D-Alanyl-D-Lactate and D-Alanyl-D-Alanine Synthesis by D-Alanyl-D-Alanine Ligase from Vancomycin-resistant Leuconostoc mesenteroides

EFFECTS OF A PHENYLALANINE 261 TO TYROSINE MUTATION*

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The Gram-positive bacterium Leuconostoc mesenteroides, ATCC 8293, is intrinsically resistant to the antibiotic vancomycin. This phenotype correlates with substitution of D-Ala-D-lactate (D-Ala-D-Lac) termini for D-Ala-D-Ala termini in peptidoglycan intermediates in which the depsipeptide has much lower affinity than the dipeptide for vancomycin binding. Overproduction of the L. mesenteroides D-Ala-D-Ala ligase (LmDdl) 2 in E. coli and its purification to ~90% homogeneity allow demonstration that the LmDdl2 does have both depsipeptide and dipeptide ligase activity. Recently, we reported that mutation of an active site tyrosine (Tyr), Tyr261, to phenylalanine (Phe) in the E. coli DdB leads to gain of D-Ala-D-Lac depsipeptide ligase activity in that enzyme. The vancomycin-resistant LmDdl2 has a Phe at the equivalent site, Phe261. To test the prediction that a Tyr residue predicts dipeptide ligase while an Phe residue predicts both depsipeptide and dipeptide ligase activity, the F261Y mutant protein of LmDdl2 was constructed and purified to ~90% purity. F261Y LmDdl2 showed complete loss of the ability to couple D-Lac but retained D-Ala-D-Ala dipeptide ligase activity. The Tyr→Phe substitution on the active site omega-loop in D-Ala-D-Ala ligases is thus a molecular indicator of both the ability to make D-Ala-D-Lac and intrinsic resistance to the vancomycin class of glycopeptide antibiotics.

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‡ The abbreviations used are: PG, peptidoglycan; D-Lac, D-lactate; Ddl, D-Ala-D-Ala ligase; Hbut, hydroxybutyrate; LmDdl, Leuconostoc mesenteroides D-Ala-D-Ala ligase; MurNAc, N-acetylmuramic acid; PCR, polymerase chain reaction; TLC, thin-layer chromatography.

creased susceptibility to osmotic lysis and bacterial death.

Clinically significant vancomycin resistance has been detected in pathogenic enterococci in three phenotypic forms, designated VanA, VanB, and VanC type resistance (2). The VanA phenotype has been best studied and found to require the expression of five genes where all five encoded proteins VanR, -S, -H, -A, -X have enzymatic activities (2, 4). VanS and -A act as partners in a two-component regulatory system, VanS as a transmembrane sensor kinase (5, 6) and VanR as a response regulator (6, 7), which account for inducible transcriptional activation of VanH, -A, and -X. VanH encodes a D-Lac dehydrogenase, functioning as a D-specific pyruvate reductase (8) to provide D-Lac for VanA, which is a D-Ala-D-Ala ligase homolog that has gained D-Ala-D-Lac depsipeptide ligase activity (8, 9). In a cell producing both D-Ala-D-Ala and D-Ala-D-Lac (Scheme 1, a and b), VanX acts selectively as a D-Ala-D-Ala dipeptidase. Thus, D-Ala-D-Lac accumulates (10) and serves as a substrate for the MurF enzyme that normally adds D-Ala-D-Ala as a unit to a UDP-N-acetylmuramic acid (MurNAc) tripeptide. Instead of the normal UDP-MurNAc pentapeptide terminating in D-Ala-D-Ala, a UDP-MurNAc tetrapeptide ester terminating in D-Ala-D-Lac is produced in such a vancomycin-resistant Enterococcus sp. Vancomycin binds with three orders of magnitude lower affinity to the D-Ala-D-Lac terminus versus the D-Ala-D-Ala terminus, accounting quantitatively for observed resistance levels (8).

