

Isolation, Cloning, and Sequencing of the *Salmonella typhimurium* *ddlA* Gene with Purification and Characterization of Its Product, D-Alanine:D-Alanine Ligase (ADP Forming)[†]

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ABSTRACT: A gene coding for D-alanine:D-alanine (D-Ala-D-Ala) ligase (ADP forming) (EC 6.3.2.4) activity has been isolated from a λ library of *Salmonella typhimurium* DNA. Insertion mutations in the gene indicate that the gene is not essential for growth of the bacterium. The encoded enzyme was purified from an overproducing strain of *S. typhimurium*. D-Ala-D-Ala ligase is a protein of 39 271 molecular weight and has a k_{cat} of 644 min^{-1} at pH 7.2. A 2.4-kilobase *SalI*-*SphI* fragment containing the gene was sequenced, and the *ddlA* gene consists of 1092 nucleotides. The gene sequence was compared to the sequence of the *ddl* gene of *Escherichia coli* [Robinson, A. C., Kenan, D. J., Sweeney, J., & Donachie, W. D. (1986) *J. Bacteriol.* 167, 809-817]. Because of differences between the *S. typhimurium* gene and the *E. coli* *ddl* gene, the *S. typhimurium* gene has been named *ddlA*.

D-Alanine is synthesized and incorporated into the *N*-acetylmuramyl-pentapeptide precursor of the cell wall by the action of three bacterial enzymes: alanine racemase, D-Ala-D-Ala ligase, and the D-Ala-D-Ala adding enzyme. These three enzymes in *Escherichia coli* are products of the *dadX* (*dadB*) (Wild et al., 1985) and *alr* genes (Wijsman 1972), the *ddl* gene, and the *murF* gene (Miyakawa et al., 1972), respectively. Alanine racemase and D-Ala-D-Ala ligase make up the D-alanine branch of the peptidoglycan biosynthetic route, synthesizing D-alanyl-D-alanine from L-alanine. The D-Ala-D-Ala adding enzyme catalyzes the addition of the D,D dipeptide to the UDP-*N*-acetylmuramyl-tripeptide to form the pentapeptide.

Interest in this pathway stems from the participation of these enzymes in unique bacterial biosynthetic reactions, and the development of specific inhibitors of these enzymes could lead to a new series of antibiotic agents (Neuhaus & Hammes, 1981). For example, both the alanine racemase and D-Ala-D-Ala ligase are inhibited by D-cycloserine; alanine racemase is inhibited by a mechanism-based inactivation reaction, while ligase is reversibly inhibited (Neuhaus & Lynch, 1964; Neuhaus & Hammes, 1981). To enable further enzymological and genetic studies of D-alanine metabolism and of the biosynthetic pathway for the cell wall, a selection for the gene coding for D-Ala-D-Ala ligase in *Salmonella typhimurium* was undertaken utilizing an *E. coli* mutant for the selection. We have previously reported genetic and enzymological studies for alanine racemase in *S. typhimurium* (Wasserman et al., 1983, 1984; Galakatos et al., 1986).

The *E. coli* strain ST640 was originally isolated by Matsuzawa et al. (1969) as a temperature-sensitive, osmotically

fragile strain. Further investigation by these (Miyakawa et al., 1972) and other authors (Lugtenberg & van Schijndel-van Dam, 1973) indicated that the defect in the strain was in D-Ala-D-Ala ligase, as alanine racemase activity was normal in cell extracts and exogenous [¹⁴C]D-Ala-D-Ala was incorporated into UDP-*N*-acetylmuramyl-pentapeptide in cell extracts. Genetic mapping showed that the mutation is closely linked to several other cell wall genes and less tightly linked to the *leu* locus at minute 2 (Miyakawa et al., 1972). By using a series of cell wall and cell division mutants in complementation studies, and analysis of *leu*-transducing λ recombinant phage (Fletcher et al., 1978; Lutkenhaus et al., 1980; Lutkenhaus & Wu, 1980), it was concluded in *E. coli* that the D-Ala-D-Ala ligase gene, the *ddl* gene, is linked to the *leu* operon and is in a transcriptional unit with the *murC* gene (see Figure 1). The temperature-sensitive mutation in ST640, however, was never backcrossed into a nonmutagenized background. Thus, it has not been shown whether or not *ddl* is an essential structural gene for cell wall synthesis and cell viability in *E. coli*.

