Existence of Two D-Alanine:D-Alanine Ligases in *Escherichia coli*: Cloning and Sequencing of the *ddlA* Gene and Purification and Characterization of the DdlA and DdlB Enzymes[†,‡]

Laura E. Zawadzke,[§] Timothy D. H. Bugg, and Christopher T. Walsh*

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 240 Longwood Avenue, Boston, Massachusetts 02115

Received August 23, 1990; Revised Manuscript Received November 2, 1990

ABSTRACT: Two distinct genes encoding D-alanine:D-alanine (D-Ala-D-Ala) ligase (ADP forming) activity in *Escherichia coli* have been cloned by complementation of *E. coli* strain ST640(λ112) deficient in D-Ala-D-Ala ligase activity with a λ library of *E. coli* DNA. One of the two genes, designated as *ddlB*, is identical with the *ddl* gene already sequenced [Robinson, A. C., Kenan, D. L., Sweeney, J., & Donachie, W. D. (1986) *J. Bacteriol. 167*, 809–817]. We describe the subcloning and DNA sequencing of the other gene, designated as *ddlA* on the basis of similarities with the *Salmonella typhimurium ddlA* gene [Daub, E., Zawadzke, L. E., Botstein, D., & Walsh, C. T. (1988) *Biochemistry 27*, 3701–3708]. The predicted amino acid sequence of the *E. coli* DdlA enzyme shows 90% homology with the *S. typhimurium* DdlA sequence. The *ddlB* gene was subcloned by use of the polymerase chain reaction into an expression vector containing an optimized ribosome binding site, which expressed the DdlB enzyme to >50% soluble cell protein. Both DdlA and DdlB enzymes were purified to >90% homogeneity and characterized kinetically.

The dipeptide D-Ala-D-Ala occupies a pivotal role in the assembly of the peptidoglycan layer of bacterial cell walls. It is synthesized from two molecules of D-alanine by the enzyme D-Ala-D-Ala ligase and is subsequently incorporated into the cell wall precursor UDPMurNAc-L-Ala-γ-D-Glu-*m*-DAP-D-Ala-D-Ala by the action of D-Ala-D-Ala adding enzyme (Walsh, 1989; see Figure 1). Since these enzymes are unique to bacteria and utilize D-amino acids, they constitute potential targets for selective antibacterial action by inhibitors based on D-amino acids (Neuhaus & Hammes, 1981).

D-Ala-D-Ala ligase is an ATP-dependent enzyme whose mechanism is thought to proceed via phosphorylation of the carboxylate of D-alanine to give D-alanyl phosphate. This intermediate is subsequently attacked by a second molecule of D-alanine, followed by loss of inorganic phosphate, leading to product formation. Evidence in support of this mechanism has come from positional isotope exchange and molecular isotope exchange experiments (Mullins et al., 1990). This enzyme is one site of action of the antibiotic D-cycloserine (Neuhaus & Lynch, 1964) and is also inactivated by a series of aminoalkyl phosphinate analogues of D-Ala-D-Ala (Parsons et al., 1988), which act as ATP-dependent slow-binding inhibitors for this enzyme (Duncan & Walsh, 1988). The mechanism of slow-binding inhibition has been determined to proceed via phosphorylation at the active site to give a phosphinophosphate transition-state analogue, which dissociates very slowly from the enzyme (McDermott et al., 1990).

Cloning of the *ddlA* gene encoding D-Ala-D-Ala ligase in *Salmonella typhimurium* led to the overexpression and purification of this enzyme to homogeneity (Daub et al., 1988). Transposon mutagenesis was carried out on the *ddlA* gene, and the insertional mutants obtained were transduced onto the *S. typhimurium* chromosome, with the objective of testing whether this was an essential gene for *S. typhimurium*. Surprisingly, the resulting strains were still viable, suggesting the existence of a second gene, termed *ddlB*, in *S. typhimurium* (Daub et al., 1988). Furthermore, significant differences between the *ddlA* gene from *S. typhimurium* and the published DNA sequence of a *ddl* gene from *Escherichia coli* (Robinson et al., 1986) raised the possibility that the *E. coli ddl* gene corresponded to the hypothesized second gene *ddlB* and that an *E. coli ddlA* equivalent might also exist. As precedent, we have previously demonstrated the existence of two genes in *S. typhimurium* encoding the previous enzyme in the pathway, alanine racemase, one of which is inducible and one constitutively expressed (Wasserman et al., 1983, 1984; Galakatos et al., 1986).

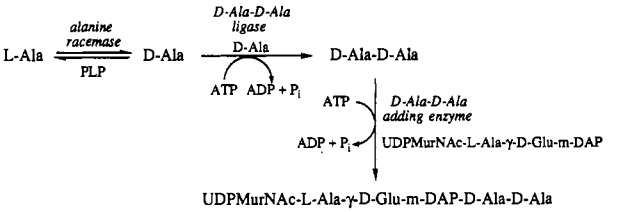


FIGURE 1: Pathway for assembly of UDP-*N*-acetylmuramylpentapeptide from D-alanine.

The existence of two D-Ala-D-Ala ligase genes would raise the question of similarities between the two encoded enzymes, which would be relevant to the status of this enzyme as a target for antibacterial action. Studies to confirm or deny the existence of two genes encoding D-Ala-D-Ala ligase were therefore commenced, by use of a λ library of *E. coli* to complement a temperature-sensitive strain of *E. coli* deficient in D-Ala-D-Ala ligase activity. By this method we now describe the cloning of two distinct genes encoding D-Ala-D-Ala ligase activity in *E. coli* and the subsequent expression, purification, and kinetic characterization of the two *E. coli* D-Ala-D-Ala ligases.

Materials and Methods

Bacterial Strains, Phage, and Plasmids. *E. coli* strain ST640 has a temperature-sensitive mutation in the gene(s)

[†]Supported in part by NSF Grants DMB 8308969 and DMB 8917290 and a SERC/NATO Postdoctoral Fellowship (T.D.H.B.).

[‡]The nucleic acid sequence in this paper has been submitted to GenBank under Accession Number J05319.

* Author to whom correspondence should be addressed.

[§]Present address: Department of Chemistry, Johns Hopkins University, Baltimore, MD 21218.

0006-2960/91/0430-1673$02.50/0

Table I: Oligonucleotide Primers

P1	5′-GCATGCTCTAGAGGAGGAAGAACAACATGACT-3′
P2	5′-GCATGCTCTAGAAGGAGATATACATATGACTGATAAAATCGCGGT-3′
P3	5′-TGACATAAGCTTAGTCCGCCAGTTCCAGAATT-3′
P4	5′-AAATTGCGGGTAGGAATAGT-3′

coding for D-Ala-D-Ala ligase activity (Miyakawa et al., 1972) and was obtained from B. Bachmann (Yale University). *E. coli* strains JM105, W3110, and XA90 (from G. Verdine, Harvard University) were used for cloning work and expression of *ddl* constructs; XL1-Blue was used as a host for M13 constructs. Plasmid pDK30 containing the *E. coli ddlB* gene was a gift of W. Donachie (Edinburgh University). Vector pKK223-X was constructed by insertion of an *Xba*I linker into the *Eco*RI site of pKK223-3 and was a gift of J. Liu (Harvard Medical School). An *E. coli* (W3110) λSE6 library was purchased from the American Type Culture Collection (Rockville, MD). This library was constructed by a partial *Sau*3A1 digest with insert sizes of 12–17 kb (Elledge & Walker, 1985) and bears the *npt* gene for kanamycin resistance (Km^r). The library used in this study was the second amplification. As λSE6 cannot lysogenize, λ112 was used as the helper phage for lysogenization (Maurer et al., 1980).

Media. LB broth and λ media are described in Davis et al. (1980). Most λ-plates and top agar solutions were made with agarose, and not agar, as described in Davis et al. (1980). Maltose was added to a final concentration of 0.2% to media when cells were being grown for subsequent λ phage infection. Antibiotic concentrations were as follows: ampicillin, 100 μg/mL; kanamycin, 50 μg/mL; tetracycline, 12.5 μg/mL. X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) was dissolved in dimethylformamide before use and was used at a concentration of 0.8 mg/mL in top agar.

Materials. The following materials were obtained from Sigma Chemical Co: tris(hydroxymethyl)aminomethane base (Tris), ethylenediaminetetraacetic acid (EDTA), ampicillin, tetracycline hydrochloride, kanamycin, D-alanine, D-Ala-D-Ala, and D-cycloserine. The following materials were obtained from Boehringer Mannheim Biochemicals: phosphoenolpyruvate (PEP), pyruvate kinase/lactate dehydrogenase (PK/LDH), reduced nicotinamide adenine dinucleotide (NADH), ATP, isopropyl thiogalactoside (IPTG), X-Gal, Klenow fragment, and T4 DNA ligase. 4-(2-Hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) and Sequenase Version 2.0 DNA sequencing kits were purchased from U.S. Biochemicals. GeneAmp DNA amplification kits were purchased from Perkin-Elmer Cetus. All restriction enzymes and M13mp18 were purchased from New England Biolabs. The cloning vector pBR322 was purchased from Pharmacia. Agarose was purchased from BRL. Other chemicals and solvents were of reagent grade. Oligonucleotide primers (synthesized by A. Nussbaum, Harvard Medical School) used in this work are shown in Table I.