The molecular analysis of vancomycin resistance has led to the similarities and differences between the dipeptide forming D-Ala-D-Lac ligases and the ~28% identical 38 kDa D-Ala-D-Ala ligase homolog VanA, whose gain of depsipeptide ligase activity is crucial for phenotypic resistance (8, 9). The x-ray structure of the DdB isomeric Escherichia coli in complex with a phosphonophosphate analog of a dipeptidyl reaction intermediate has allowed definition of the ligase active site (11) and predicted functions for several residues that were validated by mutagenesis (12). Most intriguingly, mutations at Tyr216 (Y216F) or Ser150 (S150A) in the DdlB mobile omega-loop, and Ser150 participate, with Glu15, in wild-type E. coli DdB convert the dipeptide ligase to an enzyme that has now gained substantial depsipeptide ligase activity (13) that is the hallmark of a VanA -S, -H, -A, -X type dipeptide/depsipeptide ligase. An x-ray structure of E. coli Y216F DdB has been obtained (14). Both Tyr216, on a mobile omega-loop, and Ser150 participate, with Glu15, in wild-type E. coli DdB in a hydrogen bonding array that fixes the omega-loop to cover the substrates and intermediates in the active site and to hydrogen bond (Glu15) to the amino group of D-Ala, to orient this electrophilic substrate (Fig. 1). It was this hydrogen bonding array that suggested Tyr216 as a potentially important residue.

In addition to studying the vanR, -S, -H, -A, and -X operon
function, the structure of PG intermediates in Gram-positive bacteria with intrinsic vancomycin resistance such as *Lactobacillus*, *Pediococcus*, and *Leuconostoc* species has been analyzed (16–19). In these cases, PG intermediates terminating in D-Ala-D-Lac were also detected (20, 21), suggesting a common evolutionary mechanism and a possible origin for at least the VanH and -A genes. Polymerase chain reaction (PCR) analysis has been used to identify and sequence fragments of the ddl genes in such organisms (22), and there is a correlation of Tyr/Phe in the D-Ala-D-Ala ligases at the position corresponding to Tyr216 in *E. coli* DdlB with sensitivity/resistance phenotypes (13). To test the prediction that a phenylalanine in the omega-loop region does indeed predict a D-Ala-D-Ala ligase with depsipeptide ligase activity, d-Ala-d-Ala ligase from vancomycin-resistant *Leuconostoc mesenteroides* ATCC 8293 was overproduced, purified from *E. coli* extract, and characterized for D-Ala-D-Ala and D-Ala-D-Lac ligase activities. When Phe261 was then mutated to Tyr, the resultant enzyme shows retention of D-Ala-D-Ala depsipeptide ligase activity but loss of D-Ala-D-Lac depsipeptide ligase activity.

**EXPERIMENTAL PROCEDURES**

**Materials—** *L. mesenteroides* ATCC 8293 was from American type culture collection (ATCC). Oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA), and restriction enzymes and polymers were from U. S. Biochemical, Corp. (Cleveland, OH). ATP, d-Ala, dl-α-hydroxybutyrate (Hbut), d-Lac, phosphoenolpyruvate, and HEPES were purchased from Sigma. NADH, L-Lac dehydrogenase, and polymerases were from U. S. Biochemical, Corp. (Cleveland, OH). D-[14C]-Ala and D-[14C]-Lac were from American Radiolabeled Chemicals Inc. (St. Louis, MO), and thin layer chromatography (TLC) cellulose sheets were from Kodak (Rochester, NY).

**Cloning, Subcloning, and Site-directed Mutagenesis—** Cloning was carried out by amplification of a DNA fragment by using two rounds of PCR of genomic DNA purified from *L. mesenteroides* ATCC 8293. Three PCR oligonucleotides (oligomer 1, CATAC AAGGT GAGGA CGGAA AGATG; oligomer 2, GCCGA TCCTT AGTTA AACTT CCCTA TCTTT GAGATG; oligomer 2, GCGGA TCCTT AGTTA AACTT CCCTA TCTTT GAGATG; oligomer 3, GCACA TTCTA GAAGG AGATG; oligomer 2, GCGGA TCCTT AGTTA AACTT CCCTA TCTTT GAGATG; oligomer 3, GCACA TTCTA GAAGG AGATG) used in this reaction were designed on the basis of the published *L. mesenteroides* CIP 16407 sequences (22). The first round of PCR with oligomers 1 and 2 was for the first round of PCR with oligomers 1 and 2 was for the amplification of the corresponding genomic DNA fragment. Oligomer 1 was designed to hybridize to the upstream region of the *lmddl* gene, and oligomer 2 contains the 3′ terminal 57 bases of the gene and additional sequences to introduce a BamHI restriction site. Three sequential steps were required to obtain the full-length insert. The cloning was then performed by inserting oligomer 3 and the above amplified fragment into the BamHI site of pET22b (Novagen, Madison, WI) and transformed into *E. coli* BL21(DE3). Site-directed mutagenesis at the Phe261 site was carried out by using two-round megaprimer PCR (24). The oligonucleotides used were the above two oligomers 2 and 3 and a mutated sequence oligomer (GGAAT TATCA CTTAT TATTA TAATC ATACC). After mutagenesis the sequences were confirmed by DNA sequencing.

