser) was 36-fold for the R322M mutant and 44-fold for the F250Y mutant (Table 2).

The ability to process D-Thr was 12-fold higher in the F250Y mutant and 13-fold higher in the R332M mutant than in wild-type VanC2, using k_{cat}/K_m criterion, split between a twofold k_{cat} increase and about a fivefold improvement in K_m. The recognition of D-Allothr and D-Leu is improved in the two single mutants by about fivefold, and recognition of D-Homoser some 17–23-fold, predominantly as a result of lowering the K_m. Not only are the β- and γ-hydroxy D-amino acids more efficiently recognized

Table 1

Relaxation of specificity for D-X recognition by VanC2 mutant ligases.

Substrate	Kinetic parameters	Wild-type VanC2	R322M VanC2	F250Y VanC2
D-Ser	K_m Ala_1	1.6	1.7	1.4
	K_m Ser_2	2.6	23	17
	k_{cat}	1092	780	679
	k_{cat}/K_m	700	57	67
	K_m ATP	0.42	0.25	0.30
D-Ala	K_{m2}	>100*	>100*	>100*
	k_{cat}	>178†	>760†	>1130†
	k_{cat}/K_{m2}	2.9	8.7	12
D-Thr	K_{m2}	>100*	16.8	18.7
	k_{cat}	>480[b]	1210	1280
	k_{cat}/K_{m2}	9.3	120	1 20
D-Allothr	K_{m2}	>42*	>40*	>45*
	k_{cat}	>124†	>569†	>629†
	k_{cat}/K_{m2}	4.5	24	27
D-Homoser	K_{m2}	241	37	25
	k_{cat}	290	770	690
	k_{cat}/K_{m2}	2.0	35	47
D-Asn	K_{m2}	11.5	10.4	14.6
	k_{cat}	1620	950	1360
	k_{cat}/K_{m2}	230	150	160
D-Leu	K_{m2}	>48*	>84*	>84*
	k_{cat}	>29†	>247†	>211†
	k_{cat}/K_{m2}	1.0	5.0	4.5

*highest concentration tested; †activity at highest concentration tested: K_m in units of mM, k_{cat} in units of min^{-1}, k_{cat}/K_{m2} in units of 10^{-5} $M^{-1}s^{-1}$. For assays that were performed in duplicate, the measured values of k_{cat} and K_m from each independent experiment were within 20% of each other; the values of k_{cat}/K_m were within 5% of each other. Assays were conducted using 15–20 µg enzyme in 1 ml reaction mixtures as described in the Materials and methods section.

as nucleophilic substrates by the mutants, but the hydrophobic D-amino acids, D-Leu, D-Ile, D-Phe, and even D-Trp can be accommodated in the second amino-acid subsite of these two VanC2 mutant ligases.

Although activation of most of the D-X amino acids is clearly dependent on D-Ala, we wanted to determine whether the VanC2 ligase could also utilize D-Ser in place of D-Ala at subsite 1. This activity can be assayed by D-Ser-dependent ADP production in the ADP-coupled assay (ADP production became substantial at high concentrations of D-Ser). As shown in Figure 4, the ADP production eventually begins to saturate around 100 mM for wild-type VanC2, but not for F250Y or R322M VanC2. The greater D-Ser–D-Ser activity of wild-type VanC2 is consistent with the greater D-Ala–D-Ser activity measured for wild-type VanC2 compared to the other mutants. Confirmation that a D-Ser–D-Ser dipeptide was formed came from TLC of assays containing ^{14}C-D-Ser in the absence of any D-Ala

Table 2

VanC2 mutants with reversed specificity for D-Ala–D-Ala ligase activity.

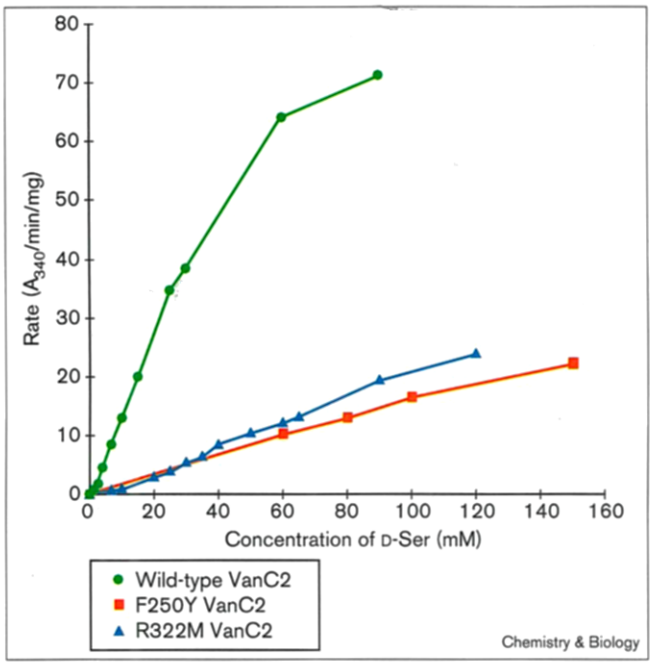
Enzyme	$\frac{k_{cat}/K_{m2}\ (D\text{-Ala})}{k_{cat}/K_{m2}\ (D\text{-Ser})}$	Switch in specificity
Wild-type VanC2	0.0042	1-fold
His-tagged wild-type VanC2	0.0017	0.40-fold
F250Y VanC2	0.19	44-fold
R322M VanC2	0.15	36-fold
His-tagged F250Y R322M VanC2	26	6,200-fold