DNA Preparation. Plasmid DNA was prepared as described in Kraft et al. (1988); λ DNA was prepared as described in Davis et al. (1980).

Isolation of λSE6 Recombinant Phage. Strain ST640 was lysogenized with λ112 (Maurer et al., 1980) and checked for lysogenization by picking and stabbing on a lawn of LE392 cells. The lawn contained X-Gal for selection. The strain ST640(λ112) was restreaked on LB plates, and single colonies were retested for λ112 lysogeny. Complementation of ST640(λ112) with the λSE6 *E. coli* library is described as follows: A 1.5-mL overnight culture of ST640(λ112) was grown in LB plus maltose and concentrated in 0.4 mL of λ dil. Then, 50 μL of cells was mixed with 50 μL of the phage library (1×10^6 pfu/mL) and incubated at 24 °C for 20 min. The 100 μL of library-infected ST640(λ112) cells was supplemented with 1 mL of LB and slowly shaken at 30 °C for 1 h. A 100-μL aliquot of the culture was plated on λ-plates with kanamycin and incubated immediately at 42 or 30 °C. Typically, several thousand colonies would grow at 30 °C, but only one to ten colonies appeared at 42 °C. Single colonies, from the 42 °C plates, were purified by restreaking at 42 °C on λ-plates with kanamycin. These candidates are referred to as ST640(λ112:λSE6 clone).

Since λSE6 contains the pDPT427 replication of origin (Elledge & Walker, 1985), the recombinant phage will replicate in the cell as a large plasmid, or "phasmid", and double lysogens cannot be selected for by inactivation of the *lacZ* gene on λ112. Selecting for kanamycin resistance, however, assures that only double lysogens can survive. For clear restriction enzyme analysis, however, we found that phasmid DNA yields were too low, but recombinant DNA purified from a standard λ DNA preparation (Maurer et al., 1980) resulted in excellent yields of clean DNA.

In order to separate the λSE6 recombinant phage from λ112, the following procedure was performed: A lawn was made with 0.2 mL of an LE392 overnight culture, 50 μL of X-Gal (2%), 1.5 μL of kanamycin (100 mg/mL), and 3 mL of λ top agarose. The mixture was plated onto λ-plates with kanamycin. After the top agarose had just solidified, single colonies of ST640(λ112:λSE6 clone) were stabbed onto the prelawn, and the plate was incubated at 37 °C. Although the LE392 cells could not grow in kanamycin, the ST640-(λ112:λSE6 clone) stabs grew as blue colonies with a blue halo. These blue colonies and halos were picked as plugs and eluted into 1 mL of λ dil buffer with 10 μL of $CHCl_3$. These crude lysates were titered on LE392 cells in the presence of X-Gal, with λ media. Blue and clear plaques arose, and only distinct clear plaques were reextracted into 1 mL of λ dil with 10 μL of $CHCl_3$ to make λSE6 clone lysates. These lysates were titered on LE392 and retested for complementation by infecting ST640(λ112). High titers of the complementing λSE6 clone lysates were used to isolate the λ phage DNA.

Construction of Plasmid pEZ1. The purified phage DNA from six *E. coli* λSE6 clone lysates were digested with *Eco*RI and *Hin*dIII. Three out of the six clearly had the ca. 800-bp *Eco*RI/*Eco*RI internal fragment of the *ddlB* gene already sequenced (Robinson et al., 1986). To subclone the other complementing factor, *Hin*dIII digests were performed on the remaining three and the pBR322 cloning vector. After the digests were heat treated at 85–90 °C for 10 min to inactivate *Hin*dIII activity, the vector DNA (0.1 μg) and the phage DNA (0.4–1.2 μg) were mixed together and treated with T4 DNA ligase at 24 °C, overnight. The DNA ligation mixtures were used to transform ST640 to ampicillin resistance (Ap^r) at 42 °C on λ-plates. DNA preparations from seven individual colonies resulted in plasmid DNA which had identical *Hin*dIII digestion patterns, with an insert in pBR322 on the order of ~12 kb. This plasmid is designated as pEZ1 and is presumed to carry the *ddlA* gene, as determined below. pEZ1 was determined to be Ap^r and Km^r.

Subcloning of ddlA Gene. Digestion of pEZ1 with *Bgl*II followed by religation with T4 DNA ligase gave plasmid pEZ4 containing 4.0 kb of insert DNA, which was found to com-

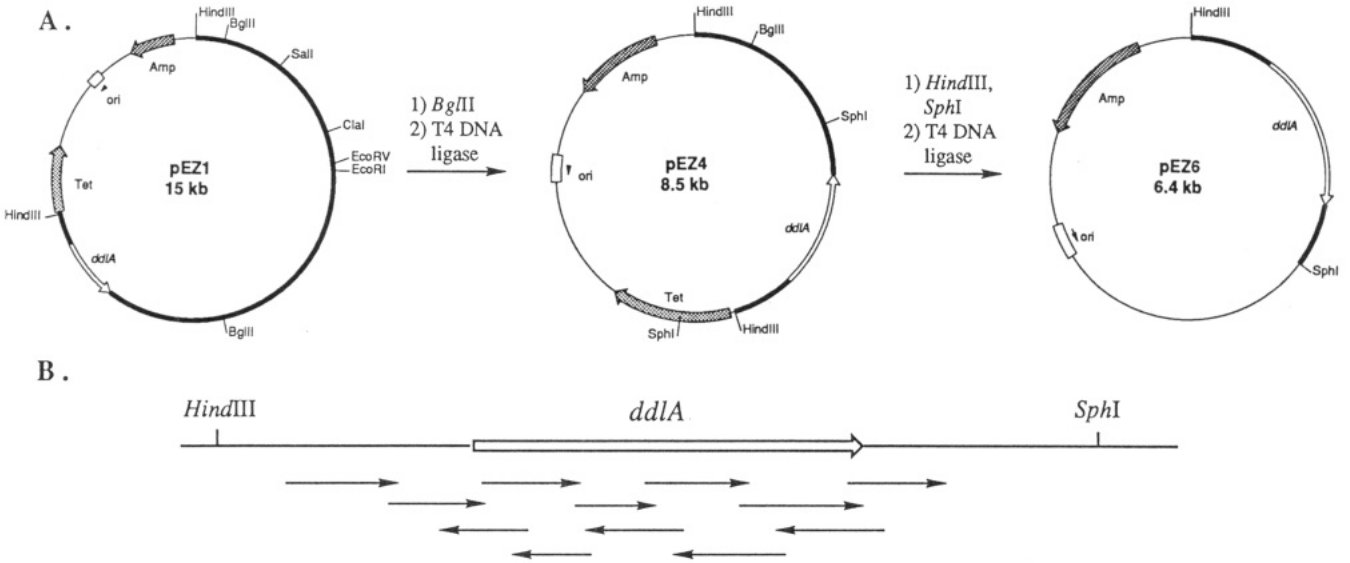


FIGURE 2: Subcloning (A) and sequencing strategy (B) for the *E. coli ddlA* gene. Methods are described under Materials and Methods. (A) Known restriction sites are indicated, insert DNA is marked in black, and the location and direction of the *ddlA* gene in each plasmid are also shown. (B) The 2.4-kb *Hin*dIII/*Sph*I fragment used for DNA sequencing is shown on the sequencing figure. The length and direction of DNA sequences obtained are indicated as arrows.

plement ST640 by transformation into competent ST640 cells and growth on λ-plates at 42 °C. A further complementing subclone was obtained by digestion of pEZ4 with *Hin*dIII and *Sph*I, followed by religation with T4 DNA ligase, yielding pEZ6, containing 2.4 kb of insert DNA encompassing the *ddlA* gene (see Figure 2).

DNA Sequencing of ddlA Gene. A 2.4-kb DNA fragment containing the *ddlA* gene was prepared by digestion of pEZ6 with *Hin*dIII and *Sph*I. This fragment was ligated by use of T4 DNA ligase with M13mp18 cut with *Hin*dIII and *Sph*I, and recombinant M13 were selected by transformation into XL1-Blue and plating out in the presence of IPTG and X-Gal, as described in "M13 Cloning and Sequencing Handbook" by Amersham Corp. ssDNA was purified as described by Amersham, and DNA sequencing was carried out with the dideoxynucleotide chain termination method (Sanger et al., 1977). Oligonucleotide primer P4 (see Table I), based on the DNA sequence near the N-terminus of the *S. typhimurium ddlA* gene, was found to prime DNA sequencing efficiently, and by use of the DNA sequence obtained, a series of specific oligonucleotide primers were synthesized and used to sequence the *ddlA* open-reading frame. The opposite DNA strand was determined by sequencing pEZ6 dsDNA, the reverse complements of the primers used to sequence in the forward direction being used. The DNA sequence and translated amino acid sequence are shown in Figure 4.