**Overexpression and Purification of Protein—** Cell culture and purification of DdlB, Y216F, and VanA were performed as described previously (12). Those of wild type and F261Y LmDdl2 are essentially the same as the above proteins with minor modifications. Briefly, *L. mesenteroides* ATCC 8293 and *E. coli* BL21(DE3) were grown in Luria-Bertani medium was inoculated by 1/40 volume of the overnight culture of the corresponding strain and incubated at 30 °C to *A*° of 0.6. At this time, 0.4 ml (final concentration) isopropyl-thio-β-d-galactoside was added to induce *lmddl* gene expression, and the culture was further incubated for ~3 h. The harvested cells in buffer P containing 50 mM Hepes buffer, pH 7.2, and 10 mM MgCl₂, were disrupted by French press (18,000 psi/in²), and the supernatant was obtained after 30 min of centrifugation of the resulting cell extract at 100,000 g. Most of the LmDdl2 protein was recovered in the supernatant. Purification of the protein was followed by ammonium sulfate fractionation (25–50% fraction), ACA54 gel (BioSepara, Marlborough, MA) filtration, and Q-Sepharose chromatography (column size, 80 ml; elution, 400 ml of buffer P; gradient, 0–1 mM KCl). In the gel filtration chromatography, the LmDdl2 protein (~42 kDa) was recovered in similar fractions as DdlB, which is a dimer of a 32 kDa polypeptide. Thus, LmDdl2 also appears to be a protein (~42 kDa) because it was a dimer of a 32 kDa polypeptide. To determine the specific activity of the LmDdl2 protein, 14C-Ala (0.1 mCi/ml, 55 Ci/mmol) or 0.2 mM d-[1-14C]-Lac (0.1 mCi/ml, 55 Ci/mmol), 100 mM HEPES, pH 7.5, 10 mM KCl, 10 mM MgCl₂, 10 mM ATP, and additional unlabeled d-Ala, d-Lac, or d-Hbut with enzyme (~10 μg), which was incubated at room temperature for 3 h. Three μl of each sample was analyzed on TLC cellulose plate as described previously (8, 9).

**Enzyme Assay by Coupled ADP Release—** The TLC assay was not suitable for measurement of kinetic parameters of LmDdl2 because it gives poor results.
was necessary to incubate the reaction mixture for more than 30 min to detect any turnover, and it was difficult to find the linear region of enzyme activity. For these reasons, the ADP release-coupled assay (25) was routinely used for evaluating kinetic parameters. The reaction mixture was composed of 100 mM HEPES, pH 7.5, 10 mM KCl, 10 mM MgCl₂, 10 mM ATP, 2.5 mM phosphoenolpyruvate, 0.15—0.2 mM NADH, 50 units/ml L-Lac dehydrogenase, 50 units/ml pyruvate kinase, D-Ala and D-Lac. The assay was initiated by adding enzymes at 30 °C, and reaction progress was monitored at 340 nm.