(data not shown). Even D-Homoser was able to support a low level of ATPase activity in wild-type and mutant VanC2 ligases (data not shown).

The F250Y R322M double mutant of VanC2 ligase

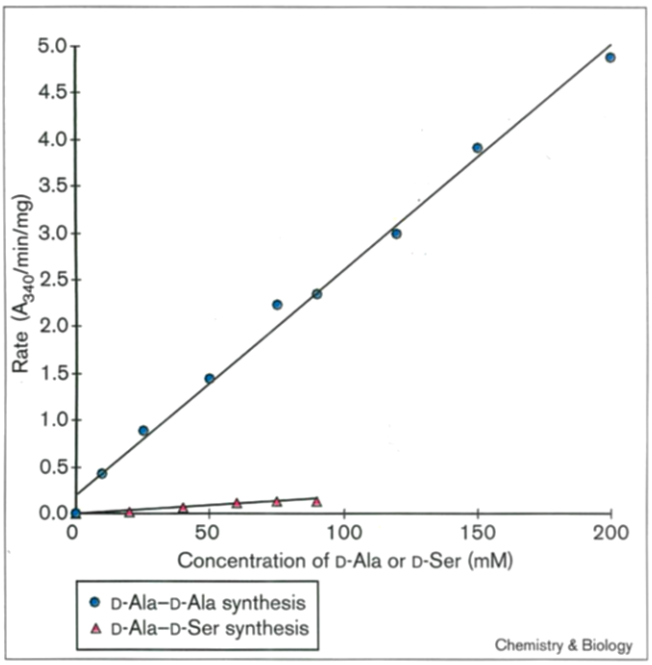
The double mutant F250Y R322M of VanC2 ligase was constructed, expressed, and purified to see whether the two mutations had any synergistic effects on the specificity switch from a D-Ala–D-Ser ligase to a D-Ala–D-Ala ligase. Under a variety of induction conditions, the double mutant enzyme was greater than 90% insoluble. Because a histidine (His)-tag does not affect the activity of wild-type VanC2, we attempted to purify a small amount of soluble His-tagged protein; but the protein could not be purified to homogeniety and no significant amino-acid-dependent ATPase activity could be detected above the amino-acid-independent background ATPase activity, which was presumably due to contaminating proteins. Thus, the insoluble His-tagged protein was solubilized with urea and the protein refolded on a nickel column using a decreasing urea gradient, providing soluble, active F250Y R322M ligase for assays. As a D-Ala–D-Ala ligase, the double-mutant enzyme has a k_{cat}/K_{m2} of 2.57×10^{-6} $M^{-1}s^{-1}$, a level readily measurable over the amino-acid-independent ATPase background (0.52 min^{-1} background compared to 15.5 min^{-1} activity in the presence of 100 mM D-Ala), and about sevenfold lower than unmodified His-tagged VanC2 (1.70×10^{-5} $M^{-1}s^{-1}$; Figure 5). In contrast, F250Y R322M has been essentially fully disabled as a D-Ala–D-Ser ligase. A k_{cat}/K_{m2} of 1×10^{-7} $M^{-1}s^{-1}$ was measured (Figure 5), barely above background activity, and 26-fold below the level of D-Ala–D-Ala ligase activity. The drop from 9.77×10^{-3} to 1×10^{-7} $M^{-1}s^{-1}$ corresponds to a 100,000-fold loss in catalytic efficiency for D-Ala–D-Ser formation. Because the presence of D-Ser did not inhibit enzyme activity at 50 or 100 mM D-Ala, as would be expected for a low k_{cat} substrate, it is likely that the decrease in catalytic efficiency is primarily a K_m effect. Both D-Ala–D-Ala and D-Ala–D-Ser activities were confirmed by TLC (data not shown). Although it is not yet clear if the renatured double mutant has properly folded to its fully native conformation and full activity, nonetheless the specificity switch for D-Ala–D-Ser to D-Ala–D-Ala is reversed by

Figure 4



D-Ser–D-Ser ligase activity of wild-type and mutant VanC2 ligases. The dependence of ADP formation by wild-type VanC2, F250Y VanC2, and R322M VanC2 on D-Ser concentration in the absence of any D-Ala is shown. The assay conditions are as described in the Materials and methods section using 15–20 μg enzyme.