Subcloning of ddlB Gene. Plasmid pDK30 (Robinson et al., 1986) containing the *E. coli ddlB* gene was used as a template for the polymerase chain reaction, on a Perkin-Elmer Cetus thermal cycler, according to the manufacturer's instructions. Reaction mixtures (100 μL) contained 0.1 μg of pDK30 DNA template, 200 μM dideoxynucleotide triphosphates, 1 μM of each primer, 2.5 units of AmpliTaq DNA polymerase, and GeneAmp reaction buffer. Primers P1 and P3 were used in construction of pTB1 and primers P2 and P3 for pTB2 (see Table I). A total of 20 cycles of 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 2 min were followed by slow cooling to room temperature and treatment with Klenow fragment for 30 min at 37 °C. After phenol/chloroform extraction and ethanol precipitation, the resulting 0.9-kb fragment was cut with *Xba*I and *Hin*dIII and purified by electrophoresis in a 1% low melting point agarose gel, followed by elution from an Elutip minicolumn (Schleicher & Schuell). Vector pKK223-X was also cut with *Xba*I and *Hin*dIII, purified as above, and ligated with the 0.9 kb cut fragment by use of T4 DNA ligase. Recombinant DNA was transformed into JM105 and subsequently into W3110. dsDNA was prepared from each construct, and digestion of both constructs with *Xba*I and *Hin*dIII gave a 0.9-kb band as visualized by agarose gel electrophoresis. dsDNA sequencing by the chain termination method revealed the anticipated sequence at the start and end of the gene and no mistakes within the sequence of the *ddlB* gene (Robinson et al., 1986).

Purification of D-*Ala*-D-*Ala Ligase A* (*See Table IIA*). D-Ala-D-Ala ligase A was purified from XA90/pEZ1. All steps were performed at 4 °C unless otherwise specified. Enzyme activity was followed by the D-alanine-dependent P_i assay (Daub et al., 1988). Protein concentrations were determined by the Bradford (1976) assay. The standard column buffer consisted of 50 mM HEPES (pH 7.2), 5 mM $MgCl_2$, and 1 mM EDTA.

The cells were grown to saturation at 37 °C in LB with ampicillin, collected by centrifugation, washed with ice-cold column buffer, and frozen in liquid nitrogen. Twenty-five grams of cells was used for purification.

The cells were thawed by grinding with a mortar and pestle, with the addition of 50 mL of lysis buffer [100 mM HEPES (pH 7.2), 5 mM $MgCl_2$, and 1 mM EDTA]. After two passes through a French press at 1000 psi, the cell debris was removed by centrifugation at 19000 rpm for 30 min. Protamine sulfate (2% w/v) was added dropwise to a final concentration of 0.4% (w/v) with stirring, and the solution was stirred for another 30 min on ice. The precipitate was removed by centrifugation at 11000 rpm for 30 min.

Solid ammonium sulfate was gradually added as a fine powder to the supernatant solution to a final concentration of 45% saturation, and the solution was stirred an additional 30 min on ice. The precipitate was collected by centrifugation, as above, and resuspended overnight in 15 mL of column buffer.

The resuspended pellet was loaded onto an Ultrogel AcA54 gel filtration column (2.5 × 108 cm) and eluted isocratically at ~65 mL/h with column buffer. The active fractions were pooled and applied to a MonoQ HR16/10 FPLC column

Table II: Purification of D-Ala-D-Ala Ligases A and B

	vol (mL)	protein (mg/mL)[a]	total protein (mg)	activity (units/mL)[b]	sp act. (units/mg)[b]	yield (%)	purification (x-fold)
(A) *E. coli* D-Ala-D-Ala Ligase A from XA90/pEZ1							
crude extract	66.0	18.7	1232	2.8[d]	0.15	100	1.0
$(NH_4)_2SO_4$ ppt	15.0	ND[c]	ND	10.0[d]	ND	81	ND
AcA54 gel filtration	16.0	ND	ND	1.2[d]	ND	10	ND
MonoQ (pH 7.2)	17.0	0.080	1.35	0.7[d]	8.8	6.5	58
MonoQ (pH 8.5)	10.0	0.060	0.60	0.74[d]	12.3	4.0	82
(B) *E. coli* D-Ala-D-Ala Ligase B from W3110/pTB2							
crude extract	20.9	42.6	890	579[e]	13.6	100	1.0
$(NH_4)_2SO_4$ ppt	11.0	64.0	704	1084[e]	16.9	98.5	1.24
AcA54 gel filtration	64.0	6.7	430	205[e]	30.6	108.5	2.25
MonoQ FPLC	48.0	6.8	326	210[e]	31.0	83.4	2.28

[a] Determined by Bradford (1976) protein assay. [b] One unit is defined as the activity required to convert 1 μmol of substrate to product per minute. [c] ND = not determined. [d] Determined by P_i release assay (Daub et al., 1988). [e] Determined by coupled spectrophotometric assay (Daub et al., 1988).

(Pharmacia) anion exchange column. The column was eluted with a 150–300 mM gradient of KCl in 650 mL of column buffer (pH 7.2). Active fractions eluted to 180 mM KCl and were analyzed by SDS–polyacrylamide gel electrophoresis (9%). Two active fractions (8 mL) were concentrated and desalted with a Centricon 30 unit (Amicon). Each sample was further purified by application to a MonoQ HR5/5 FPLC column in column buffer (pH 8.5). A gradient of 0–300 mM KCl was applied, and active enzyme eluted at 215 mM KCl.

Aliquots of the active D-Ala-D-Ala ligase fractions were electrophoresed on a 9% SDS–polyacrylamide gel, and the protein was transferred to a PVDF membrane by electroblotting, with a Bio-Rad Trans-Blot apparatus. The membrane was stained with Ponceau Red and destained with 10% acetic acid and water, and a single protein band was excised for N-terminal sequence analysis. This band migrated as a MW 44000 protein.

Purification of E. coli D-*Ala*-D-*Ala Ligase B* (*See Table IIB*). D-Ala-D-Ala ligase B was purified from W3110/pTB2. All steps were performed at 4 °C unless otherwise specified. Enzyme activity was monitored by either D-alanine-dependent P_i assay or continuous ADP release coupled assay (Daub et al., 1988). Protein concentration was determined by the method of Bradford (1976). The standard column buffer consisted of 50 mM HEPES, 5 mM $MgCl_2$, and 1 mM EDTA, pH 7.2.

W3110/pTB2 was grown at 37 °C in 1 L of LB containing ampicillin to an A_{595} of 0.6, whereupon IPTG was added to a final concentration of 1 mM, to induce the *tac* promoter. Cells were then grown for a further 5 h at 37 °C and harvested by centrifugation at 7000 rpm for 10 min. The cell pellet (5.4 g) was resuspended in 20 mL of lysis buffer (100 mM HEPES, 5 mM $MgCl_2$, 1 mM EDTA, pH 7.2) and was passed twice through a French press at 1000 psi. Cell debris was removed by centrifugation at 18000 rpm for 30 min.

Powdered ammonium sulfate was gradually added to the supernatant to a final concentration of 25% saturation, and the solution was stirred for 1 h. The solution was cleared by centrifugation at 10000 rpm for 30 min, and ammonium sulfate was added to the supernatant to a concentration of 50% saturation. After being stirred for a further 1 h, the precipitate was removed by centrifugation at 10000 rpm for 30 min. The precipitate was resuspended overnight in 5 mL of column buffer.

The resuspended pellet was loaded onto an Ultrogel AcA54 gel filtration column (2.5 × 108 cm) and eluted at 60 mL/h with column buffer. Active fractions were pooled and applied directly to a MonoQ ion exchange FPLC column (Pharmacia) in column buffer. Enzyme was eluted at 4.0 mL/min with a 200–300 mM gradient of KCl in the same buffer. The activity eluted at 210 mM KCl. The purified protein appeared as a 32-kDa band on SDS–polyacrylamide gel electrophoresis (9%), and the specific activity of the purified protein was 31.0 units/mg.

N-Terminal Sequence Determination. Amino acid analysis, N-terminal sequence determinations (using the the Edman degradation procedure), and proteolytic digestions were carried out on either an SDS–PAGE blot (DdlA) or dialyzed purified protein (DdlB) by William Lane at Harvard Microchemistry Facility, Harvard University, Cambridge, MA.

Native Molecular Weight Determination. Native molecular weights of the D-Ala-D-Ala ligases were determined by gel filtration on Superose 6 FPLC (Pharmacia). Protein samples (0.1–1 mg) were eluted (20 mM Tris, 50 mM KCl, pH 7.4) at 0.5 mL/min, and the retention time at 50% peak front was measured. Ribonuclease A (13700), chymotrypsinogen A (25000), and bovine serum albumin (67000) were run separately under the same conditions as molecular weight standards. The native molecular weights were determined from a linear plot of ln M_r versus retention time.

