Gain of D-Alanyl-D-lactate or D-Lactyl-D-alanine Synthetase Activities in Three Active-Site Mutants of the *Escherichia coli* D-Alanyl-D-alanine Ligase B†

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ABSTRACT: *Escherichia coli* D-Ala-D-Ala ligase (Ddl) and the vancomycin resistance-conferring protein VanA are homologues, but VanA has gained the ability to activate D-lactate (D-Lac) and make the depsipeptide D-Ala-D-Lac as well as D-Ala-D-Ala. This depsipeptide ligase activity of VanA is its crucial catalytic function necessary for phenotypic vancomycin resistance. We report here that three *E. coli* DdlB active-site mutants that we made previously based on X-ray structure/function predictions have gained interesting new ligase activities. Y216, S150, and E15 form a hydrogen-bonding triad that orients an ω-loop to close over the active site and also to orient substrate D-Ala$_1$. Mutants Y216F and S150A have gained depsipeptide (D-Ala-D-Lac, D-Ala-D-hydroxybutyrate) ligase activity with dipeptide/depsipeptide partition ratios that mimic the pH behavior of VanA. E15Q has negligible depsipeptide synthetase activity but now uniquely activates D-Lac as the electrophilic rather than the nucleophilic partner for condensation with D-Ala to make a regioisomeric D-Lac-D-Ala, an amide rather than an ester product. These results provide insights into the active-site architecture of the ligases and the subsites for recognition of D-Ala vs D-Lac and predict the Y216F substitution will impart D-Ala-D-Lac synthetase activity to Ddls from Gram-positive bacteria with intrinsic resistance to vancomycin.

As the glycopeptide antibiotic vancomycin has come into widespread use as a front-line agent in the treatment of Gram-positive bacterial infections, clinically significant resistance has arisen in enterococcal strains (Wright & Walsh, 1992; Arthur & Courvalin, 1993; Walsh, 1993). Inducible, high-level resistance has been associated with five genes, *vanR*, *-S*, *-H*, *-A*, and *-X*, on a transposable element (Arthur et al., 1993), which encode a two-component regulatory system (VanS and -R) (Arthur & Courvalin, 1992; Wright et al., 1993; Holman et al., 1994) and three enzymes (VanH, -A, and -X) (Dutka-Malen et al., 1990; Bugg et al., 1991a,b; Wu et al., 1995) that provide for the formation and selective accumulation of the depsipeptide D-Ala-D-Lac[1] in the cytoplasmic phase of peptidoglycan biosynthesis. D-Ala-D-Lac is incorporated in place of the D-Ala-D-Ala, produced by the chromosomal D-Ala-D-Ala ligase, in cell wall intermediates to yield a muramyl tetrapeptide ester terminating in D-Ala-D-Lac rather than a muramyl pentapeptide terminating in D-Ala-D-Ala (Bugg et al., 1991a,b; Handwerger et al., 1992). Vancomycin binds to such peptidoglycan termini and prevents subsequent transpeptidative cross-linking that yields cross-linked peptidoglycan. Reduction in cell wall cross-links reduces tensile strength and leaves the bacterium susceptible to osmotic lysis. The vancomycin affinity for *N*-acyl-D-Ala-D-Lac is 1000-fold less that for *N*-acyl-D-Ala-D-Ala (Bugg et al., 1991a,b), accounting for the 1000-fold difference in minimal inhibitory concentrations observed in resistant and sensitive entercocci (Arthur & Courvalin, 1993).

We have focused on the similarities and differences between the *Escherichia coli ddlB*-encoded D-Ala-D-Ala ligase (DdlB, eq 1) and the 26% homologous VanA that can activate both D-Lac and D-Ala as nucleophiles to yield both depsipeptide D-Ala-D-Lac and dipeptide D-Ala-D-Ala (eq 2). To understand how the ester bond in the depsipeptide product is formed by VanA, we have obtained a crystal structure of *E. coli* DdlB complexed with ADP and a phosphinophosphate transition-state analog (Fan et al., 1994) which has identified several active-site residues. We subsequently mutated several of these amino acids to assess predicted function in the various stages of the Ddl-catalyzed dipeptide-forming reaction, including a trio of residues, Y216, S150, and E15, that form a hydrogen-bonded network, schematized in Figure 1. The hydrogen bonds of this triad connect a mobile ω-loop containing Y216, via S150, and E15 to the binding of the α-amino group of the first molecule of substrate D-Ala (D-Ala$_1$) as it undergoes activation as the acyl phosphate D-alanyl phosphate. Three mutants that interrupt or modulate the hydrogen-bond network, Y216F, S150A, and E15Q of *E. coli* DdlB, all retained significant Ddl activity (Shi & Walsh, 1995) with k_{cat} values ranging from 38% to 110% of wild type, although K_m values for both the first and second D-Ala substrates were elevated 1−2 orders of magnitude from the wild-type K_m values of 1.2 μM (D-Ala$_1$) and 1.1 mM (D-Ala$_2$).

In this study we have analyzed these three *E. coli* DdlB mutants further and found that two of the mutants, Y216F

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[1] Abbreviations: D-Lac, D-lactate; Ac_2O, acetic anhydride; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; Ddl, D-Ala-D-Ala ligase; DL-Hbut, DL-hydroxybutyrate; DMAP, (*N*,*N*-dimethylamino)pyridine; EDTA, ethylenediaminetetraacetic acid; EtOAc, ethyl acetate; HEPES, *N*-(2-hydroxyethyl)piperazineethanesulfonic acid; HOBT·H_2O, 1-hydroxybenzotriazole hydrate; LDH, L-lactate dehydrogenase; MES, 2-(*N*-morpholino)ethanesulfonic acid; NADH, reduced nicotinamide adenine dinucleotide; NMM, *N*-methylmorpholine; PEP, phosphoenolpyruvate; PK, pyruvate kinase; PyBOP, benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane hydrochloride.

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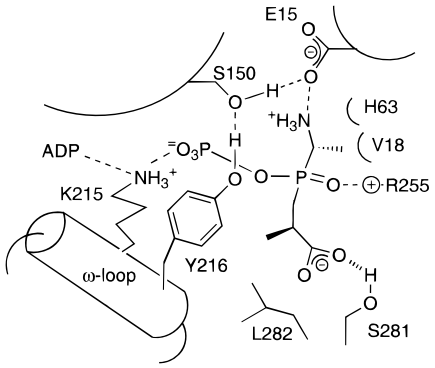


FIGURE 1: Schematic diagram of the residues around the D-Ala$_1$ and D-Ala$_2$ site of DdlB complexed with ADP and phosphinophosphate, a transition-state analog. Dashed lines indicate hydrogen-bond or electrostatic interactions.

and S150A, have gained VanA-type activity, the ability to make significant quantities of depsipeptide products D-Ala-D-Lac and D-Ala-D-hydroxybutyrate (Hbut) (eq 2b). The third *E. coli* Ddl mutant, E15Q, has gained a novel activity. It too can now activate D-Lac but as an electrophilic, not nucleophilic, substrate to make the regioisomer D-Lac-D-Ala, a novel amide product (eq 3b).