Figure 5



D-Ala–D-Ala and D-Ala–D-Ser ligase activities in the double-mutant F250Y R322M VanC2 ligase. D-Ala–D-Ala synthesis and D-Ala–D-Ser synthesis were assayed at different D-Ala or D-Ser concentrations in the standard coupled spectrophotometric assay as described in the Materials and methods section.

about 6,000-fold compared to untagged wild-type VanC2 and 15,000-fold compared to His-tagged but otherwise wild-type VanC2 (Table 2).

The Tyr256→Phe mutant of VanC2 ligase

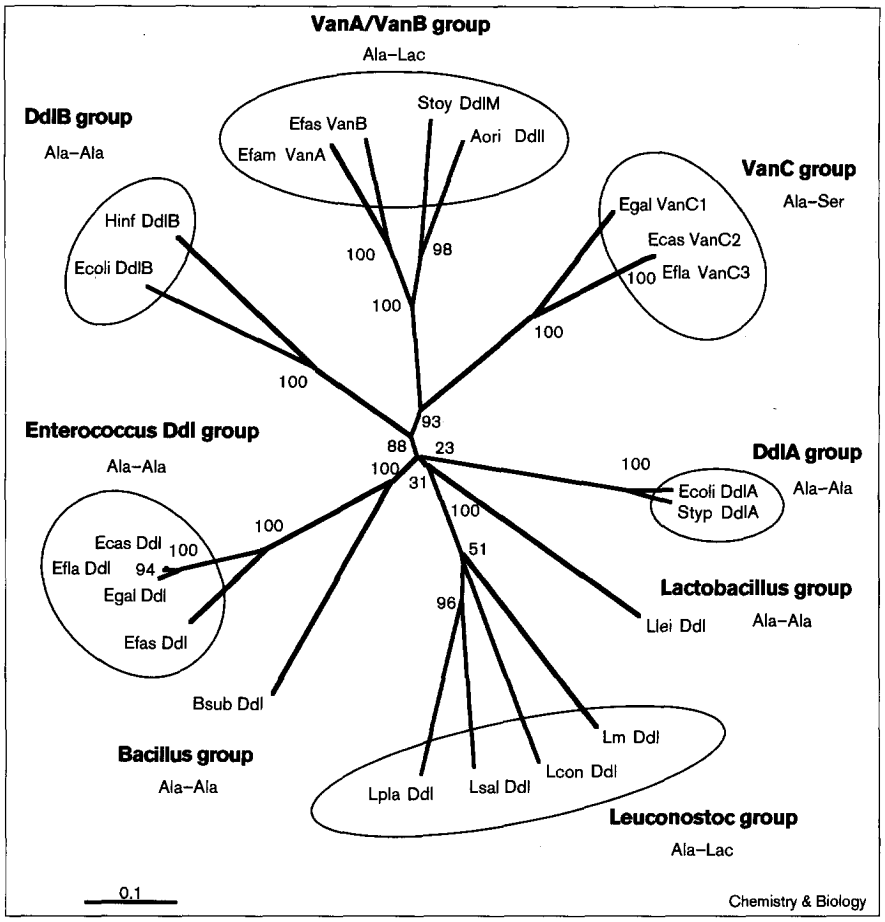
Tyr256 in VanC2 corresponds to Tyr216 in *E. coli* D-Ala–D-Ala ligase (Figure 2a,b), a residue identified in the crystal structure as part of a hydrogen-bonded triad (with Ser150 and Gln15) that stabilizes the mobile Ω loop (residues 205–227) and sets up the catalytic active site geometry (Lys215 required for γ-PO_3 transfer) in the enzyme–substrate complex [22]. A survey of D-, D-ligases shows either tyrosine or phenylalanine at this position in all D-Ala–D-X ligases except the depsipeptide-forming VanA and VanB ligases, which appear to have a different Ω loop structure (Figure 2a). When tyrosine is present (*E. coli* DdlA and DdlB; see Figure 2a), dipeptide ligase activity and vancomycin sensitivity are observed [19,20]. When phenylalanine is present (for example, Lm Ddl, Figure 2a), there is phenotypic vancomycin resistance and gain of depsipeptide ligase activity. In particular, the Tyr216→Phe (Y216F) mutant of *E. coli* DdlB gains depsipeptide ligase activity [19] whereas the Phe216→Tyr (F216Y) mutant of *Leuconostoc mesenteroides* (a naturally vancomycin-resistant organism) retains D-Ala–D-Ala ligase activity but loses D-Ala–D-Lac synthetic ability [20].