Kinetic Analysis of D-*Ala*-D-*Ala Ligase.* Kinetic assays on the purified D-Ala-D-Ala ligases were carried out with the continuous ADP release coupled assay (Daub et al., 1988), the assay being monitored at 340 nm. Since D-Ala-D-Ala ligase binds two molecules of D-alanine in two distinct binding sites, two D-alanine K_m values are measurable. Applying the steady-state approximation to system 1, a rate equation 2 can be derived, which predicts parabolic Lineweaver–Burk plots (eq 3), from which $1/v_{max}$ can be measured as the y intercept (see Figure 3). A plot of $[S](1/V - 1/V_{max})$ against $1/[S]$ then gives a straight line (eq 4), whose y intercept (K_2/V_{max}) and gradient (K_1K_2/V_{max}) provide the two K_m values (Neuhaus, 1962b).

$$E + S \underset{}{\overset{K_1}{\rightleftharpoons}} ES + S \overset{K_2}{\rightleftharpoons} ES_2 \xrightarrow{k_{cat}} E + P \qquad (1)$$

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

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_2}{V_{max}}\frac{1}{[S]} + \frac{K_1K_2}{V_{max}}\frac{1}{[S]^2} \qquad (3)$$

$$[S]\left(\frac{1}{V} - \frac{1}{V_{max}}\right) = \frac{K_2}{V_{max}} + \frac{K_1K_2}{V_{max}}\frac{1}{[S]} \qquad (4)$$

Results

Complementation of ST640 with the E. coli λ Library. A λ library was used to complement the temperature-sensitive strain ST640(λ112) in a lysogenic selection. Previously, it has

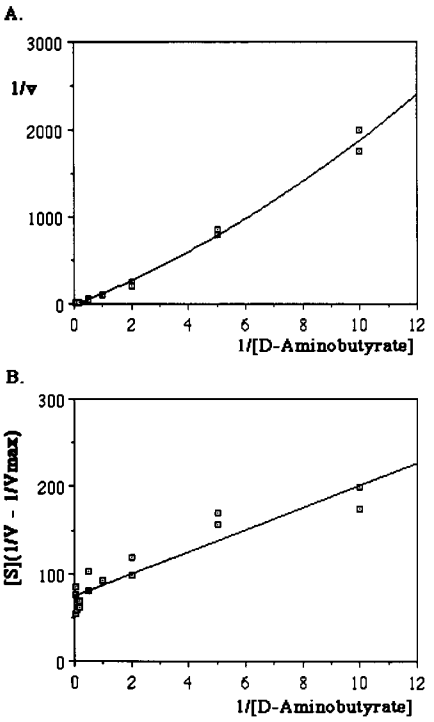


FIGURE 3: Determination of two K_m values for D-Ala-D-Ala ligase. Kinetic analysis is described under Materials and Methods. Data shown are for D-aminobutyrate, assayed against *E. coli* DdlB. Graph A shows parabolic variation of $1/V$ against $1/[S]$, from which the y intercept $1/V_{max}$ can be measured. Graph B shows the linear variation of $[S](1/V - 1/V_{max})$ against $1/[S]$. If no second-order effects were present, graph A would be linear and graph B horizontal.

been reported that strain ST640 does not have a tight temperature-sensitive mutation (Daub et al., 1988; Lugtenberg & van Schijndel-van Dam, 1973) and that survivors grown on LB media at 42 °C, albeit poorly. To avoid this problem, the complementation experiments were performed on the less rich λ-media, which provided tight selection.

From 32 candidates, six clones were randomly selected for λ DNA isolation, as described under Materials and Methods. Conclusively, *Eco*RI digests of the λ DNA revealed that three of the six clones contained the 800-bp *Eco*RI/*Eco*RI internal fragment (data not shown) also found in the previously sequenced *ddl* gene (Robinson et al., 1986). We propose to rename this gene the *ddlB* gene, in view of our previously published sequence of the *ddlA* gene from *S. typhimurium* (Daub et al., 1988) and the DNA sequence data discussed below. The remaining three clones clearly had different *Eco*RI digest patterns. Digests with *Hin*dIII revealed that these three clones exhibited a common large band of ~12 kb and that two of the clones had a 7-kb fragment in common.

By a shotgun cloning approach, the larger of the two *Hin*dIII fragments was ligated into pBR322 by transforming ST640 to Ap^r at 42 °C on λ-plates. This plasmid was designated pEZ1 and transformed into another *E. coli* strain to look for D-Ala-D-Ala ligase activity in crude cell extracts. The XA90/pEZ1 cells grew quickly to high densities, and the D-Ala-D-Ala ligase activity was determined to be 5-fold higher than background activity in XA90/pBR322, consistent with the expectation that a second ligase structural gene had been cloned. We propose to call this gene the *ddlA* gene in *E. coli*, on the basis of the DNA primary sequence and N-terminal protein sequence data below.

The location of the *ddlA* gene on plasmid pEZ1 was investigated by restriction enzyme mapping and the ability of subclones to confer temperature-sensitive resistance to ST640, as described for pEZ1. The plasmid pEZ4 resulted from a *Bgl*II digest of pEZ1, and plasmid pEZ6 resulted from digesting pEZ4 with *Hin*dIII and *Sph*I (see Figure 2). Therefore, the location of the *E. coli ddlA* gene was narrowed down to the 2.4-kb insert of pEZ6 for DNA sequence analysis.

DNA Sequencing of the ddlA Gene. The 2.4-kb *Hin*dIII/*Sph*I fragment from pEZ6 containing the *ddlA* gene was cloned into the polylinker site of M13mp18, and ssDNA purified from the recombinant M13 construct was used to sequence the *ddlA* gene, according to the chain termination method (Sanger et al., 1977). Since the N-terminal sequence of the *E. coli* DdlA enzyme was known (see below) to show a high degree of homology to the N-terminus of *S. typhimurium* DdlA, sequencing primer P4 (see Table I) was synthesized, on the basis of the DNA sequence near the start of the *S. typhimurium ddlA* gene. This primer was found to prime DNA synthesis, and from the resulting DNA sequence, a series of oligonucleotide primers were synthesized and used to sequence both DNA strands of the *ddlA* gene (see Figure 2).

The 1092-bp *ddlA* open-reading frame, shown in Figure 4, encodes a 364 amino acid protein of predicted M_r 39 322, in agreement with the observed mobility of DdlA by SDS–PAGE (see Figure 5). The predicted amino acid sequence is in exact agreement with the N-terminal sequence determined by Edman degradation and shows 90% homology with the amino acid sequence of *S. typhimurium* DdlA (see Figure 6). No extensions were found at the N- or C-termini of *E. coli* DdlA as compared to *S. typhimurium* DdlA, thus both enzymes contain 364 amino acids but show slightly different mobilities by SDS–PAGE (see Figure 5), perhaps due to slight differences in structure.

Purification of D-Ala-D-Ala Ligase A. The D-Ala-D-Ala ligase activity encoded on pEZ1 was purified by a six-step procedure, analogous to ligase purification schemes previously described (Daub et al., 1988; Knox et al., 1989). The enzyme was purified 82-fold to a final specific activity of 12.3 μmol min^{-1} mg^{-1}, as determined by the coupled assay (see Table IIA). The overall yield was 4%; however, only the purest fractions from each column was processed, in order to obtain a homogeneously pure protein band for N-terminal sequence analysis. The N-terminal sequence obtained was as follows: M-E-K-L-R-V-G-I-V-F-G-G-K-S-A-E-H-E-V-S-L-Q-S-A-K-N-I-V-D-A-I-D-K-S-(R)-F-(D)-(V). Of the 38 amino acid residues obtained, 36 matched the N-terminal sequence of *S. typhimurium* DdlA (Daub et al., 1988).