DdlB

$$\text{D-Ala} + \text{D-Ala} + \text{ATP} \longrightarrow \text{D-Ala}_1\text{-D-Ala}_2 + \text{ADP} + \text{P}_i \quad (1)$$

VanA, DdlB Y216F, and S150A

$$\text{D-Ala} + \text{D-Lac} + \text{ATP} \xrightarrow{\text{a}} \text{D-Ala}_1\text{-D-Ala}_2 + \text{ADP} + \text{P}_i \quad (2)$$
$$\text{D-Ala} + \text{D-Lac} + \text{ATP} \xrightarrow{\text{b}} \text{D-Ala-D-Lac} + \text{ADP} + \text{P}_i \quad (2)$$

E150Q DdlB

$$\text{D-Ala} + \text{D-Lac} + \text{ATP} \xrightarrow{\text{a}} \text{D-Ala}_1\text{-D-Ala}_2 + \text{ADP} + \text{P}_i \quad (3)$$
$$\text{D-Ala} + \text{D-Lac} + \text{ATP} \xrightarrow{\text{b}} \text{D-Lac-D-Ala} + \text{ADP} + \text{P}_i \quad (3)$$

MATERIALS AND METHODS

Materials. ATP, D-Ala, DL-Hbut, D-Lac, phosphoenolpyruvate (PEP), 2-(N-morpholino)ethanesulfonic acid (MES), 2-(*N*-cyclohexylamino)ethanesulfonic acid (CHES), *N*-(2-hydroxyethyl)piperazineethanesulfonic acid (HEPES), tris-(hydroxymethyl)aminomethane hydrochloride (Tris), and D-alanyl benzyl ester were purchased from Sigma. Reduced nicotinamide adenine dinucleotide (NADH), L-lactate dehydrogenase (LDH), and pyruvate kinase (PK) were from Boehringer Mannheim Biochemicals. D-[^{14}C]Ala and D-[^{14}C]-Lac were from American Radiolabeled Chemicals Inc. (St. Louis, MO) and thin-layer chromatography (TLC) cellulose sheets were from Kodak.

Cell Culture and Protein Purification. Cell culture and purification of wild-type DdlB, Y216F, S150A, E15Q, and VanA were performed as described previously (Shi & Walsh, 1995).

Enzyme Assay by TLC. The assay mixture included 0.2 mM D-[1-^{14}C]Ala (0.1 mCi/mL, 55 μCi/μmol), buffer (see below), 10 mM KCl, 10 mM $MgCl_2$, 10 mM ATP, additional unlabeled D-Ala, and D-Lac or DL-Hbut with enzyme ($\sim$10 μM) which was incubated at room temperature for various times. Three microliters of each sample was analyzed on a TLC cellulose plate as described previously (Bugg et al., 1991a,b). To quantitate the amount of each product, the dried TLC plate was exposed to Storage Phosphor Screen system (Molecular Dynamics, Sunnyvale, CA) and the resulting image was analyzed by Image Quant program (Molecular Dynamics, Sunnyvale, CA). Because radiolabeled D-[^{14}C]Ala-D-[^{14}C]Ala dipeptide contains two radioactive labels, the observed values were divided by 2 to calculate the amount of product formed.

Enzyme Assay by Coupled ADP Release. The ADP release coupled assay (Daub et al., 1988) was also used to measure the kinetic parameters of DdlB and VanA enzymes. The reaction mixture was composed of buffer (see below), 10 mM KCl, 10 mM $MgCl_2$, 10 mM ATP, 2.5 mM PEP, 0.15−0.2 mM NADH, 50 units/mL LDH, 50 units/mL PK, D-Ala, and D-Lac. The assay was initiated by adding enzymes at 37 °C and monitored at 340 nm.

Enzyme Activity Dependent on pH. A composite buffer system containing MES, HEPES, Tris, and CHES (50 mM each) was used for reactions in which pH range studies were conducted (Figures 3 and 4; in Figure 7, wild-type result). In all other cases, either MES, HEPES, Tris, or CHES buffer at 100 mM was used for pH 6−6.5, 7.5−7.9, 8.3−8.6, and 9.2, respectively. In the ADP coupled assay, saturating amounts of LDH and PK were determined and included in each pH condition.

Kinetic Analysis. The kinetic analysis was carried out as described previously (Neuhaus, 1962a,b; Segel, 1975; Zawadzke et al., 1991). The basic equation for D-Ala-D-Ala ligase (eq 5 or eq 6 in its reciprocal form) was derived on the basis of the steady-state kinetics for two identical substrate molecules of D-Ala (eq 4):

$$\text{E} + \text{S} \overset{K_1}{\rightleftharpoons} \text{ES} + \text{S} \overset{K_2}{\rightleftharpoons} \text{ES}_2 \xrightarrow{k_{cat}} \text{E} + \text{P} \quad (4)$$

$$V = \frac{V_{max}[\text{S}]^2}{K_1K_2 + K_2[\text{S}] + [\text{S}]^2} \quad (5)$$

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_2}{V_{max}}\frac{1}{[\text{S}]} + \frac{K_1K_2}{V_{max}}\frac{1}{[\text{S}]^2} \quad (6)$$

In a case of $K_1 \ll [\text{S}]$, eq 7 was used instead of eq 6 because the last term becomes very small compared to the second term.