We examined a possible comparable role for Tyr256 by constructing the Tyr256→Phe (Y256F) VanC2 mutant as described in the Materials and methods section. When we overproduced the *E. casseliflavus* Y256F VanC2, it was expressed at low levels in *E. coli* but could be purified and analyzed. The Y256F mutant protein is substantially disabled as a D-Ala-D-Ser ligase. Although the binding site for the first D-Ala (D-Ala_1) appears to be intact (K_{m1} = 0.6 mM), and D-Ala–D-Ser activity is still measurable, no saturation was seen up to 100 mM D-Ser (υ_0 at 20 min^{-1} at this concentration of D-Ser) so the K_m for D-Ser (2.6 mM in wild-type VanC2) may have been increased by at least two orders of magnitude. Likewise, Y256F VanC2 showed no tendency to saturate with D-Ala (K_m D-Ala_2 > 180 mM) or D-Thr (K_m D-Thr > 150 mM), suggesting a broadly incapacitated catalyst.

Finally, the question of whether the mutant had gained detectable depsipeptide ligase activity was assayed using D-lactate, DL-hydroxybutyrate, and D-glycerate as D-X substrates. Under conditions that resulted in significant D-Ala–D-lactate and D-Ala–D-hydroxybutyrate production by VanA, corresponding production by Y256F VanC2 was not detected (data not shown). Even at pH 6.0, which results in almost 100% depsipeptide activity by VanA [19], D-Ala–D-glyceric acid was not detected in reactions incubated

Figure 6

Phylogenetic tree of the D-, D-ligase family. The alignment was obtained using the program ClustalX(1.5) [32], as described in the Materials and methods section. Aori, *Amycolatopsis orientalis*; Baci, *Bacillus subtilis*; Ecas, *Enterococcus casseliflavus*; Efam, *E. faecium*; Efas, *E. faecalis*; Efla, *E. flavesceus*; Egal, *E. gallinarium*; Hinf, *Haemophilus influenza*; Lcon, *Lactobacillus confusus*; Llei, *Lactobacillus leichmannii*; Lm, *Leuconostoc mesenteroides*; Lpla, *Lactobacillus plantarum*; Lsal, *Lactobacillus salivarius*; Stoy, *Streptomyces toyocaensis*; Styp, *Salmonella typhimurium*.



with VanA or Y256F VanC2; D-Ala-D-Ala and D-Ala–D-Ser activities were confirmed by TLC, however (data not shown). Thus, this tyrosine to phenylalanine mutation is not sufficient for VanC2 to gain depsipeptide ligase activity.

The Leu282→Arg mutant of *E. coli* DdlB

We made a comparable mutant in *E. coli* DdlB, namely Leu282→Arg (L282R), in which the arginine sidechain corresponding to Arg322 in VanC2 (Figure 2c) was engineered into *E. coli* D-Ala–D-Ala ligase. We had previously noted [23] a decline in catalytic efficiency of *E. coli* L282R DldB as a D-Ala–D-Ala ligase. Repetition of these experiments confirmed the 18-fold increase in K_m for D-Ala_2 (1.13 mM to 20.9 mM) in L282R and an 11-fold decrease in k_{cat} (1870 min^{-1} to 158 min^{-1}) for a net drop in k_{cat}/K_m of $2.76 \times 10^{-2}\,M^{-1}s^{-1}/1.27 \times 10^{-4}\,M^{-1}s^{-1}$ of 210-fold.

In contrast, when assayed as a D-Ala–D-Ser ligase the *E. coli* enzyme shows an improvement in D-Ser K_m of 24-fold (56 to 2.4 mM) resulting in a D-Ser K_{m2} similar to that of wild-type VanC2 (2.6 mM). There was a ninefold drop in k_{cat} (157 to 17.2 min^{-1}), however, for a net gain of only threefold in catalytic efficiency as a D-Ala-D-Ser ligase (this activity was confirmed by TLC using ^{14}C-Ser). Examining the ratio of $[k_{cat}/K_m(\text{D-Ala}_2)]:[k_{cat}/K_m(\text{D-Ser})]$, there is about a 560-fold switch towards selective D-Ala–D-Ser ligase activity, driven mostly by the 210-fold decline in D-Ala–D-Ala ligase catalytic efficiency. In contrast to the results for D-Ser, the catalytic efficiency for D-aminobutyric acid, which lacks a hydroxy group, decreased 30-fold from $1.78 \times 10^{-3}\,M^{-1}s^{-1}$ to $5.88 \times 10^{-5}\,M^{-1}s^{-1}$ for DdlB L282R, suggesting a switch to preferential recognition of the CH_2OH sidechain of D-Ser.