Overexpression of ddlB Gene. Plasmid pDK30 was known to contain the *E. coli ddlB* gene, whose DNA sequence had been determined and which was known to complement ST640, but whose encoded protein had not been purified (Robinson et al., 1986). However, expression of D-Ala-D-Ala ligase activity in crude extracts of JM105/pDK30 was undetectable by D-alanine-dependent P_i assay. In an attempt to increase the expression of the *ddlB* gene, the 1.2-kb *Bam*H1 insert containing the *ddlB* gene was cloned into vector pKK223-3 containing the *tac* promoter, giving a construct which also complemented ST640 but also showed essentially no D-Ala-D-Ala ligase activity in crude extracts (E. Ullian and L. Zawadzke, unpublished results). This suggested that the 120 bp of DNA between the *Bam*HI site and the start of the *ddlB* gene were involved in preventing or regulating its expression. The possibility of catabolite activation of the *ddlB* gene was tested by growth of JM105/pDK30 in the presence of 10 mM L-alanine or 10 mM D-alanine, which resulted in no detectable change in D-Ala-D-Ala ligase activity.

```
-24  GTGTTAGACGGGATAGGTTTTTAAGATGGAAAAACTGCGGGTAGGAATCGTTTTTGGTGGTAAATCAGCGGAACATGAAGTGTCTCTGCA
                              M  E  K  L  R  V  G  I  V  F  G  G  K  S  A  E  H  E  V  S  L  Q

66   ATCGGCAAAAAACATTGTCGATGCCATTGATAAAAGTCGCTTCGACGTTGTGCTGCTGGGCATTGATAAACAAGGGCAATGGCACGTCAG
       S  A  K  N  I  V  D  A  I  D  K  S  R  F  D  V  V  L  L  G  I  D  K  Q  G  Q  W  H  V  S

156  CGATGCCAGCAATTATCTGCTAAATGCAGACGATCCTGCCCATATTGCGTTGCGCCCTTCGGCGACCAGCCTTGCGCAGGTGCCAGGTAA
       D  A  S  N  Y  L  L  N  A  D  D  P  A  H  I  A  L  R  P  S  A  T  S  L  A  Q  V  P  G  K

246  ACATGAGCATCAGCTTATCGACGCGCAAAACGGTCAGCCGTTGCCGACGGTGGATGTCATTTTCCCGATTGTCCACGGTACGCTGGGCGA
       H  E  H  Q  L  I  D  A  Q  N  G  Q  P  L  P  T  V  D  V  I  F  P  I  V  H  G  T  L  G  E

336  AGATGGTTCCTTGCAGGGAATGCTGCGGGTCGCCAATTTACCGTTTGTAGGTTCTGATGTTCTGGCTTCAGCAGCCTGTATGGACAAAGA
       D  G  S  L  Q  G  M  L  R  V  A  N  L  P  F  V  G  S  D  V  L  A  S  A  A  C  M  D  K  D

426  TGTCACCAAACGTCTGCTACGCGATGCCGGGCTGAACATTGCGCCATTTATTACCCTGACGCGCGCCAATCGTCACAACATTAGTTTTGC
       V  T  K  R  L  L  R  D  A  G  L  N  I  A  P  F  I  T  L  T  R  A  N  R  H  N  I  S  F  A

516  CGAAGTGGAGTCTAAACTGGGGTTACCACTGTTTGTAAAACCGGCTAATCAGGGCTCTTCTGTTGGTGTCAGCAAAGTAACCAGTGAAGA
       E  V  E  S  K  L  G  L  P  L  F  V  K  P  A  N  Q  G  S  S  V  G  V  S  K  V  T  S  E  E

606  ACAGTACGCAATTGCCGTCGATCTGGCGTTCGAGTTCGATCATAAAGTGATCGTTGAGCAAGGGATCAAAGGTCGTGAGATCGAATGCGC
       Q  Y  A  I  A  V  D  L  A  F  E  F  D  H  K  V  I  V  E  Q  G  I  K  G  R  E  I  E  C  A

696  AGTTCTGGGCAACGACAATCCGCAAGCCAGCACCTGTGGAGAGATCGTACTCACCAGCGATTTCTATGCCTACGACACCAAGTACATTGA
       V  L  G  N  D  N  P  Q  A  S  T  C  G  E  I  V  L  T  S  D  F  Y  A  Y  D  T  K  Y  I  D

786  CGAAGATGGCGCGAAAGTGGTAGTTCCGGCAGCCATTGCGCCAGAAATCAACGATAAGATCCGGGCGATTGCCGTTCAGGCTTATCAAAC
       E  D  G  A  K  V  V  V  P  A  A  I  A  P  E  I  N  D  K  I  R  A  I  A  V  Q  A  Y  Q  T

876  GTTGGGATGCGCAGGCATGGCGCGTGTAGACGTGTTTTTAACCCCAGAGAACGAAGTGGTGATCAACGAGATCAACACCCTGCCTGGCTT
       L  G  C  A  G  M  A  R  V  D  V  F  L  T  P  E  N  E  V  V  I  N  E  I  N  T  L  P  G  F

966  CACTAATATCAGTATGTATCCGAAGCTGTGGCAAGCCAGCGGTCTGGGTTACACCGATCTGATCACACGTTTGATTGAACTGGCGCTGGA
       T  N  I  S  M  Y  P  K  L  W  Q  A  S  G  L  G  Y  T  D  L  I  T  R  L  I  E  L  A  L  E

1056 GCGTCACGCTGCGGATAACGCATTGAAAACCACAATGTAATATTTTAACGCCCGATAATATTCTTATTGGGCGTTTTCAGTTTCTTCATC
       R  H  A  A  D  N  A  L  K  T  T  M  *
```

FIGURE 4: DNA sequence of the *ddlA* gene and predicted amino acid sequence of DdlA. The *ddlA* DNA sequence, given on the first line, is numbered so that the A in the ATG start codon is in position 1. The predicted amino acid sequence is shown on line two, and the extent of the N-terminal sequence obtained is indicated by underlining. The N-terminal sequence obtained matches the predicted sequence exactly.

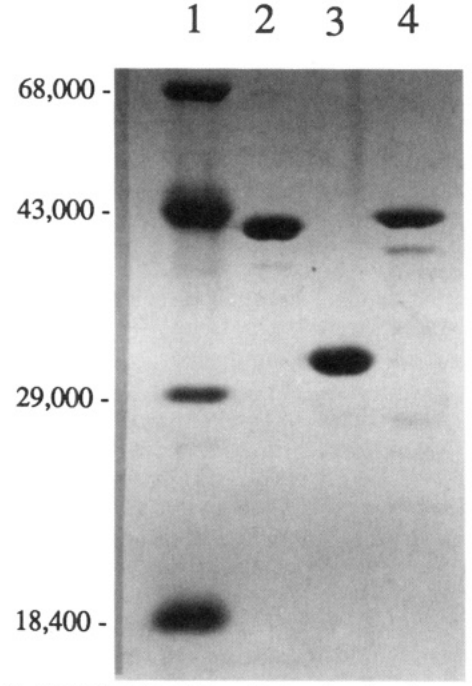


FIGURE 5: SDS–PAGE (11% gel) showing the three purified D-Ala-D-Ala ligase enzymes: lane 1, molecular weight standards; lane 2, *S. typhimurium* DdlA (5 μg); lane 3, *E. coli* DdlB (10 μg); lane 4, *E. coli* DdlA (6 μg).

Therefore, specific amplification of the *ddlB* gene from pDK30 was carried out, by use of the expression-cassette polymerase chain reaction (Macferrin et al., 1990). Oligonucleotide primers P1 and P2 consisted of sequences in the start region of the *ddlB* gene, preceded by an *Xba*I site, and six additional bases. Primer P1 encompassed the existing Shine–Dalgarno sequence of the *ddlB* gene, while primer P2 contained a modified Shine–Dalgarno sequence, *AGGAG*A-TATACAT, derived from the T7 gene 10 protein (Dunn & Studier, 1983), in place of the existing sequence. Primer P3 was complementary to the end region of the *ddlB* gene, followed by a *Hin*dIII site and six additional bases.

PCR amplification from pDK30 using either P1 and P3 (for pTB1) or P2 and P3 (for pTB2) gave the expected 0.9-kb DNA fragment, which was cut with *Xba*I and *Hin*dIII and cloned into pKK223-X, a pKK223-3 derivative containing an additional *Xba*I site. Both constructs were found to express D-Ala-D-Ala ligase activity in crude extracts after induction with IPTG: W3110/pTB1 to approximately 2% of soluble cell protein and W3110/pTB2 to approximately 50% of soluble cell protein. Analysis of both crude extracts by SDS–PAGE revealed the anticipated 32-kDa protein as the major band. The dramatic increase in expression of pTB2 over pTB1 by optimization of the Shine–Dalgarno sequence suggests that this may be a useful strategy for improving the expression of weakly expressed proteins in *E. coli*.

Purification of D-*Ala*-D-*Ala Ligase B.* *E. coli* D-Ala-D-Ala ligase B was purified from W3110/pTB2 by a three-step procedure. After the cells were harvested and broken, a 25–50% ammonium sulfate fractionation was applied, followed by elution from an Ultrogel AcA54 gel filtration column, after which the enzyme was approximately 99% pure. The remaining contaminant was removed by MonoQ FPLC, giving a yield of 326 mg of pure enzyme from 1 L of cell culture (see

Table III: Kinetic Parameters for *E. coli* DdlA and DdlB and *S. typhimurium* DdlA[a]

		S. typhimurium DdlA	*E. coli* DdlA	*E. coli* DdlB
D-alanine	k_{cat} (min^{-1})	644	444	1018
	K_1 (μM)	1.9	5.7	3.3
	K_2 (mM)	0.54	0.55	1.2
	K_m(ATP) (μM)	38	38	40
D-amino-butyrate	V_{rel}	1.27	1.09	0.86
	K_1 (mM)	0.22	0.12	0.17
	K_2 (mM)	15	8.9	9.7
glycine	V_{rel}	0.63	0.63	0.97
	K_1 (mM)	14	2.3	2.5
	K_2 (mM)	75	79	170
D-Ala-D-Ala	$(K_m)_{rev}$ (μM)	23	ND	18
	V_{rev}/V_{for}	5.8×10^{-4}	ND	1.5×10^{-3}
	$(K_i)_{for}$ (μM)	61 (NC)	49 (NC)	70 (NC)
D-cycloserine	K_i (μM)	14 (C)	8.9 (C)	27 (C)
aminoalkyl phosphinate inhibitor[b]	K_i (μM)	250 (C)	130 (C)	28 (C)
	k_{inact} (min^{-1})	2.5	2.4	2.3

[a] K_1 and K_2 refer to the Michaelis constants for the binding of the first (N-terminal) and second (C-terminal) molecules of substrate, as described under Materials and Methods. V_{rel} refers to the value of V_{max}, relative to that of D-alanine. NC = noncompetitive inhibition, C = competitive inhibition, and ND = not determined. [b] $R_1 = R_2 = Me$, racemic (McDermott et al., 1990).