**Kinetic Analysis**—The kinetic analysis was carried out as described previously (13, 26—29). The basic equation for D-Ala-D-Ala ligase (Equation 2) was derived based on the steady-state kinetics for two identical substrate molecules of D-Ala (Equation 1).

\[
E + S \overset{K_{n1}}{\rightarrow} ES + S \overset{K_{n2}}{\rightarrow} ESS \rightarrow E + P
\]  
(Eq. 1)

\[
v = \frac{V_{\text{max}}[S]}{K_{n1}[S] + [S]^2}
\]  
(Eq. 2)

For several different conditions of \([S]<K_{n1}, K_{n1}<[S]<K_{n2}, K_{n2}<[S]<K_{n3}, K_{n3}<[S]\) (or \(K_{n3}<[S]<K_{n4}\) or \(K_{n4}<[S]\)), Equation 2 can be approximated by Equations 3, 4, 5, or 7, respectively.

\[
v = \frac{V_{\text{max}}[S]}{K_{n2}}
\]  
(Eq. 3)

\[
v = \frac{V_{\text{max}}[S]}{K_{n2} + [S]}
\]  
(Eq. 4)

\[
v = \frac{V_{\text{max}}[S]}{K_{n2} + [S]}
\]  
(Eq. 5)

\[
v = V_{\text{max}}
\]  
(Eq. 6)

Because of lack of saturation by D-Ala as shown in Fig. 4, it was not possible to measure \(V_{\text{max}}\) and \(K_{n2}\) values for the D-Ala-D-Ala ligase activity of LmDdl2 wild-type and F261Y mutant proteins. According to the above approximations, it is apparent that in Fig. 4, the concentrations of D-Ala vary from around \(K_{n3}\) to much less than \(K_{n2}\). \(h_{\text{cat}}/K_{n2}\) values were therefore obtained by measuring the slope values of the linear portions of reaction time courses.

D-Ala-D-Lac ligase activities were measured as described previously (13). Briefly, Equation 8 was used based on Equation 7, where \(S_1\) and \(S_2\) are D-Ala and D-Lac, respectively. Those activities were measured by the ADP release-coupled assay. Because this method cannot discriminate D-Ala-D-Lac ligase activity from D-Ala-D-Ala ligase activity (paths a and b in Scheme 1), the velocity values were corrected. The value obtained from the observed activity minus D-Ala-D-Ala ligase activity in the absence of D-Lac was regarded as D-Ala-D-Lac ligase activity because, in the conditions used (for D-Ala-D-Lac ligase activity of LmDdl2 wild-type), the D-Ala-D-Ala ligase activity is less than 5% of the maximum observed activity.

**Results**

Purification and Characterization of LmDdl2—The gene encoding the putative D-Ala-D-Ala ligase from the vancomycin-resistant *L. mesenteroides* ATCC 8293 (reported MIC 1012

![Image](http://www.jbc.org/)

**FIG. 3.** Enzyme activity analysis of wild-type (Wt) and mutant proteins by TLC. See “Experimental Procedures” for reaction and TLC conditions. The reaction mixtures contain 10 mM D-Ala and 20 mM Dl-Hbut (A) or 10 mM D-Ala and 10 mM D-Lac (B).

| Protein          | Activity | \(K_{n1}\) | \(K_{n2}\) | \(k_{\text{cat}}\) | \(k_{\text{cat}}/K_{n2}\) | \(k_{\text{cat}}/K_{n2}\) | \(K_{m}\) (ATP) |
|------------------|----------|------------|------------|----------------|-------------------------|-------------------------|---------------|
| LmDdl2 Wt        | A-A\(^d\)| ND\(^e\)    | \(\geq 150\)\(^e\) | ND\(^e\) | 0.35 | 3          | 0.71\(^f\) |
| LmDdl2 F261Y     | A-A\(^d\)| 14         | 130        | 140        | 1.1       | 0.49\(^e\) |
| DdB              | A-A\(^d\)| 0.003      | 2          | 880        | 440     | 0.04       | 0.033         |
| DdB Y216F        | A-A\(^d\)| 0.33       | 16         | 630        | 39       | 0.04       | 0.033         |
| VanA Wt          | A-A\(^d\)| 1.6        | 27         | 27         | 1.6      | 30         | 0.116         |

\(^a\) Measured by ADP coupled assay.

\(^b\) \(K_{n1}\) for D-Ala.

\(^c\) \(K_{n2}\) for D-Ala or D-Lac at subsite 2.

\(^d\) ND, not determined.

\(^f\) The highest concentrations of D-Ala used.

\(^g\) Apparent values determined with 100 mM D-Ala.

\(^h\) D-Ala-D-Lac ligase.