Discussion

Analysis of the specificity of D-, D-dipeptide formation by the D-, D-ligases involved in providing the dipeptidyl unit at the termini of peptidoglycan intermediates in bacterial cell wall biosynthesis has taken on some immediacy with the recent realization that vancomycin resistance, both in opportunistic clinical pathogenic gram-positive bacteria (VRE) and in the natural antibiotic immunity in the producer organisms (streptomycetes) [24], is due to altered ligase specificity. The family of D-Ala-D-X ligases can be subdivided into eight subgroups (Figure 6), five of which are D-Ala–D-Ala ligases physiologically, two D-Ala–D-Lac

ligases and the eighth, the VanC subgroup, has a D-Ala–D-Ser ligase. A change from D-Ala_2 to either D-Lac or D-Ser leads to a lowered affinity for vancomycin when the D-Ala–D-X moiety is displayed on peptidoglycan termini on the outer face of the cytoplasmic membrane of resistant enterococci [25,26], lactobacilli [27], or leuconosti [27]. The vancomycin resistance engendered by the switch from the peptidoglycan terminus D-Ala–D-Ala-COO^- to D-Ala–D-Lac-COO^- is thought to be due in substantial part to loss of a hydrogen bond between the dipeptide NH and one of the backbone carbonyls of vancomycin (Figure 1), whereas the D-Ala–D-Ser-COO^- alteration preserves the hydrogen-bonding network between the dipeptide and the antibiotic, but the CH_2OH sidechain of D-Ser is presumed to pack less well against the concave surface of the rigid cup-shaped vancomycin.

A starting point for structure–function analysis of the switch in D-Ala–D-X ligase selectivity has been the X-ray structure of the *E. coli* DdlB D-Ala–D-Ala ligase complexed with a tightly binding phosphinate analog of a tetrahedral reaction intermediate [22], which has allowed definition of the ligase active site and led to structure-driven mutagenesis studies of *E. coli* DdlB [23]. The VanC ligase (D-Ala–D-Ser) and *E. coli* Ddl ligase subgroups have a very strong conservation of residues found within a 4 Å sphere of the *E. coli* DdlB active site with the two changes noted in Figure 2a,b. The Tyr210 and Leu282 residues in the *E. coli* Ddl active-site structure are 3.7 and 4.3 Å, respectively, from the methyl sidechain of the cocrystallized transition-state analog inhibitor (Figure 2c). Modeling a similar arrangement for the VanC2 ligase active site suggested the two distinct residues Phe250 and Arg322 would occupy comparable positions and, in particular, that the amidino sidechain could hydrogen bond to the CH_2OH sidechain of a D-Ala–D-Ser tetrahedral intermediate (putatively at 2.8 and 2.7 Å in the model of Figure 2c). This model formed the basis of the choice of F250Y and R322M mutants constructed in this work.

We had anticipated that elimination of the guanidinio moiety in R322M would decrease any favorable interaction with the CH_2OH sidechain of D-Ser in the second subsite of a D-Ala–D-X ligase. Indeed the observed result is a 10–12-fold drop in the k_{cat}/K_m ratio for D-Ala–D-Ser catalytic efficiency, and the D-Ala–D-Ala production rose three- to four-fold. But, more significantly, the replacement of the long cationic arginine sidechain of residue 322 by the shorter linear methionine sidechain should create space for improved recognition of bulkier, larger D-X groups, and a significant broadening of specificity was observed, as noted in Figure 3. In particular, both the 2*R*,3*R* and 2*R*,3*S* diastereomers of threonine (D-Thr, D-Allothr) were activated with 12- and 5-fold improvement in catalytic efficiency. Likewise, activation of D-Homoser, which is very poorly incorporated by wild-type VanC2 ligase, is increased 18-fold. The other single mutant, F250Y, shows equivalent trends: a comparable lowering of activity as a D-Ala–D-Ser ligase, marginal gain as a D-Ala–D-Ala ligase, and a net promiscuity in D-X activation. It is not obvious exactly how F250Y substitution relaxes D-X specificity; this will require X-ray structures of VanC2 and/or these mutants.

Even though each single mutant had a 36–44-fold alteration in favor of D-Ala–D-Ala formation over D-Ala–D-Ser, initial studies with the F250Y R322M VanC2 double mutant showed about a 6,000-fold switch, largely due to selective loss of D-Ala–D-Ser ligation. It is not yet clear whether the double mutant F250Y R322M is properly folded and fully native; at worst it has become a much better D-Ala–D-Ala ligase than a D-Ala–D-Ser ligase with decreased catalytic efficiency.

Neither VanC2 nor the F250Y or R322M mutants have detectable ability to activate D-Lac, confirming that the ability to turn D-hydroxy acids into effective nucleophiles is not a property of this ligase subgroup. Although we have noted an experimental correlation of D-Lac activation with a Y216F mutant (DdlB numbering) in *E. coli* Ddl and Lm Ddl [19,20], mutation of the corresponding tyrosine in VanC2 to phenylalanine (Y256F) did not produce detectable depsipeptides, either from D-Lac or the CH_2OH containing cognate, D-glycerate. Thus, we do not understand yet how to get D-, D-depsipeptide ligase activity in a VanC ligase framework.