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_2}{V_{max}}\frac{1}{[\text{S}]} \quad (7)$$

For D-Ala-D-Lac ligase activity, eqs 9 and 10 were used, based on eq 8, where S_1 and S_2 are D-Ala and D-Lac, respectively. D-Ala-D-Lac ligase activities were measured by ADP release coupled assay. Because that method cannot discriminate D-Ala-D-Lac ligase activity from D-Ala-D-Ala ligase activity (paths a and b in eq 2), the following corrections were employed. The velocity value obtained from observed activity minus D-Ala-D-Ala ligase activity in the absence of D-Lac was regarded as D-Ala-D-Lac ligase activity if D-Ala-D-Ala ligase activity is less than 10% of the maximum observed activity (e.g., at pH 6.0 of S150A and Y216F in Table 1 and Figure 6 and at pH 6.0, 6.5, and 7.5 of VanA in Table 1 and Figure 7). If the D-Ala-D-Ala flux exceeds 10%, the D-Ala-D-Lac ligase flux (path b of eq 2) was obtained from total activity minus D-Ala-D-Ala ligase activity multiplied by an inhibition factor measured at each concentration of D-Lac. In the presence of high amounts of D-Ala, equal to K_m for D-Ala$_2$, at pH 7.5 in the case of S150A

and Y216F and at pH 8.3 in VanA, the flux of D-Ala-D-Ala ligase activity greatly exceeds the D-Ala-D-Lac activity flux by raw values of 980/3.4 (S150A), 630/4.2 (Y216F), and 900/67 (VanA) (see Table 1). Thus flux via the bottom arm (path b) of eq 2 is minuscule and D-Lac, as a low V_{max} substrate, acts as an inhibitor of total flux (data not shown). We have used the percent inhibition of flux to D-Ala-D-Ala at a given concentration of D-Lac to correct what otherwise would be an underestimate of flux to D-Ala-D-Lac from ADP formation rates. For example, the corrections were about 6% at pH 6.0 and 17% at pH 7.5 both for S150A (at pH 7.5, k_{cat} adjustment from apparent 2.9 min^{-1} to 3.4 min^{-1}) and for Y216F (at pH 7.5, k_{cat} adjustment from apparent 36 min^{-1} to 42 min^{-1}). The corrections for VanA are <5% except at pH 8.3, where correction from an apparent k_{cat} of 58 min^{-1} to 67 min^{-1} resulted. The corrections also apply to K_m and k_{cat}/k_m estimates.

$$E + S_1 \overset{K_1}{\rightleftharpoons} ES_1 + S_2 \overset{K_2}{\rightleftharpoons} ES_1S_2 \xrightarrow{k_{cat}} E + P \qquad (8)$$

$$V = \frac{V_{max}[S_1][S_2]}{K_1K_2 + K_2[S_1] + [S_1][S_2]} \qquad (9)$$

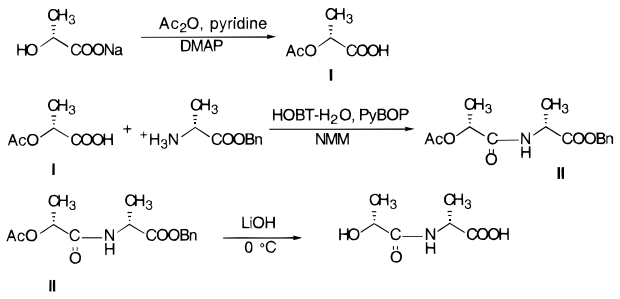
$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_2}{V_{max}}\frac{1}{[S_2]} + \frac{K_1K_2}{V_{max}}\frac{1}{[S_1][S_2]} \qquad (10)$$

Synthesis of Authentic D-*Lac*-D-*Ala*. Sodium D-lactate (376 mg, 3.36 mmol) was acetylated with acetic anhydride (Ac_2O, 15 mL), pyridine (15 mL), and a catalytic amount of (*N*,*N*-dimethylamino)pyridine (DMAP) at room temperature. The reaction was monitored by TLC with ethyl acetate (EtOAc)−hexane (4:1) and a phosphomolybdic acid stain solution. After 6 h, the reaction solution was evaporated to give a dry residue which was partitioned between EtOAc and 0.1 M HCl. The organic phase was washed three times with 0.1 M HCl and four times with saturated NaCl, dried over anhydrous $MgSO_4$, and concentrated. The product (compound **I**) was used in the next step without further purification.

Compound **I** (3.36 mmol), D-alanyl benzyl ester (*p*-toluenesulfonate salt, 1.18 g, 3.36 mmol), 1-hydroxybenzotriazole hydrate (HOBT·H_2O, 499 mg, 3.69 mmol), benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBOP, 1.92 g, 3.69 mmol), and *N*-methylmorpholine (NMM, 0.85 mL, 7.72 mmol) were dissolved in DMF (2 mL) and dichloromethane (25 mL) with stirring. The solution was stirred at room temperature overnight. After the solvent was removed by rotary evaporation under high vacuum, the residue was redissolved in EtOAc. The resulting solution was washed with 0.5 M H_2SO_4, saturated $NaHCO_3$, and saturated NaCl, dried over anhydrous $MgSO_4$, and freed of solvent. The resulting residue was chromatographed on a silica gel column with EtOAc−hexane (1:2) to give 817 mg of the product **II** (83%).

An aqueous solution of LiOH was added dropwise to a solution containing compound **II** (300 mg, 1.02 mmol) in methanol−H_2O (3:1, 20 mL) at 0 °C. From TLC analysis [with $CHCl_3$−methanol (1:1) or 1-propanol−acetate−H_2O (10:1:2)], the reaction was complete within 30 min. Amberlite IRC-50 [H^+] resin was added to adjust the solution to pH 4, the resin was filtered off, and the filtrate was concentrated. The product was purified with Sephadex G-10 chromatography using water as eluent to give 150 mg of the final product D-Lac-D-Ala (91%). ^1H NMR, field = 500 MHz (D_2O, HOD = 4.67 ppm) δ 4.307 (1 H, quartet, J = 7.0 Hz), 4.195 (1 H, quartet, J = 7.0 Hz), 1.334 (3 H, d, J = 7.0 Hz), and 1.258 (3 H, d, J = 7.0 Hz).

Scheme 1



HPLC, NMR, and Mass Spectrometry. A DdlB E15Q reaction mixture (5 mL) contained 100 mM Tris, pH 8.3, 10 mM KCl, 3 mM ATP, 25 mM D-Lac, 0.2 mM D-Ala, and 1 μM enzyme. After reaction for ~6 h at 30 °C, 0.2 mM D-Ala, 1 μM enzyme, and 7 mM ATP were further added and the mixture was incubated for an additional 16 h. The mixture was applied to a 0.1% trifluoroacetic acid-equilibrated HPLC reversed-phase column (C_{18}, 250 × 21.5 mm, Bio-Rad). Isocratic elution with the same solvent system at a speed of 8 mL/min eluted a peak whose retention time corresponds to that of authentic D-Lac-D-Ala (24 min). When the purified product was coinjected with authentic D-Lac-D-Ala into HPLC, only a single peak was observed. The peak was collected, dried, and dissolved in D_2O for ^1H NMR (Varian VXR-5005) analysis. The enzymatic product as well as a mixture containing both the enzymatic product and authentic D-Lac-D-Ala had the same ^1H NMR signals as authentic D-Lac-D-Ala. The purified product was also analyzed by positive electrospray mass spectrometry (ammonia CI-MS) at Harvard Microchemistry Facility: unit mass calculated for $C_6H_{12}NO_4$ ($M + H^+$) 162, found 162; calculated for $C_6H_{15}N_2O_4$ ($M + NH_4^+$) 179, found 179.