Table IIB). The specific activity of the purified enzyme was 31.0 units/mg.

A sample of purified DdlB was submitted for N-terminal sequencing, which was carried out by Dr. William Lane (Harvard University). Although the sample gave satisfactory amino acid analysis (data not shown), no N-terminal sequence was obtained, suggesting that the N-terminus is blocked in some way. Digestion with trypsin, followed by HPLC purification and sequencing of a major tryptic peptide, yielded the sequence Ile-Gln-Pro-Ser-Gly-Thr-Phe-Tyr-Asp-Tyr-Glu-Ala-Lys, which is identical with peptide Ile(203)–Lys(215) of the predicted amino acid sequence.

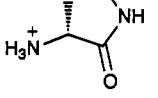
Determination of Physical Properties of DdlA and DdlB Enzymes. Comparison of purified DdlA and DdlB from *E. coli* and DdlA from *S. typhimurium* by SDS–PAGE shows that the two DdlA enzymes are of similar size, close to 40 kDa, whereas the *E. coli* DdlB enzyme is significantly smaller at 32 kDa (see Figure 5), as predicted by their DNA sequences. Measurement of the respective native molecular masses by gel filtration FPLC yielded values of 55–60 kDa for *S. typhimurium* DdlA, 58–62 kDa for *E. coli* DdlA, and 63–65 kDa for *E. coli* DdlB. These data suggest the DdlB enzyme is a dimer (1.9–2.0 monomer subunits/native enzyme) but are inconclusive regarding the subunit composition of the DdlA enzymes (both 1.4–1.5 monomer subunits/native enzyme).

Determination of Kinetic Properties of DdlA and *DdlB Enzymes.* The kinetic parameters for the three D-Ala-D-Ala ligases were determined with respect to substrates D-alanine and ATP and alternative substrates D-aminobutyrate and glycine (see Table III). The turnover numbers for the two DdlA enzymes are comparable (444 and 644 min^{-1}), but the k_{cat} value for DdlB is somewhat higher at 1018 min^{-1}. These values are similar to the k_{cat} values of alanine racemase and D-Ala-D-Ala adding enzyme, the other enzymes of the pathway (Walsh, 1989). Since D-Ala-D-Ala ligase binds two molecules of D-alanine in two distinct binding sites, two K_m values for D-alanine can be measured. For all three enzymes, the K_m value for the first (N-terminal) D-alanine is in the range 1.9–5.7 μM, whereas the K_m for the second (C-terminal) D-alanine is between 100-fold and 300-fold higher: close to 0.6 mM for the DdlA enzymes and 1.2 mM for DdlB. All three enzymes have similar K_m values for ATP, close to 40 μM (see Table III).

D-Aminobutyrate and glycine are alternative substrates for all three enzymes, with maximal velocities similar to that of D-alanine, but higher K_m values. The substrate specificity profiles found are similar to that reported for partially purified *Streptococcus faecalis* D-Ala-D-Ala ligase (Neuhaus, 1962a), suggesting that the active sites of Gram-positive D-Ala-D-Ala ligases are similar to those of *E. coli* and *S. typhimurium*. The observation that the maximal velocity is very similar for D-alanine, D-aminobutyrate, and glycine may indicate that the rate-determining step of the mechanism does not involve D-alanine but could rather be a physical step or binding of ATP.

D-Ala-D-Ala acts as a substrate for the reverse reaction (i.e., ADP/P_i-dependent phosphorylysis of D-Ala-D-Ala), which is assayed by a hexokinase/glucose-6-phosphate dehydrogenase coupled assay (Mullins et al., 1990), at a V_{max} of less than 1% of the forward reaction. D-Ala-D-Ala also acts as a reversible inhibitor of the forward reaction: for all three enzymes noncompetitive inhibition was observed, with K_i values in the range 49–70 μM (see Table III), in contrast to the competitive inhibition reported for the *S. faecalis* enzyme (Neuhaus et al., 1969). The reason for noncompetitive inhibition rather than competitive inhibition is not clear and may indicate that the mode of binding of D-Ala-D-Ala to the active site is different as a substrate and as an inhibitor.

D-Cycloserine acts as a competitive inhibitor for all three ligases, with K_i values in the range 9–27 μM. Assaying in the presence of large amounts of enzyme, no phosphorylation of D-cycloserine was found, and no irreversible enzyme inhibition. In view of the two K_m values measured for D-alanine, it seems likely that the low K_i for D-cycloserine is simply due to preferential binding to the first (N-terminal) D-alanine site. Each enzyme is inactivated in an ATP-dependent slow-binding manner by the aminoalkyl phosphinate inhibitor ($R_1 = R_2 =$ Me; McDermott et al., 1990), with varying K_i values but similar rates of onset of slow-binding inhibition (k_{inact}) (see Table III).



D-cycloserine

Discussion

To prove the existence of two D-Ala-D-Ala ligase genes in *E. coli*, a λ library of *E. coli* DNA was used to complement a temperature-sensitive strain deficient in D-Ala-D-Ala ligase activity. Two distinct complementing activites were cloned, and we show that both of these structural genes code for active D-Ala-D-Ala ligase enzymes. One of the genes, designated *ddlB*, is identical with the sequenced *ddl* gene found in the minute 2 region of the *E. coli* chromosome, situated in a transcriptional unit with several other genes involved in cell division and peptidoglycan biosynthesis (Robinson et al., 1986). We describe here the subcloning and DNA sequencing of the other gene, designated *ddlA* on the basis of similarities with the *ddlA* gene of *S. typhimurium* (Daub et al., 1988).

Alignment of the predicted amino acid sequences of *E. coli* DdlA, *E. coli* DdlB, and *S. typhimurium* DdlA reveals 90% homology between the two DdlA sequences and 35% homology between the *E. coli* DdlA and DdlB enzymes (and 36% homology between *S. typhimurium* DdlA and *E. coli* DdlB).

```
STDDLA: M A K L R V G I V F G G K S A E H E V S L Q S A K N I V D A I D K T R F D V V L L G I D K
ECDDLA: M E K L R V G I V F G G K S A E H E V S L Q S A K N I V D A I D K S R F D V V L L G I D K
ECDDLB: M T D . K I A V L L G G T S A E R E V S L N S G A A V L A G L R E G G I D A Y P V D P K E
        *       + + + + +   * *   * * *   * * * *   * +     + +   + +           *       +

STDDLA: A G Q W H V N D A E N Y L Q N A D D P A H I A L R P S A I S L A Q V P G K H Q H Q L I N A
ECDDLA: Q G Q W H V S D A S N Y L L N A D D P A H I A L R P S A T S L A Q V P G K H E H Q L I D A
ECDDLB: V D V T Q L K S M G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

STDDLA: Q N G Q P L P T V D V I F P I V H G T L G E D G S L Q G M L R V A N L P F V G S D V L S S
ECDDLA: Q N G Q P L P T V D V I F P I V H G T L G E D G S L Q G M L R V A N L P F V G S D V L A S
ECDDLB: . . . . . . . . F Q K V F I A L H G R G G E D G T L Q G M L E L M G L P Y T G S G V M A S
                              + *     + * *     * * * * + * * * * *   +     * * +   * *   *   + *

STDDLA: A A C M D K D V A K R L L R D A G L N I A P F I T L T R . T N R H A F S . . . F A E V E S
ECDDLA: A A C M D K D V T K R L L R D A G L N I A P F I T L T R A . N R H N I S . . . F A E V E S
ECDDLB: A L S M D K L R S K L L W Q G A G L P V A P W V A L T R A E F E K G L S D K Q L A E I . S
        *     * * *       *   *       * * *   + * *   +   * * *               *         * * +   *

STDDLA: R L G L P L F V K P A N Q G S S V G V S K V A N E A Q Y Q Q A V A L A F E F D H K V V V E
ECDDLA: K L G L P L F V K P A N Q G S S V G V S K V T S E E Q Y A I A V D L A F E F D H K V I V E
ECDDLB: A L G L P V I V K P S R E G S S V G M S K V V A E N A L Q D A L R L A F Q H D E E V L I E
          * * * * +   * * * +     * * * * *   * * *     *           * +   * * *     *     * + + *

STDDLA: Q G I K G R E I E C A V L G N D N P Q A S T C G E I V L N S E F Y A Y D T K Y I D D N G A
ECDDLA: Q G I K G R E I E C A V L G N D N P Q A S T C G E I V L T S D F Y A Y D T K Y I D E D G A
ECDDLB: K W L S G P E F T V A I L G E E . I L P S I R I Q P S G T . . F Y D Y E A K Y L S D E T .
            +   *   *       * + * *   +         *                     * *   * +   * * +   +

STDDLA: Q V V V P A Q I P S E V N D K I R A I A I Q A Y Q T L G C A G M A R V D V F L T A D N E V
ECDDLA: K V V V P A A I A P E I N D K I R A I A V Q A Y Q T L G C A G M A R V D V F L T P E N E V
ECDDLB: Q Y F C P A G L E A S Q E A N L Q A L V L K A W T T L G C K G W G R I D V M L D S D G Q F
                * *   +               +   * +   +   *     * * * *   *   + * + * *   *     +

STDDLA: V I N E I N T L P G F T N I S M Y P K L W Q A S G L G Y T D L I S R L I E L A L E R H T A
ECDDLA: V I N E I N T L P G F T N I S M Y P K L W Q A S G L G Y T D L I T R L I E L A L E R H A A
ECDDLB: Y L L E A N T S P G M T S H S L V P M A A R Q A G M S F S Q L V V R I L E L A D
          +   *   * *   * *   *     *     *           + *     + +   * +   * + + * * *

STDDLA: N N A L K T T M
ECDDLA: D N A L K T T M
```

FIGURE 6: Alignment of amino acid sequences of *E. coli* DdlA (ECDDLA; this work), *E. coli* DdlB (ECDDLB; Robinson et al., 1986), and *S. typhimurium* DdlA (STDDLA; Daub et al., 1988). Alignment was carried out with the DOOLITTLE alignment program and was subsequently refined by hand. Absolutely conserved residues are marked with an asterisk (*), and functionally conserved residues (allowed replacements: I/L/V, Y/F, K/R, G/A, A/S, S/T, and D/E) are marked by a plus sign (+).