\(^i\) Apparent values determined with 10 mM D-Ala.
$\mu$g/ml) (20, 21) was subcloned into plasmid pET22b behind the T7 promoter as noted under "Experimental Procedures," expressed in E. coli BL21(DE3), and purified as summarized in Fig. 2. Overproduction of the $\sim 42$ kDa LmDdl2 was obtained in soluble form and was readily purified by gel filtration and ion exchange chromatography. A yield of 30 mg of enzyme from $\sim 5 g$ (wet weight) of E. coli was obtained. Fig. 3A shows the profile of products from pure LmDdl2 by TLC analysis in which radio-labeled D-[14C]-Ala was the tracer substrate and D-[14C]-Ala-D-[14C]-Ala and D-[14C]-Ala-D-Hbut products were detected. D-Hbut was utilized as a surrogate substrate for D-Lac in these assays as in earlier studies (8, 9, 13) since D-Ala-D-Lac comigrates with D-Ala-D-Ala on TLC while D-alapha-D-Hbut depsipeptide migrates with higher mobility. The enzyme LmDdl2 makes D-[14C]-Ala-D-Hbut as does VanA and, albeit more slowly, E. coli Y216F DdlB, shown as positive controls for depsipeptide ligase activity, while wild-type E. coli DdlB makes only D-Hbut. In Fig. 3B, cognate incubations with D-[14C]-Lac as tracer show the depsipeptide ligase capacity with D-Lac as a nucleophile hydroxy acid cosubstrate for LmDdl2.

Table I summarizes steady-state kinetic data for LmDdl2 for D-Ala$_1$, D-Ala$_2$, and D-Lac, using a continuous coupled assay for ADP production as previously reported (25). The affinity for D-Ala$_1$ and D-Ala$_2$ could not be readily determined in contrast to several of the other enzyme forms in the table. It is clear that $K_{\text{m,2}}$ for D-Ala$_i$ is very high and probably non-physiological. The $k_{\text{cat}}$ values for D-Ala-D-Lac ligase activity of LmDdl2 (23 min$^{-1}$) and of the E. coli Y216F DdlB mutant (42 min$^{-1}$) approximate those previously reported for VanA (45 min$^{-1}$) (13). Typically, these $k_{\text{cat}}$ values are less than those for D-Ala$_i$/D-Ala$_i$ dipeptide synthesis, perhaps reflecting the weaker nucleophile D-Lac versus D-Ala$_i$ in capturing the D-Ala$_i$-PO$_4$ intermediate. The best determined steady-state parameter for wild-type LmDdl2 was $k_{\text{cat}}/K_{\text{m,2}}$ of 0.35 min$^{-1}$ mM$^{-1}$ for D-Ala$_2$ as the nucleophile cosubstrate and 1.2 for D-Lac as the nucleophile substrate for a catalytic efficiency ratio of $\sim 3/1$ in favor of depsipeptide. By comparison, the corresponding $k_{\text{cat}}/K_{\text{m,2}}$(D-Lac)] (D-Ala$_2$) for VanA is $\sim 30/1$. The E. coli DdlB Y216F mutant that has gained D-Ala$_2$-D-lac depsipeptide ligase activity has a corresponding ratio of 0.4. Thus, the LmDdl2 is intermediate in its preferential catalytic efficiency to make D-Ala$_2$-D-Lac instead of D-Ala$_2$-D-Ala. Given the vancomycin resistance phenotype and the exclusive detection of D-Ala$_2$-D-Lac termini in UDP-MurNAc peptide intermediate (20, 21), it may be that LmDdl2 functions in depsipeptide ligase mode in vivo.

In the LmDdl2 F261Y mutant (see below) in which no residual activity to synthesize D-Ala-D-Lac is detectable, the $k_{\text{cat}}/K_{D, \text{Ala2}}$ value has increased three-fold (to 1.1 mM$^{-1}$ min$^{-1}$), as shown by the data of Fig. 4C.