Protein Quantitation. Protein concentrations were determined by the method of Bradford with bovine serum albumin as standard (Bradford, 1976).

RESULTS

E. coli DdlB Mutants Y216F and S150A Have Gained Depsipeptide Ligase Activity. The initial determination that VanA had the ability to activate D-hydroxy acids such as D-Lac and D-Hbut and couple them to D-Ala (eq 2) utilized D-[^{14}C]Ala as labeled substrate and a TLC assay to separate labeled substrate, dipeptide, and depsipeptide products (Bugg et al., 1991a,b). We have regularly used D-Hbut, available commercially as DL-Hbut, because of superior resolution, in comparison to D-[^{14}C]Ala-D-Lac, of the D-[^{14}C]Ala-D-Hbut from D-[^{14}C]Ala-D-[^{14}C]Ala. The autoradiogram of Figure 2 shows the unanticipated result that Y216F and S150A mutants of *E. coli* DdlB form D-[^{14}C]Ala-D-Hbut and behave like VanA-type catalysts under conditions where wild-type DdlB makes only D-Ala-D-Ala. As shown in Figure 2, VanA makes predominantly the depsipeptide product (eq 2) under these pH conditions (pH 7.8), while DdlB Y216F and S150A make both dipeptide and depsipeptide products.

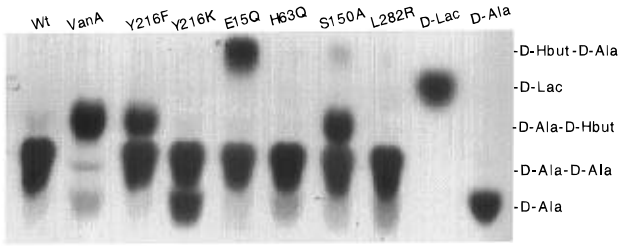


FIGURE 2: Enzymatic analysis of DdlB wild-type (Wt) and mutant proteins and VanA by TLC methods. The reaction mixtures (pH 7.8) including 0.2 mM D-[^{14}C]Ala and 50 mM DL-Hbut (see Materials and Methods for concentrations of other compounds) were incubated at room temperature for 16 h. The products were visualized by autoradiography after TLC.

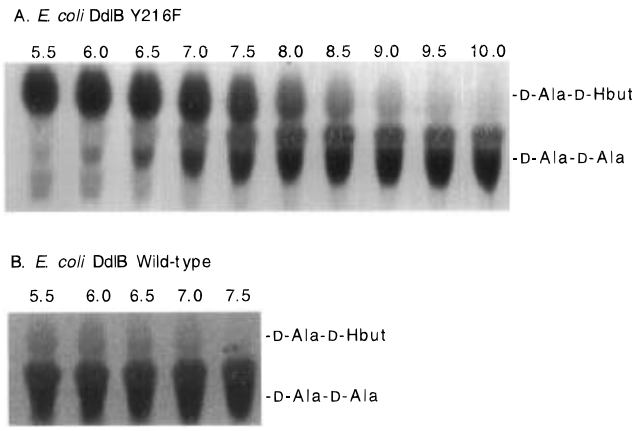


FIGURE 3: Product partitioning data of D-Ala-D-Ala and D-Ala-D-Hbut ligase activities of (A) DdlB Y216F and (B) wild-type DdlB enzymes at different pHs. Reaction and TLC were performed as in Figure 2. The numbers 5.5−10.0 are the pH values of each incubation.

pH-Dependent Partitioning between Dipeptide and Depsipeptide Formation. Figure 3 shows autoradiograms for the Y216F DdlB mutant that established a clear pH-dependent partitioning between the preferred depsipeptide product at low pH and the dipeptide product at high pH. This product switching was quantitated over the pH range 5.5−10 for Y216F, S150A, VanA, and wild-type *E. coli* DdlB and is displayed in Figure 4, panels A−D, respectively. In this set of experiments, D-[^{14}C]Ala was held at 0.2 mM for the DdlB (10 mM for VanA) and DL-Hbut at 50 mM (10 mM for VanA) to maximize the chance for D-Hbut to outcompete D-Ala$_2$ and observe the depsipeptide catalytic capacity.

VanA (and related VanB; Meziane-Cherif et al., 1994) has been the only case heretofore of an ATP-dependent depsipeptide ligase. Figure 4C shows that under the given conditions VanA produces a 1:1 ratio of D-Ala-D-Hbut/D-Ala-D-Ala at pH 7.5. At more acidic pH, the depsipeptide predominates such that at pH 6, the corresponding optimum for its formation, it is the sole product. The pH optimum for dipeptide D-Ala-D-Ala formation by VanA is pH 9 where there is still about 20% depsipeptide formation.

The VanA-like pH-dependent product partitioning of dipeptide/depsipeptide was followed by both Y216F and S150A mutants of *E. coli* DdlB as seen in Figure 4A,B. Again at pH 6 the D-Ala-D-Hbut depsipeptide is the sole product. The amount of depsipeptide falls in both absolute and relative terms, again with a midpoint of pH 7.5, for both Y216F and S150A (at substrate concentrations of 0.2 mM D-Ala and 50 mM DL-Hbut). The D-Ala-D-Ala fraction rises

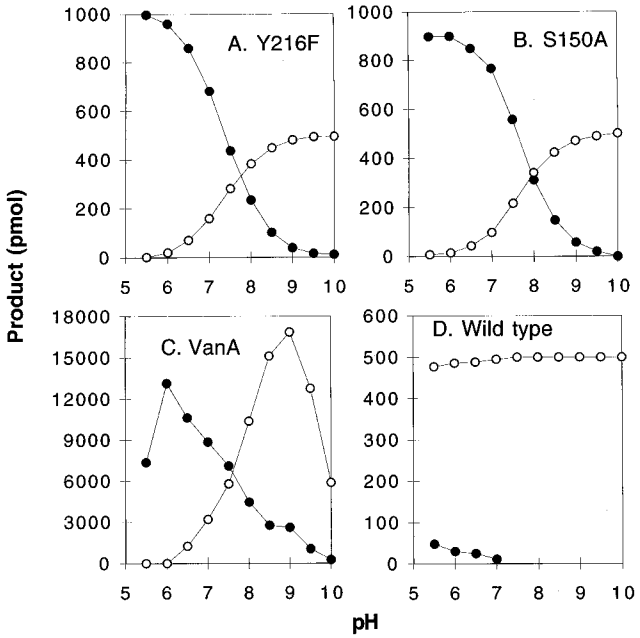


FIGURE 4: Partitioning of D-Ala-D-Ala (open circles) and D-Ala-D-Hbut (closed circles) ligase activities of Y216F (A), S150A (B), and wild-type DdlB (D) and of VanA (C) at different pHs. The reactions were carried out as in Figure 2 except panel C, in which 10 mM DL-Hbut and 10 mM D-Ala were used in order to visualize the partitioning clearly. After TLC, the amounts of product were evaluated as described in Materials and Methods.

from undetectable levels at pH 6, to about 50% at pH 7.5−8, to be the sole product by pH 10.