The mutant in *E. coli* DdlB that should go the 'other way', and turn a D-Ala–D-Ala ligase into a D-Ala–D-Ser ligase, is L282R. Indeed this *E. coli* DdlB mutant ligase is 210-fold disabled in catalytic efficiency for making D-Ala–D-Ala and about threefold improved for yielding D-Ala–D-Ser, for a net swing of about 560-fold for selecting D-Ser over D-Ala in the second position of the D-Ala–D-X dipeptide. It may be worth crystallizing and determining the structure of this mutant, preferably with a CH_2OH-containing phosphinate analog, to see whether the guanidino sidechain of the newly introduced arginine residue is indeed in contact with the CH_2OH substituent.

Significance

The D-alanyl–D-alanine ligases of both gram-negative and gram-positive bacteria occupy a crucial step in bacterial cell-wall peptidoglycan assembly, and are themselves antibacterial targets. In the gram-positive soil bacteria that produce glycopeptide antibiotics [24] and in opportunistic human pathogens (such as the vancomycin-resistant enterococci, VREs) [11–13], these D-, D-ligases also serve as switches that confer antibiotic resistance on the bacteria themselves. The cell-wall peptidoglycan termini of vancomycin-sensitive bacteria, for example, end in the motif

D-Ala–D-Ala. In contrast, vancomycin-resistant peptidoglycans end in D-Ala–D-hydroxy acid (in the case of the VanA and VanB phenotypes) or D-Ala–D-Ser in the VanC phenotype. These differences are due to a change in the D-, D-ligase. Understanding the molecular basis of the D-X switching among members of the D-Ala–D-X ligase family should help in deciphering antibiotic resistance and immunity mechanisms and in the design of effective ligase inhibitors. Using the *Escherichia coli* D-Ala–D-Ala ligase (Ddl) X-ray structure as a guide, we have identified and mutated active-site residues in the VanC2 and Ddl ligases that alter D-X recognition in both D-, D-dipeptide and D-, D-depsipeptide formation (two peptidoglycan synthesis steps that involve the D-, D-ligases). Our findings confirm that certain D, D-ligase residues are critical for determining D-X recognition, and might provide a way to combat bacterial antibiotic resistance in pathogens.

Materials and methods

Materials

Culture media was obtained from Difco Laboratories. The expression vectors pET22b and pET15b, and the competent *E. coli* strains BL21(DE3) and BL21(DE3) pLysS were obtained from Novagen. The competent *E. coli* strain DH5α was purchased from GibcoBRL. Oligonucleotides were obtained from Integrated DNA Technologies, Inc. Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs, Inc., and *pfu* DNA polymerase from Strategene. ATP, amino acids, L-Ala–L-X dipeptides, phospho(enol)pyruvate, chloramphenicol, ampicillin, bovine serum albumin, dithiothreitol (DTT), and buffers were purchased from Sigma. D-lactate was obtained from Fluka, and D-glyceric acid from Aldrich. Isopropyl-thio–β-D-galactoside and L-Ser–L-Ser were purchased from Bachem Biosciences. Reduced nicotinamide adenine dinucleotide (NADH), lactate dehydrogenase (LDH), and pyruvate kinase (PK) were obtained from Boehringer Mannheim Biochemicals. Radiolabeled ^{14}C-D-alanine, ^{14}C-D-serine, ^{14}C-D-lactate, and ^{14}C-DL-glyceric acid (0.1 mCi/mL, 55 μCi/μmol) were purchased from American Radiolabeled Chemicals Inc. Thin-layer chromatography (TLC) cellulose plates were obtained from Kodak, and TLC silica plates from Whatman. Plasmid pVANC2 expressing the *E. casseliflavus* ATCC 25788 VanC2 gene has been described previously [21].

Recombinant DNA methods

Recombinant DNA techniques were performed as described elsewhere [28]. Preparation of plasmid DNA, gel purification of DNA fragments, and purification of polymerase chain reaction (PCR)-amplified DNA fragments were performed using a QIAprep spin plasmid miniprep kit, a QIAEX II gel extraction kit, and a QIAquick PCR purification kit, respectively (QIAGEN).

Site-directed mutagenesis

Site-directed mutants F250Y and R322M were constructed using the megaprimer method [29] with pVANC2 as the template [21]. In the first round, a DNA fragment was amplified for the F250Y mutant using the primer F1 (5′-GTAGACGGCTTTT**A**CGATTTTGAAG-3′–mismatch in bold) and primer wt1 (5′-GCGGATCCCTCATTTGACTTCCTCCT-TTGCTAAGACAAGTTTTTGG-3′).