**Construction, Purification, and Characterization of LmDdl2 F261Y Mutant—To test the proposition that Phe$_{261}$ in LmDdl2, analogous to the Y216F mutant of E. coli DdlB (13), is involved in the gain of function depsipeptide ligase activity of wild-type LmDdl2, the LmDdl2 F261Y mutant was constructed, and the enzyme was purified to $\sim 90\%$ homogeneity with the prediction that it should retain depsipeptide ligase activity but be selectively ablated for depsipeptide ligase activity. This enzyme has the activity shown in Fig. 4B as a function of added D-Lac. While wild-type LmDdl2 (Phe$_{261}$) has both D-Ala$_2$-D-Ala ligase activity and D-Ala$_2$-D-Lac ligase activity, the F261Y enzyme has no detectable ability to utilize D-Lac (at up to 100 mM concentration) either at 3 or 10 mM concentrations of cosubstrate D-Ala$_2$. Wild-type LmDdl2 saturates at about 50 mM D-Lac. While the F261Y LmDdl2 is inactive with D-Lac, it actually has a more robust D-Ala$_2$-D-Ala ligase activity (Fig. 3C).
This work describes the purification and initial kinetic characterization of a D-Ala-d-Ala ligase for the first time from a Gram-positive bacterium, *L. mesenteroides*, known to possess intrinsic chromosomally mediated resistance to the antibiotic vancomycin (20, 21). As previously demonstrated for the enterococcal VanA enzyme (8, 9), LmDdl2 does indeed possess both dipeptide ligase (d-Ala-d-Ala) and depsipeptide ligase activity (d-Ala-d-Lac, d-Ala-d-Hbut), consistent with both the vancomycin-resistance phenotype and the detection of cell wall PG intermediates terminating in d-Ala-d-Lac\(^2\) (20, 21). These activities pinpoint this chromosomal d-Ala-d-Ala ligase as a key molecular determinant in the antibiotic resistance phenotype. The gain of depsipeptide ligase activity of LmDdl2 generalizes previous observations on VanA and VanB-containing drug-resistant enterococci to the naturally resistant Gram-positive soil bacterium and increases the probability that this will be the immunity mechanism for vancomycin producing staphylocyces.

It has been noted that a striking correlation exists for the occurrence of Tyr/Phe at position 216 with the vancomycin sensitivity/resistance phenotype (13). In the comparison of the Tyr\(^{216}\) or equivalent residue (Fig. 5), it is conceivable that there are three classes. The first class, with Tyr in that position, includes 11 proteins. Among them, eight proteins are known or proposed to be d-Ala-d-Ala ligases (8, 9, 25, 29), whereas VanC1, 2, 3 were predicted to be d-Ala-d-Ser ligases since d-Ala-d-Ser terminating UDP-MurNAc was detected in enterococci containing these proteins (37). The lower affinity for d-Ala-d-Ser (amide) versus d-Ala-d-Ala (amide) is ascribed to steric clash of the hydroxymethyl side chain of d-Ser in the complex with vancomycin.

The second group includes four proteins from Gram-positive bacteria (Lc, Lm, Lp, Ls) which are d-Ala-d-Ala ligases in Fig. 5) that contain Phe instead of Tyr. The Tyr/Phe\(^7\) replacement has previously been tested in one direction, by converting wild-type *E. coli* DdlB to the Y216F mutant (13). As summarized in Table II, this results in specific gain of function of depsipeptide ligase activity. Wild-type LmDdl2 with the Phe residue similarly has lost all detectable depsipeptide ligase activity. X-ray structures are available for wild-type *E. coli* DdlB (11) and now for the *E. coli* Y216F DdlB (14), but the altered ability to activate d-Lac C\(_2\)-OH as a nucleophile is not yet clear. Crystal structures for the wild-type and mutant pair of LmDdl2 may also be needed to decipher the amine versus hydroxyl specificity in d-Ala\(_2\) versus d-Lac.

In VanA and B isoforms, which may comprise the third group, the homology suggests a slightly altered loop region and no obviously discernible aromatic residue that is isostructural to Phe\(^{216}\) or Phe\(^{261}\) of *E. coli* DdIB or LmDdls. This may indicate a microscopically different structural solution for a loop in VanA and VanB, permitting d-Lac to function as a nucleophilic cosubstrate. d-Ala-d-Lac has 800–1000-fold lower affinity for vancomycin compared with d-Ala-d-Ala because of the ester for amide substitution (8, 9).

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