Given the profiles of DdlB Y216F and S150A, we were led to explore the pH-dependent ability of wild-type *E. coli* DdlB to generate any detectable D-Ala-D-Hbut depsipeptide at the low pH extreme. Figures 3B and 4D reveal that indeed wild-type DdlB possesses the intrinsic, albeit weak, ability to make depsipeptide in the range pH 5.5−6.5. At pH 6 depsipeptide is about 6% of the flux; at pH 5.5, about 10%. For comparison, VanA makes only depsipeptide at these pHs. At pH 7.0 DdlB makes 2% D-Ala-D-Hbut while VanA makes almost 100% under the same conditions (data not shown) for about a 50-fold depsipeptide/dipeptide ratio difference.

Dependence of Depsipeptide/Dipeptide Partitioning on D-Hbut. To assess the recognition of D-Hbut vs D-Ala (D-Ala$_2$, eq 2) as the nucleophilic cosubstrate for Y216F and S150A DdlB mutants, the dependence of dipeptide/depsipeptide product on D-Hbut concentration was also evaluated. Figure 5 shows TLC-derived rate data for Y216F at pH 6, where D-Ala-D-Hbut is the sole product, and at pH 7.5, where D-Ala-D-Hbut/D-Ala-D-Ala is 1/1. An initial estimate of EC_{50} for D-Hbut is 20−40 mM from such data.

Catalytic Efficiency for Dipeptide vs Depsipeptide Formation. The TLC experiments described to this point were designed to maximize detection of depsipeptide production capacity by utilizing the fact that K_m for D-Ala$_1$ in wild-type DdlB is ca. 1000-fold lower than K_m for D-Ala$_2$ (3 μM vs 2 mM at pH 7.5; see Table 1), permitting use of a concentration (0.2 mM) of D-Ala that populated the D-Ala$_1$ subsite but left the D-Ala$_2$ subsite available for competition between D-Ala$_2$ and D-Hbut or D-Lac. To obtain a more quantitative measure of VanA and the DdlB mutants to discriminate among these competitions at the D-Ala$_2$ subsite, we turned to a continuous spectrophotometric assay, coupling ADP production via pyruvate kinase and L-Lac dehydrogenase to NADH forma-

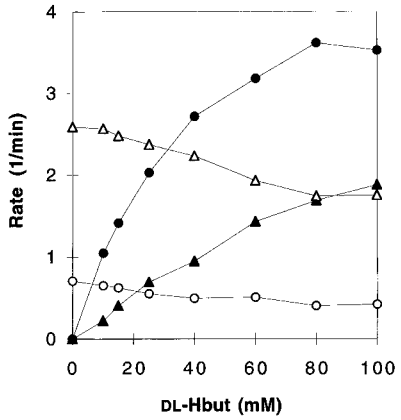


FIGURE 5: D-Ala-D-Ala (open symbols) and D-Ala-D-Hbut (closed symbols) ligase activities of Y216F DdlB. The reactions were performed as in Figure 2 at pH 6 (circles) and 7.5 (triangles) with varying concentrations of DL-Hbut. After TLC, the amounts of product were evaluated as described in Materials and Methods.

Table 1: Kinetic Parameters of DdlB and VanA Proteins[a]

protein	activity	pH	K_{m1}[b] (mM)	K_{m2}[c] (mM)	k_{cat} (min^{-1})	k_{cat}/K_{m2} (mM^{-1} min^{-1})
DdlB Wt	A-A[d]	6.0	0.11	8.0	710	89
		7.5	0.003	2.0	880	440
		9.2	0.004	1.1	1600	1500
S150A	A-A[d]	6.0	6.2	140	200	1.4
		7.5	0.85	80	980	12
		9.2	0.26	44	1100	26
	A-L[e]	6.0	ND[f]	1.6	1.4	0.87
		7.5	ND	2.2	3.4	1.5
	A-H[g]	6.0	ND	5.3	1.1	0.21
Y216F	A-A[d]	6.0	0.26	150	450	3.0
		7.5	0.33	16	630	39
		9.2	0.17	4.8	1200	260
	A-L[e]	6.0	ND	5.1	7.8	1.5
		7.5	ND	27	42	1.6
	A-H[g]	6.0	ND	8.9	5.3	0.60
		7.5	ND	40	6.2	0.16
VanA	A-A[d]	6.4	ND	$\gg$100[h]	ND	0.14
		7.5	0.10	$\gg$100[h]	940	1.7
		7.9	0.37	260	940	3.6
		8.3	0.31	69	900	13
		8.6	0.67	74	1000	14
		9.2	0.21	47	1100	24
	A-L[e]	6.0	4.9	1.3	39	30
		6.5	1.9	1.1	40	38
		7.5	0.23	0.88	45	51
		8.3	ND	1.5	67	45
E15Q	A-A[d]	6.0	11	82	200	2.4
		7.5	0.51	74	820	11
		9.2	0.51	65	1100	17

[a] Measured by ADP coupled assay except *g*. [b] K_m for D-Ala_1. [c] K_m for D-Ala_2 or D-Lac or D-Hbut activity at subsite 2. [d] D-Ala-D-Ala ligase. [e] D-Ala-D-Lac ligase; see Materials and Methods for calculations. [f] ND, not determined. [g] D-Ala-D-Hbut ligase, measured by TLC method. [h] The highest concentration of D-Ala used.

tion to obtain initial rates, K_m values for D-Ala_2 or D-hydroxy acids (K_{m2} in Table 1), and k_{cat} values. The data of Figure 6 show the dependence of ADP production on D-Lac by *E. coli* DdlB Y216F at two different pHs, 6 and 7.5. In each instance, the filled symbols represent the observed activity and the open symbols are the values corrected for flux to D-Ala-D-Ala as noted in the Kinetic Analysis section under Materials and Methods. Double-reciprocal plots (not shown) yield k_{cat} values, 8 min^{-1} at pH 6 and 42 min^{-1} at pH 7.5, and K_m values, K_{m2} (for D-Lac), of 5 mM and 27 mM, listed in Table 1.[2]