Carbamoyl phosphate	(*E. coli*)	160-183	A D V G F P C I I R P S F T M G G S G G G I A Y
synthetase		706-729	K E I G Y P L V V R P S Y V L G G R A M E I V Y
Pyruvate carboxylase	(*S. cerevisiae*)	169-192	N E Y G Y P V I I K A A F G G G G R G M R V V R
Acetyl CoA carboxylase	(chicken)	302-325	E E V G Y P V M I K A S E G G G G K G I R K V N
D-Ala-D-Ala ligase A	(*S. typhimurium*)	176-199	S R L G L P L F V K P A N Q G S S V G V S K V A
D-Ala-D-Ala ligase A	(*E. coli*)	176-199	S K L G L P L F V K P A N Q G S S V G V S K V T
D-Ala-D-Ala ligase B	(*E. coli*)	135-158	S A L G L P V I V K P S R E G S S V G M S K V V

FIGURE 7: Alignment of D-Ala-D-Ala ligase sequences with the ATP-binding domains [as proposed by Post et al. (1990)] of *E. coli* carbamoyl phosphate synthetase (Nyunoya & Lusty, 1983), *S. cerevisiae* pyruvate carboxylase (Lim et al., 1988), and chicken acetyl-CoA carboxylase (Takai et al., 1988). Identical or functionally similar residues found in at least four sequences are boxed. Conservative replacements allowed are I/L/V, K/R, G/A, and A/S.

These data imply that the gene duplication event giving rise to the *ddlA* and *ddlB* genes occurred considerably earlier than the point of species divergence of *E. coli* and *S. typhimurium*, which suggests that two *ddl* genes may also be found in other enteric bacteria. Alignment of the three amino acid sequences (see Figure 6) shows several strongly conserved regions, which may comprise parts of the enzyme active site.

Both *E. coli* D-Ala-D-Ala ligase enzymes DdlA and DdlB were purified to homogeneity and compared with the *S. typhimurium* DdlA enzyme. The two DdlA enzymes show similar specific activities and subunit and native molecular weights, whereas the DdlB enzyme possesses a smaller subunit molecular weight and higher specific activity. Despite the differences in size, all three enzymes show very similar kinetic constants and substrate specificity, and similar susceptibility to inhibition by known reversible and time-dependent inhibitors of D-Ala-D-Ala ligase. Kinetic analysis shows a striking difference in K_m values for the two D-alanine binding sites—2–6 μM for the first D-alanine and 0.6–1.2 mM for the second—which rationalizes the tight inhibition of this enzyme by D-cycloserine.

The amino acid sequence of the two DdlA enzymes contains a consensus sequence, Gly-$(X)_4$-Gly-Lys-Thr, found in many ATP-utilizing enzymes (Walker et al., 1982) at positions 7–14, suggesting that this region might comprise part of the ATP-binding site. However, the central lysine residue is not conserved in DdlB, appearing instead as a threonine. Moreover, site-directed mutagenesis carried out on other ATP-utilizing enzymes containing this motif has revealed that the central lysine residue is essential for activity (Xia & Storm, 1990; Tian et al., 1990). The fact that the *E. coli* DdlB enzyme has a high specific activity and similar K_m for ATP suggests that Lys-13 does not interact directly with ATP and therefore that this region may not be in the ATP-binding site at all. We suggest that a more likely location for the ATP-binding domain is region 176–199 of the DdlA sequence, which is strongly conserved in the DdlB sequence and which shows sequence similarity with the proposed ATP-binding domains of carbamoyl phosphate synthetase (Nyunoya & Lusty, 1983), pyruvate carboxylase (Lim et al., 1988), and acetyl-CoA carboxylase (Takai et al., 1988), as shown in Figure 7. Interestingly, all of these enzymes bind acyl phosphate interme-

diates, thus it is possible that this motif acts as an acyl phosphate binding site as well as an ATP-binding site.

The existence of two D-Ala-D-Ala ligase genes in *E. coli* rationalizes our earlier observation that *ddlA*⁻ mutants in *S. typhimurium* are viable and show wild-type phenotype, but also raises the question of why enteric bacteria such as *E. coli* should require the presence of two genes encoding apparently similar enzymes. Other examples of two (or more) genes encoding the same enzyme in the same organism are somewhat rare: usually the two enzymes are used for two different pathways which are regulated separately, for example, the two carbamoyl phosphate synthetases of *Saccharomyces cerevisiae* (Lacroute et al., 1965), the *Pseudomonas aeruginosa* anthranilate synthases *trpE* and *phnA* (Essar et al., 1990), and the two dehydroquinase activities of *N. crassa* (Giles et al., 1967); or they are isoenzymes regulated by feedback inhibition from different end products, for example, the *E. coli* isoenzymes of acetolactate synthase, DAHP synthase, and aspartate kinase (Umbarger, 1978). However, in other cases the reason for the two genes being present is unknown, for example, the two *E. coli* lysyl-tRNA synthetases (Clark & Neidhardt, 1990).

We have previously shown the existence of two genes encoding the preceding enzyme alanine racemase in *S. typhimurium*: *alr* encoding a constitutive alanine racemase and *dadB* encoding an inducible alanine racemase apparently involved in utilization of L-alanine as a sole carbon source (Wasserman et al., 1983, 1984; Galakatos et al., 1986). It is therefore conceivable that a second parallel pathway exists for peptidoglycan biosynthesis, due to the crucial importance of this pathway for bacteria, perhaps one of which is constitutively expressed and one inducible. The problems encountered in expressing the *ddlB* gene, together with observations of others concerning regulation of genes in the minute 2 region (Robinson et al., 1986), suggest that expression of the *ddlB* gene is tightly regulated in vivo and is coupled with expression of other cell wall biosynthesis genes. In contrast, the map position of the *ddlA* gene is as yet unknown, and sequences surrounding the *ddlA* gene in both *E. coli* and *S. typhimurium* show no homology with any known gene sequences (data not shown), thus shedding no light on the cellular function or metabolic regulation of the *ddlA* gene. It is also not known whether more than one *murF* gene exists to encode the third enzyme of the pathway, D-Ala-D-Ala adding enzyme, which would be predicted if two parallel pathways exist.

A second possible explanation for the existence of two genes is that the dipeptide D-Ala-D-Ala has some use for bacteria other than for peptidoglycan biosynthesis, for example, biosynthesis of a D-amino acid containing secondary metabolite, and that the second gene is involved in the biosynthesis of this hypothetical metabolite.

In the light of the existence of two *ddl* genes, one can speculate that the temperature-sensitive *E. coli* strain ST640, constructed by chemical mutagenesis (Miyakawa et al., 1972), contains a permanent mutation in one of the two genes and a temperature-sensitive mutation in the second. Such a double mutation would explain the failure of attempts to backcross the mutation in ST640 (Daub, 1986).

Finally, the existence of two genes for this enzyme may also be relevant to recent studies concerning clinically significant vancomycin resistance in Gram-positive *Enterococcus* strains. The VanA protein, responsible for high-level vancomycin resistance in *Enterococcus faecium* BM4147, has been found to show significant amino acid sequence homology to D-Ala-D-Ala ligase (36% with DdlA and 28% with DdlB; P. Courvalin, personal communication). Although the mechanism of resistance due to VanA is yet to be established, it now seems likely that the *vanA* gene has evolved from one of the two *ddl* genes. It remains to be seen, however, whether Gram-positive bacteria also possess two D-Ala-D-Ala ligase genes.

In summary, although the cellular roles and relative expression of the *E. coli ddlA* and *ddlB* genes remain to be determined, the purified DdlA and DdlB enzymes display remarkably similar catalytic efficiencies and substrate recognition features, in spite of their difference in size and only 35% amino acid sequence homology. Their similar susceptibility to D-Ala-D-Ala ligase inhibitors verifies that this enzyme is a viable target for antibacterial development, and the availability of both DdlA (from *S. typhimurium*; Knox et al., 1989) and DdlB (from *E. coli*; this work) enzymes in large quantities will facilitate further structural and active site studies on this enzyme.