For the R322M mutant, the primer R1 (5′-CGAGTCACTCC**ATG**TATC-CTGCC-3′) and primer wt1 were used. The second run was achieved using the respective gel-purified PCR-amplified DNA fragment and primer wt2 (5′-GAAACATATGAAAAAAATCGCCATTATTTTTGG-3′) with pVANC2 as template. For the Y256F mutant and the F250Y R322M double mutant, the splicing by overlap extension method [30] was used with plasmids pVANC2 and pVANC2.F250Y as templates, respectively. In the first round, the upstream and downstream sequence of the mutations were amplified using the primer pairs wt3 (5′-CGGTCTAGAGGAAGGAAGAAACATATGAAAAAAATCGCCAT-TA-3′) /Y1 (5′-GAAGAAAAGT**T**CCAGCTGATCAGCG-3′) and wt1/Y2 (5′-CGCTGATCAGCTGG**A**ACTTTTCTTC-3′) for the F250Y mutant. Primer pairs wt3/R1 and wt1/R2 (5′-GGCAGGATA**CATG**GAGT-GACTCG-3′) were used for the F250Y R322M double mutant. The second run was achieved by using an approximately equimolar ratio (total amount about 20 ng) of the two gel-purified PCR-amplified fragments to be joined as template and the primer pair wt3/wt1. The resulting purified PCR products were digested with *Xba*I and *Bam*HI, gel-purified, and subcloned into *Xba*I- and *Bam*HI-digested pET22b. The sequences were confirmed by DNA sequencing by the Molecular Biology Core Facility of the Dana Farber Cancer Institute (Boston, MA).

Construction of His-tagged fusions

pVANC2 and pVANC2.F250YR322M were digested with *Nde*I and *Bam*HI, gel-purified, and subcloned into *Nde*I- and *Bam*HI-digested pET15b, giving pHVANC2 and pHVANC2.F250YR322M, respectively. The plasmids were then transformed into *E. coli* strain DH5α and subsequently into *E. coli* strain BL21(DE3) pLysS for expression.

Overexpression and purification of protein

Overexpression and purification of wild-type VanC2, and the F250Y, R282M, and Y256F mutants were performed as previously described [20], giving respectively 40 mg, 14 mg, 19 mg, and 6 mg from 2 L cell cultures. The His-tagged VanC2 fusion was overexpressed in the same way as the untagged protein except that 34 μg/mL chloramphenicol was added to the culture media. The harvested cells were resuspended in 20 mL of buffer A (50 mM $NaPO_4$, pH 8.0, 5 mM $MgCl_2$, and 10% glycerol), disrupted by French press (18,000 psi/in^2), and centrifuged for 30 min at 100,000 $\times g$. The supernatant was applied to a Ni-NTA Superflow (Qiagen) column (8 mL) preequillibrated with buffer A at a flow rate of 1 mL/min. After washing with 40 mL of buffer A and 80 mL of buffer A containing 7.5 mM imidazole, the protein was eluted using a linear gradient of 0–0.25 M imidazole in buffer A containing 300 mM NaCl at a flow rate of 2 mL/min. The buffer was changed to buffer B (50 mM HEPES, pH 7.2, 150 mM KCl, 5 mM $MgCl_2$, 5 mM DTT, and 20% glycerol) using a Centriprep 10 concentrator (Amicon), yielding 40 mg protein from 1 L of cell culture. The F250Y R322M double mutant initially could not be overexpressed using the pET22b vector transformed into *E. coli* BL21(DE3) or BL21(DE3)pLysS. Good overexpression was obtained using pET26b vector transformed into BL21(DE3) or pET15b or pET28b transformed into BL21(DE3) pLysS. A 1 L culture of His-tagged F250Y R322M double mutant was grown, harvested, lysed, and centrifuged as described above. Over 90% of the protein remained in the pellet as assayed by SDS–PAGE (data not shown). The insoluble protein found in the cell debris pellet was solubilized using 50 mL buffer C (0.1 M $NaP0_4$, 0.01 M Tris, pH 8.0, and 8 M urea) and incubated for one hour. After centrifugation for 30 minutes at 100,000 $\times g$, the supernatant was loaded on a 8 mL Ni-NTA Superflow column preequilibrated with buffer C. The protein was refolded using a linear gradient of 6–0 M urea in 20 mM Tris-Cl, pH 7.4, 5 mM $MgCl_2$, 20% glycerol, 500 mM NaCl, and 0.5 mM ATP over 120 mL at 1 mL/min. The protein was eluted with 20 mM Tris-Cl, pH 7.4, 5 mM $MgCl_2$, 20% glycerol, and 250 mM imidazole. The protein sample was then dialyzed into buffer B and centrifuged for 30 minutes at 100,000 $\times g$, yielding 6 mg of protein. Wild-type and L282R DdlB were overexpressed and purified as described previously [23]

Protein quantitation

Protein concentrations were determined using the method of Bradford with bovine serum albumin as standard [31].

Enzyme assay by coupled ADP release

Steady state kinetic constants for the ATP and D-X substrates were determined using a spectrophotometric assay where the production of ADP is coupled to the oxidation of NADH through PK and LDH [20].