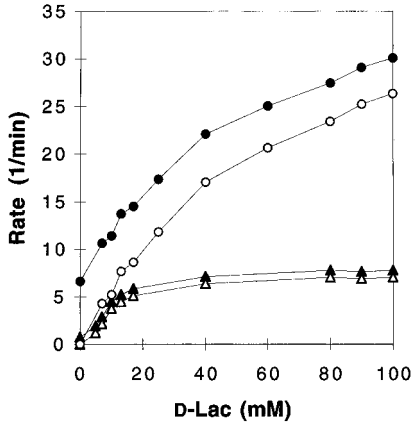


FIGURE 6: D-Ala-D-Ala and D-Ala-D-Lac ligase activities of Y216F at pH 6.0 (triangles) and pH 7.5 (circles). The reaction mixture contains 0.25 mM D-Ala and varying concentrations of D-Lac. The activities were measured at 37 °C by ADP coupled assay. D-Ala-D-Lac ligase activities (open symbols) were calculated as in Materials and Methods from observed activities (closed symbols), with correction from reductions in flux to D-Ala-D-Ala as D-lactate shunts intermediate by path b of eq 2 as described in Materials and Methods.

Comparable studies with wild-type *E. coli* DdlB (Table 1) show k_{cat} for dipeptide formation varies only 2-fold from pH 6 to 9.2 (710 to 1600 min^{-1}), while K_{m1} (D-Ala_1) is some 30−40-fold higher at lower pH. K_{m2} becomes 4−8-fold worse, at lower pH, leading to a k_{cat}/K_{m2} variation of 16-fold over the pH range. For the Y216F mutant at pH 7.5, recognition of the nucleophilic substrates D-Ala_2 and D-Lac, the K_{m2} values for D-Ala (16 mM) and D-Lac (27 mM) are about equivalent, while k_{cat} values favor dipeptide over depsipeptide by 18-fold (630 min^{-1}/42 min^{-1}) for a catalytic efficiency difference, k_{cat}/K_{m2}, of ∼15/1 for dipeptide/depsipeptide product. At pH 6.0, the D-Ala_2 K_m worsens 9-fold, where the K_m for D-Lac improved 5-fold;[2] the k_{cat} ratio still favors dipeptide by 50/1 but now k_{cat}/K_{m2} of 3/1.5 shows that, at acidic pH, Y216F has only 2-fold greater catalytic efficiency in dipeptide than depsipeptide synthesis.

The S150A DdlB enzyme at pH 7.5 has a 50-fold lower K_{m2} for D-Lac than D-Ala (2.2 mM/80 mM). As with Y216F, k_{cat} favors dipeptide synthesis (980 min^{-1} vs 3 min^{-1}) to yield a k_{cat}/K_{m2} catalytic efficiency ratio of 8/1 for dipeptide/depsipeptide. A comparison of DdlB S150A with the VanA enzyme (at pH 8.3) shows a very similar K_{m2} discrimination in favor of D-Lac (1.5 mM/69 mM). Again, the k_{cat} data show that even VanA has a higher k_{cat} for dipeptide formation over depsipeptide (900 min^{-1}/67 min^{-1}). By k_{cat}/K_{m2} ratio, though, VanA is a better depsipeptide than dipeptide synthetase by a ratio of 3.5/1 (45 mM^{-1} min^{-1}/13 mM^{-1} min^{-1}). Analysis of the pH dependence of log (k_{cat}/K_{m2}) for VanA vs DdlB, Figure 7, shows that the VanA depsipeptide ligase catalytic efficiency is invariant over pH 6−8 while its dipeptide ligase k_{cat}/K_{m2} increases with a slope of 1. The DdlB data on Figure 7 show a similar pH trend but less clearly defined.

The E15Q E. coli DdlB Mutant Can Activate D*-Hydroxy Acids as Electrophilic Substrates.* When the E15Q *E. coli* DdlB was analyzed in the TLC assay with D-[^{14}C]Ala and

[2] We have not devised an assay to determine whether D-Lac competes with D-Ala_1. However, we do not see any D-Lac-D-Lac product (see footnote 3).

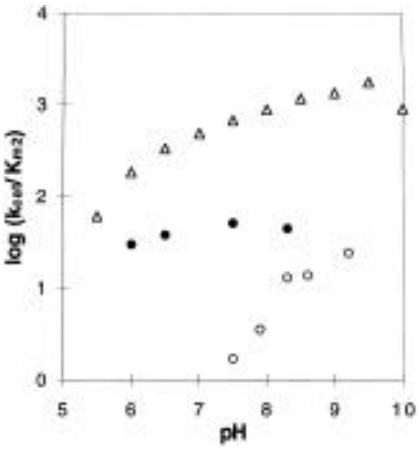


FIGURE 7: pH dependence of D-Ala-D-Ala ligase activity (open triangles) of wild-type DdlB and of D-Ala-D-Ala ligase (open circles) and D-Ala-D-Lac ligase (closed circles) activities of VanA. The reactions were analyzed by ADP coupled assay. For D-Ala-D-Lac ligase activity of VanA, a saturating amount of D-Ala (20 mM at pH 6.0, 10 mM at pH 6.5, 2 mM at pH 7.5, and 1 mM at pH 8.3) was included in the reaction mixture. The observed values were corrected from reductions in flux to D-Ala-D-Ala as D-lactate shunts intermediate by path b of eq 2 as described in Materials and Methods.

DL-Hbut (or D-Lac in other experiments), a new radioactive product spot of higher mobility was detected as shown in Figure 2. Given that the new product was not D-Ala-D-Ala or D-Ala-D-Hbut by TLC mobility, yet it required ATP, E15Q enzyme, and D-Hbut for its formation, it seemed highly likely that the new product was D-Hbut-D-Ala, an amide rather than an ester. Subsequent characterization was performed in experiments using D-Lac rather than D-Hbut to compare a C_3 substrate rather than a C_4 hydroxy acid to a C_3 amino acid. Authentic D-Lac-D-Ala was synthesized to produce a standard which migrated on TLC and HPLC identically with the E15Q reaction product. More convincingly, a large-scale E15Q incubation gave the novel amide product that had the characteristic ^{1}H NMR signals (data not shown) of the authentic D-Lac-D-Ala regioisomer (eq 3b). Only the E15Q mutant has the capacity to make this novel amide and by TLC analysis it makes no detectable ester product D-Ala-D-Hbut. It does still produce D-Ala-D-Ala in competition with D-Lac-D-Ala (eq 3a). The pH dependence of E15Q, shown in Figure 8, is distinct from the common pattern of VanA, S150A DdlB, and Y216F DdlB (Figure 4) in having no significant alternate product activity at low pH. At a substrate concentration of 0.2 mM D-Ala and 50 mM D-Lac, the D-Lac-D-Ala and D-Ala-D-Ala products rise in parallel from pH 5.5 to 6.5. Then D-Ala-D-Ala production plateaus while D-Lac-D-Ala rises until pH 8 and plateaus with a partition ratio of about 2/1 in favor of the novel amide product. Total product formation as a function of added D-Lac is constant at pH 6 but still increases at pH 9 up to 100 mM D-Lac, indicating a low affinity for hydroxy acid (data not shown).