ACKNOWLEDGMENTS

We thank Dr. W. Donachie (University of Edinburgh) for the gift of plasmid pDK30, Dr. Jun Liu for the gift of vector pKK223-X and for helpful advice concerning design of PCR cloning primers, and Dr. Qi-Zhuang Ye for the gift of synthetic aminoalkyl phosphinate inhibitor.

Registry No. ATP, 56-65-5; D-Ala, 338-69-2; Gly, 56-40-6; D-Ala-D-Ala, 923-16-0; DNA (*Escherichia coli* D-alanine:D-alanine ligase ddlA gene), 131152-50-6; D-alanine:D-alanine ligase (*Escherichia coli* reduced), 131152-51-7; D-aminobutyrate, 2623-91-8; D-cycloserine, 68-41-7; aminomethylphosphonate, 1066-51-9; D-alanyl-D-alanine ligase, 9023-63-6.

REFERENCES

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.
Clark, R. L. & Neidhardt, F. C. (1990) *J. Bacteriol. 172*, 3237–3243.
Daub, E. (1986) Ph.D. Dissertation, Massachusetts Institute of Technology.
Daub, E., Zawadzke, L. E., Botstein, D., & Walsh, C. T. (1988) *Biochemistry 27*, 3701–3708.
Davis, R. W., Botstein, D., & Roth, J. R. (1980) *Advanced Bacterial Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Duncan, K., & Walsh, C. T. (1988) *Biochemistry 27*, 3709–3714.
Dunn, J. J., & Studier, F. W. (1983) *J. Mol. Biol. 166*, 477–535.
Elledge, S. J., & Walker, G. C. (1985) *J. Bacteriol. 162*, 777–783.
Essar, D. W., Eberly, L., Hadero, A., & Crawford, I. P. (1990) *J. Bacteriol. 172*, 884–900.
Galakatos, N. G., Daub, E., Botstein, D., & Walsh, C. T. (1986) *Biochemistry 25*, 3255–3260.
Giles, N. H., Case, M. E., Partridge, C. W. H., & Ahmed, S. I. (1967) *Proc. Natl. Acad. Sci. U.S.A. 58*, 1930–1937.
Knox, J. R., Liu, H., Walsh, C. T., & Zawadzke, L. E. (1989) *J. Mol. Biol. 205*, 461–463.
Kraft, R., Tardiff, J., Krauter, K. S., & Leinwand, L. A. (1988) *BioTechniques 6*, 544–546.
Lacroute, F., Pierard, A., Grenson, M., & Wiame, J. M. (1965) *J. Gen. Microbiol. 40*, 127–142.
Lim, F., Morris, C. P., Occhiodoro, F., & Wallace, J. C. (1988) *J. Biol. Chem. 263*, 11493–11497.
Lugtenberg, E. J. J., & Schijndel-van Dam, A. (1973) *J. Bacteriol. 113*, 96–104.
Macferrin, K. D., Terranova, M. P., Schreiber, S. L., & Verdine, G. L. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1937–1941.

Maurer, R., Meyer, B. J., & Ptashne, M. (1980) *J. Mol. Biol. 139*, 147-161.
McDermott, A. E., Creuzet, F., Griffin, R. G., Zawadzke, L. E., Ye, Q.-Z., & Walsh, C. T. (1990) *Biochemistry 29*, 5767-5775.
Miyakawa, T., Matsuzawa, H., Matsuhashi, M., & Sugino, T. (1972) *J. Bacteriol. 112*, 950-958.
Mullins, L. S., Zawadzke, L. E., Walsh, C. T., & Raushel, F. M. (1990) *J. Biol. Chem. 265*, 8993-8998.
Neuhaus, F. C. (1962a) *J. Biol. Chem. 237*, 778-786.
Neuhaus, F. C. (1962b) *J. Biol. Chem. 237*, 3128-3135.
Neuhaus, F. C., & Lynch, J. L. (1964) *Biochemistry 3*, 471-479.
Neuhaus, F. C., & Hammes, W. P. (1981) *Pharmacol. Ther. 14*, 265-319.
Neuhaus, F. C., Carpenter, C. V., Miller, J. L., Lee, N. M., Gragg, M., & Stickgold, R. A. (1969) *Biochemistry 8*, 5119-5124.
Nyunoya, H., & Lusty, C. J. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4629-4633.
Parsons, W. H., Patchett, A. A., Bull, H. B., Schoen, W. R., Taub, D., Davidson, J., Combs, P. L., Springer, J. P., Gadebusch, H., Weissberger, B., Valiant, M. E., Mellin, T. N., & Busch, R. D. (1988) *J. Med. Chem. 31*, 1772-1778.
Post, L. E., Post, D. J., & Raushel, F. M. (1990) *J. Biol. Chem. 265*, 7742-7747.
Robinson, A. C., Kenan, D. L., Sweeney, J., & Donachie, W. D. (1986) *J. Bacteriol. 167*, 809-817.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463-5467.
Takai, T., Yokoyama, C., Wada, K., & Tanabe, T. (1988) *J. Biol. Chem. 263*, 2651-2657.
Tian, G., Yan, H., Jiang, R.-T., Kishi, F., Nakazawa, A., & Tsai, M.-D. (1990) *Biochemistry 29*, 4296-4304.
Umbarger, H. E. (1978) *Annu. Rev. Biochem. 47*, 533-606.
Walsh, C. T. (1989) *J. Biol. Chem. 264*, 2393-2396.
Wasserman, S. A., Walsh, C. T., & Botstein, D. (1983) *J. Bacteriol. 153*, 1439-1450.
Wasserman, S. A., Daub, E., Grisafi, P., Botstein, D., & Walsh, C. T. (1984) *Biochemistry 23*, 5182-5187.
Xia, Z., & Storm, D. R. (1990) *J. Biol. Chem. 265*, 6517-6520.

Role of Cell-Surface Lysines in Plasminogen Binding to Cells: Identification of α-Enolase as a Candidate Plasminogen Receptor†

Lindsey A. Miles,*,‡ Carol M. Dahlberg,‡ Janet Plescia,‡ Jordi Felez,‡ Kanefusa Kato,§ and Edward F. Plow‡

Committee on Vascular Biology, Research Institute of Scripps Clinic, 10666 North Torrey Pines Road, La Jolla, California 92037, and Department of Biochemistry, Institute for Developmental Research, Aichi Prefectural Colony, Kamiya, Kasugai, Aichi 480-03, Japan

Received June 15, 1990; Revised Manuscript Received October 31, 1990

ABSTRACT: Plasminogen binding to cell surfaces results in enhanced plasminogen activation, localization of the proteolytic activity of plasmin on cell surfaces, and protection of plasmin from α_2-antiplasmin. We sought to characterize candidate plasminogen binding sites on nucleated cells, using the U937 monocytoid cell as a model, specifically focusing on the role of cell-surface proteins with appropriately placed lysine residues as candidate plasminogen receptors. Lysine derivatives with free α-carboxyl groups and peptides with carboxy-terminal lysyl residues were effective inhibitors of plasminogen binding to the cells. One of the peptides, representing the carboxy-terminal 19 amino acids of α_2-antiplasmin, was ~5-fold more effective than others with carboxy-terminal lysines. Thus, in addition to a carboxy-terminal lysyl residue, other structural features of the cell-surface proteins may influence their affinity for plasminogen. Affinity chromatography has been used to isolate candidate plasminogen receptors from U937 cells. A major protein of M_r 54000 was recovered and identified as α-enolase by immunochemical and functional criteria. α-Enolase was present on the cell surface and was capable of binding plasminogen in ligand blotting analyses. Plasminogen binding activity of a molecular weight similar to α-enolase also was present in a variety of other cell types. Carboxypeptidase B treatment of α-enolase abolished its ability to bind plasminogen, consistent with the presence of a C-terminal lysyl residue. Thus, cell-surface proteins with carboxy-terminal lysyl residues appear to function as plasminogen binding sites, and α-enolase has been identified as a prominent representative of this class of receptors.

Cell-surface binding sites for components of the fibrinolytic system, plasminogen and plasminogen activators, provide a mechanism for local regulation of fibrinolysis [reviewed in Miles and Plow (1988) and Blasi (1988)]. The functional consequences of occupancy of cellular plasminogen receptors are currently being elucidated. To date, enhancement of plasminogen activation (Miles & Plow, 1985; Hajjar et al., 1986; Stephens et al., 1989), protection of cell-bound plasmin from α_2-antiplasmin (Plow et al., 1986), localization of plasmin activity (Miles & Plow, 1985; Plow et al., 1986), and promotion of the conversion of single-chain to two-chain urokinase (Ellis et al., 1989; Stephens et al., 1989) are events that have been ascribed to the occupied receptors.

Plasminogen receptors have been detected on most peripheral blood cells as well as on many transformed cell lines (Miles

† This work was supported by NIH Grants HL38272, CA23944, and HL17964. Publication No. 6394-CVB from the Research Institute of Scripps Clinic. Portions of this manuscript were presented at the Ninth International Congress on Fibrinolysis, Amsterdam, 1988.
* To whom correspondence should be addressed.
‡ Research Institute of Scripps Clinic.
§ Institute for Developmental Research.

0006-2960/91/0430-1682$02.50/0