Reaction conditions of 100 mM HEPES, pH 7.5, 10 mM KCl, 10 mM $MgCl_2$ and 5 mM ATP were used throughout this study. Kinetic parameters were determined using the equations 1 and 2 (see below) previously described [19,20]. To measure D-Ala–D-Ala activities, the enzymes were incubated in the above buffer in the presence of increasing concentrations of D-Ala; no other amino acids were present. For wild-type and mutant VanC2 ligases, D-Ala–D-X activities were determined in the presence of 10 mM D-Ala and corrected for the background D-Ala–D-Ala activity. Equation 2 was used because in the case of $K_1 \ll [S_1]$, the last term of equation 1 becomes very small compared to the second term. Because the D-Ala–D-Ala activty of the F250Y R322M VanC2 mutant is high compared to the D-Ala–D-Ser activity, it is possible that the D-Ala–D-Ser activity could be underestimated by substracting a constant value for the background D-Ala–D-Ala activity which might decrease in the presence of high D-Ser concentrations. To test this possibility, the D-Ala–D-Ala activity, of the F250Y R322M VanC2 mutant was tested in the presence of 10 mM ^{14}C-D-Ala and increasing D-Ser concentrations and analyzed by TLC as described below. The level of D-Ala–D-Ala activity was constant within the range of error of the experiment, however. For wild-type VanC2, the K_{m1} for D-Ala was determined using 15 mM D-Ser; for F250Y and R322M VanC2, 50 mM D-Ser was used. The K_m for ATP was determined in the presence of 10 mM D-Ala and 60 mM D-Ser.

For wild-type and L282R DdlB, D-Ala–D-X activities were measured using 0.5 mM D-Ala. Equation 1 was used with the reported K_{m1} values of 0.0012 and 0.49 mM, respectively [23].

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_2}{V_{max}}\frac{1}{[S_2]} + \frac{K_1K_2}{V_{max}}\frac{1}{[S_1][S_2]} \quad (1)$$

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_2}{V_{max}}\frac{1}{[S_2]} \quad (2)$$

Enzyme assay by TLC

The assay mixture included 0.2 mM ^{14}C-D-Ala, ^{14}C-D-Ser, ^{14}C-D-Lac, or ^{14}C-DL-glyceric acid (0.1 mCi/ml, 55 μCi/μmol), 100 mM HEPES, pH 7.5, 10 mM KCl, 10 mM $MgCl_2$, 5 mM ATP and additional unlabeled amino acids with enzyme at room temperature. D-Ala–D-Lac and D-Ala–D-glyceric acid activities were also tested at pH 6.5 and 6.0 using 100 mM MES buffer. D-Ala–D-Ala, D-Ala–D-Asn, D-Ala–D-Thr activities were analyzed using ^{14}C-D-Ala, 10 mM unlabeled D-Ala, and 20 mM of the second substrate incubated with enzyme for 4 h. The D-Ala–D-Ala and D-Ala–D-Asn reaction mixtures were analyzed on TLC cellulose plates as described previously [14,18]. D-Ala–D-Thr reactions mixtures were separated on TLC silica plates (thickness, 250 μm; pore size, 60 Å) using 25% 1 M ammonium acetate in 2-propanol for development (solvent A). D-Ala–D-Ser dipeptide formation was confirmed using ^{14}C-D-Ser as described previously [21]. D-Ser–D-Ser activity was measured using ^{14}C-D-Ser and 5 mM unlabeled D-Ser. Before applying the reaction mixtures to TLC silica plates and developing with solvent A, unlabeled D-Ser was added to a final concentration of 50 mM.

Phylogenetic tree of D-,D-ligase family

The alignment was obtained by using the default settings of program ClustalX(1.5) [32]. The tree was made based on the alignment and evaluated by the bootstrap method using 100 replications produced by the same program.

Modeling of the structure of VanC2

The VanC2 structure was modeled based on the known structure of DdlB [22]. Initially, an alignment of DdlB and VanC2 was performed using the ClustalX program [32] and sent to the Swiss-Model protein modeling service according to the standard procedure provided [33]. Because this service does not model ligands, the D-Ala–D-Ser analog of the phosphinophosphate was modeled by adding an oxygen atom to the D-Ala–D-Ala phosphinophosphate from the DdlB structure at the C3′ position and by changing the C1′ atom to a nitrogen. Swiss-pdbviewer [34] was used to three-dimensionally align the conserved amino acid residues of DdlB and VanC2 and to add the ligand model to the active site of VanC2 based on the position of the equivalent atoms in the D-Ala–D-Ala phosphinophosphate. One set of rotamers of F250 and R322 in VanC2 that align with those of Y210 and L282 in DdlB are shown along with their distances in angstroms to the phosphinophosphate analog in Figure 1b. Because no energy minimization calculations have been performed, this model serves only as a starting point for structure–function studies.

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