DISCUSSION

A key molecular switch that underlies the shift from vancomycin sensitivity to vancomycin resistance in Gram-positive bacteria is the expression of VanA (Bugg et al., 1991a,b; Messer & Reynolds, 1992), an enzyme homologue of the D-Ala-D-Ala dipeptide-forming ligase family, which has gained the capacity to activate D-Lac as nucleophilic

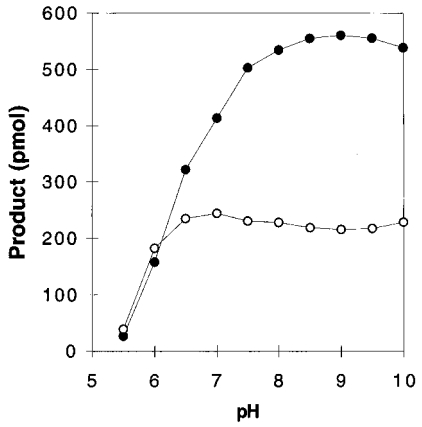


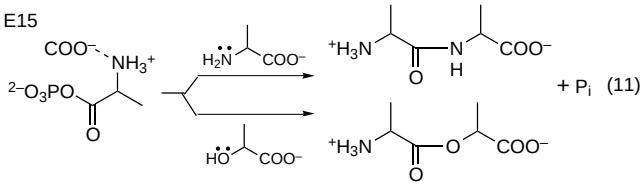
FIGURE 8: Partitioning of D-Ala-D-Ala (open circles) and D-Lac-D-Ala (closed circles) ligase activities of E15Q at different pHs. The reaction was carried out as in Figure 2 except 50 mM D-Lac was used in place of DL-Hbut. After TLC, the amounts of product were evaluated as described in Materials and Methods.

cosubstrate to yield D-Ala-D-Lac in competition with D-Ala-D-Ala product (eq 2). In a bacterial cell also expressing VanX, a D-Ala-D-Ala dipeptidase (Wu et al., 1995), only D-Ala-D-Lac accumulates and is carried forward for ligation with a UDP-muramyl tripeptide. The muramyl tetrapeptide esters so generated are incorporated at peptidoglycan termini and show three log units lower affinity for vancomycin binding.

With the solution of the three-dimensional structure of *E. coli* DdlB by X-ray crystallography of an enzyme−ADP−phosphinophosphate analog complex (Fan et al., 1994), we have been able to identify active-site residues and to test predicted function by mutagenesis (Shi & Walsh, 1995). Prime among the questions has been what distinguishes Ddl (dipeptide synthetase) and VanA (depsipeptide synthetase). We previously noted a triad of Y216, S150, and E15 (Figure 1) that may help to stabilize the flexible ω-loop closure over the active site and to bring K215, adjacent to Y216 on the loop, into the correct distance and orientation to facilitate γ-PO_3 transfer from ATP to the bound D-Ala_1 COO^- to yield the D-alanyl-PO_3 acyl phosphate intermediate. E15 is in hydrogen-bonding distance to the α-NH_3^+ of D-Ala_1, suggesting an additional substrate-orienting role of this hydrogen-bonded triad. The E15 and S150 residues of DdlB are conserved at equivalent loci in VanA, but Y216 is replaced by a K216 equivalent (in DdlB numbering). When we looked at Y216K and Y216F mutants of DdlB, they still retained dipeptide ligase activity.

In the work reported here, we have more closely examined Y216F and found a substantial gain of VanA-type depsipeptide ligase activity. Likewise, the S150A mutant had such ester synthetase activity. The E15Q mutant did not have ester synthetase activity but it had actually gained yet another variant activity, D-Lac-D-Ala synthetase activity. A close scrutiny of wild-type *E. coli* DdlB at acidic pH now revealed that it too has a very low but intrinsic depsipeptide ligase activity unmasked at pH values below 7.0. Thus, VanA, Y216F, and S150A Ddl mutants and even wild-type *E. coli* DdlB can all be analyzed in one reaction manifold. They all show D-Ala-D-Hbut (or D-Ala-D-Lac) depsipeptide ligase activity with an acid pH optimum. In VanA the pH optimum is 6 with a broad tail out to basic pH such that under the experimental conditions of Figure 4 (10 mM D-Ala and 10 mM D-Hbut) 20% of the reaction flux at pH 9 is still to make

depsipeptide. In turn, VanA makes no D-Ala-D-Ala dipeptide at pH 6, but formation of that product is optimized at pH 9, where it is 80% of the flux. For comparison, wild-type DdlB makes no detectable depsipeptide at pH 7.5 and above. Flux is only detectable at lower pH, and at pH 6 no more than 3−10% of the catalytic flux is through the lower arm of the partition in eq 11. There will be no physiological significance to this minor catalytic capacity of DdlB, but it may have profound mechanistic consequences in understanding how a Ddl could evolve to a VanA.



Hints to that end are provided by the dramatically more robust depsipeptide ligase activities that have been gained by the two single mutants of *E. coli* DdlB, Y216F and S150A, mutants that each disrupt the hydrogen-bonding triad of Y216−S150−E15. Disruption of the triad rather than a specific gain of function in Y216F or S150A may be the key event, since VanA has S150 and E15 but a K rather than a Y at 216. The pH profiles of Y216F and S150A DdlB mutants closely mirror that of VanA in the pH-dependent partitioning of flux through the upper and lower arms of eq 11 and a crossover pH of 7.5−8 under conditions as described in Figure 4.

The data both in Table 1 and in Figure 7 show that the dipeptide ligase activities of both Ddl and VanA are sensitive to pH, with log k_{cat}/K_{m2} rising as pH increases. The pH 7.5 vs pH 6 (or pH 6.4 for VanA) k_{cat}/K_{m2} comparisons show catalytic efficiency increases of 5-, 8-, 12-, and 12-fold for Ddl wt, S150A, Y216F, and VanA, respectively. This trend may reflect a disfavored productive binding of D-Ala_2 at lower pH where its required nucleophilic amino group will be as the NH_3^+ species. By contrast, the log k_{cat}/K_{m2} for depsipeptide ligase activity vs pH is much less pronounced for VanA (Table 1 and Figure 7) and for Y216F and S150A mutants of Ddl (Table 1). The pH-related changes in partitioning that favor D-Ala-D-lac formation at lower pH may thus largely be controlled by changes in the protonation state of D-Ala_2 vs D-Lac. In Y216F, S150A, and even VanA, the k_{cat} data of Table 1 show that D-Ala_2 addition is always faster than D-Lac addition, by factors of 15, 290, and 20, respectively, suggesting that the chemical step of cosubstrate nucleophilic capture of the acyl phosphate (eq 11) may be rate-determining and reflect the intrinsic nucleophilicity differences of RNH_2 vs ROH. This will require a rapid kinetic method to sort out rate- and product-determining steps, including features that yield K_{m2} values favoring D-Lac over D-Ala_2 by ratios of 25−50/1.

A separate correlation regarding the importance of Y216 vs F216 (*E. coli* DdlB numbering) in dipeptide vs depsipeptide ligase capability is noted by sequence comparisons in Table 2. Both *E. coli* DdlA and DdlB are dipeptide-only ligases, while *E. faecalis* and *L. leichmannii* are vancomycin-sensitive organisms (Ruoff et al., 1988), implying that their Ddls will not have the capacity to make depsipeptides. All these four examples have a Y216. The next four Ddl sequences from PCR analyses are from vancomycin-resistant

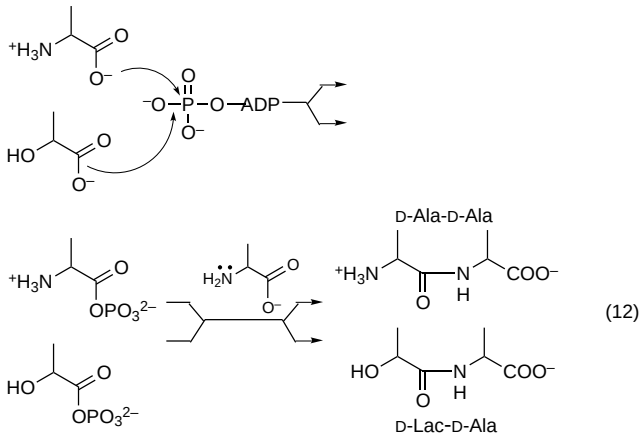
Table 2: Sequence Comparison of ω-Loop Regions of Ddl Proteins[a]

protein	sequence	vanco-mycin
	210[b] 215 220 225	
En. faecalis Ddl	FYDYDAK**Y**INNTIEMQI	sensitive
E.coli DdlA	FYAYDTK**Y**IDEDGAKVV	sensitive
E.coli DdlB	FYDYEAK**Y**LSDETQYFC	sensitive
La. leichmannii BM4285 Ddl	WYSYENK**Y**STESTTTLQ	sensitive
La. confusus CIP102578 Ddl	WYDYNNK**F**VDASGMVFE	resistant
La. salivarius CIP103140T Ddl	FYDYNNK**F**VDASGVVFE	resistant
La. plantarum CIPA159 Ddl	FYDYNNM**F**VDASGVTFE	resistant
Lw. CIP16407 Ddl	WYDYNNK**F**VDNSAVHFE	resistant
VanC	FFDFEEK**Y**QLISATITV	resistant

[a] Elisha & Courvalin (1995). [b] Amino acid residue numbering based on *E. coli* DdlB.

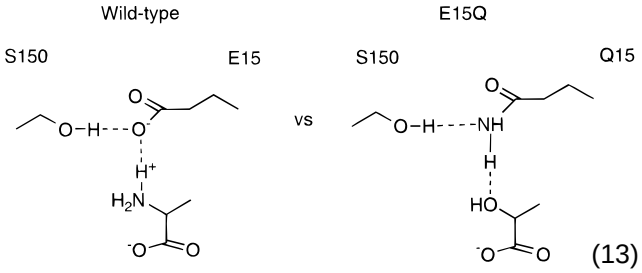
Gram-positives, all of which have F216 and are presumed to have depsipeptide ligase capacity since their peptidoglycan intermediates have D-Ala-D-Lac termini (Billot-Klein et al., 1994; Handwerger et al., 1994). Y216F mutants in Ddls could be a signature for D-Ala-D-Lac synthetase activity. VanC has a Y216 and makes a peptidoglycan intermediate containing the terminal dipeptide D-Ala-D-Ser (Reynolds et al., 1994), not D-Ala-D-Lac.

While Y216F and S150A make the ester D-Ala-D-Lac, they do not make the other regioisomeric product, the amide D-Lac-D-Ala, but uniquely E15Q does (eq 3) while failing to make ester. That is, while D-Lac and D-Ala_2 compete for capture of D-alanyl-P (eq 11) in VanA, Y216F, S150A, and even (very poorly) in wild-type DdlB, it is D-Lac and D-Ala_1 that compete in E15Q. Now this competition in D-Ala subsite 1 yields two alternate acyl phosphates, the normal D-alanyl-P and the hydroxyacylphosphate D-Lac-P, as D-Lac now competes as the electrophilic, not the nucleophilic, partner in the condensation. Only D-Ala is bound productively in subsite 2 (eq 12).[3]



This reversal of regiospecificity is understandable given the X-ray prediction that the γ-COO^- of E15 is in hydrogen-bonding distance to the α-NH_3^+ of D-Ala_1. Replacement of the γ-COO^- of E15 by the γ-$CONH_2$ of Q15 would maintain hydrogen-bonding but not the charge attraction for the NH_3^+ of D-Ala_1. Instead the HO of D-Lac could be relatively favored for coordination to Q15 as hypothesized in eq 13.

[3] In fact, there is a very slow but real D-Lac-driven ATPase activity found only in the E15Q mutant, at 10^{-3} the k_{cat}/K_{m2} for D-Ala-D-Ala (11 mM^{-1} min^{-1} vs 0.01 mM^{-1} min^{-1}), but we have not been able to detect D-Lac-D-Lac product.



(13)

In summary, mutations in a hydrogen-bonding triad of active-site residues of D-Ala-D-Ala ligase on the one hand relax selectivity with gain of function to activate the C_3 and C_4 D-hydroxy acids and on the other hand create an exclusive regiospecificity to utilize the D-Lac either as a nucleophilic cosubstrate (Y216F and S150A) or as an electrophilic cosubstrate (E15Q) in condensation with D-Ala, leading to mutually exclusive ester or amide products. These results suggest a path for acquisition of vancomycin resistance-type depsipeptide synthetase activity from dipeptide ligases.

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