The goal of this work, therefore, was 2-fold: to isolate the gene coding for the D-Ala-D-Ala ligase from *S. typhimurium* by complementation and to express the cloned gene for purification of the ligase enzyme. With characterization of the pure enzyme, together with the gene sequence, the cloned complementing activity was positively identified as a structural gene. Experiments have also been performed to determine whether or not the cloned ligase gene is essential in *S. typhimurium*. While this work was in progress, Robinson and co-workers (Robinson et al., 1986) have cloned and sequenced the *E. coli* *ddl* gene but have not isolated its encoded protein. Since differences exist between the *E. coli* gene and the *S. typhimurium* gene, as reported here, the *S. typhimurium* gene will be referred to as *ddlA*.

MATERIALS AND METHODS

Bacterial and Phage Strains. The bacterial strains used are reported in Table I. *E. coli* strain ST640 (Matsuzawa, 1969) was obtained from B. Bachmann (Yale University). This strain has a temperature-sensitive mutation in the gene coding

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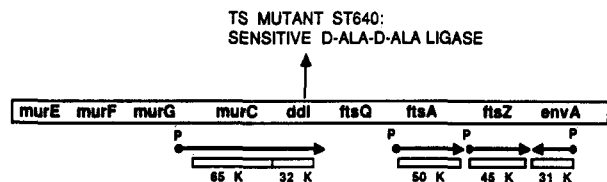


FIGURE 1: Proposed genetic map of *E. coli* near minute 2 on the chromosome. The genetic map in *E. coli* flanking the *ddl* gene is shown (Robinson et al., 1986). Depicted underneath the genetic map are some of the protein products. Approximate positions of promoters are indicated by the letter P, and arrows indicate the direction of transcription (Lutkenhaus & Wu, 1980).

Table I: Bacterial Strains

strain	relevant genotype	source
<i>E. coli</i>		
ST640	<i>ddl</i> ⁻¹	B. Bachmann
JM101	Δ <i>lacpro thi supE</i> <i>F'</i> <i>traD36 proAB lacI</i> ^q <i>ZAM15</i>	J. Messing, Amersham
HB101	<i>hsdS20 recA13 ara14</i> <i>proA2 lacY1 galK2</i> <i>rpsL20 xyl-5 mtl-1</i> <i>supE44</i>	R. Davis
DB4548	<i>hsdR514 supE44 supF58</i> <i>lacY1 galK2 galT22</i> <i>metB1 trpR55</i>	R. Davis
<i>S. typhimurium</i>		
DB7000	<i>leu</i>	D Botstein
DB7960	<i>leu ddl1::Tn10Δ16Δ17</i>	this work
DB7961	<i>leu ddl2::Tn10Δ16Δ17</i>	this work
DB7962	<i>leu</i> <i>zzz-1211::Tn10Δ16Δ17</i>	this work
DB9191	<i>thyA hisC527(am)</i> <i>galE496</i> <i>hsdL6(r^{-m+})/pSM5</i> <i>(lamB)</i>	R. Maurer

for D-Ala-D-Ala ligase activity (Miyakawa et al., 1972). Strain DB9191 (RM486; obtained from R. Maurer, Case Western Reserve University) is a λ -sensitive strain of *S. typhimurium* and was used for the transfer of *Tn10Δ16Δ17* insertions from λ recombinant phage to the *S. typhimurium* chromosome. The *S. typhimurium* strain DB7000 was used as recipient for the *Tn10Δ16Δ17* insertions. Routine growth of bacteriophage λ was done on *E. coli* strain DB4548 (BNN45; Davis et al., 1980). *E. coli* strain HB101 was used as a host for plasmid preparation.

The λ 1059 *Sau3A1* partial library of *S. typhimurium* DNA was made by Dr. R. Maurer. As λ 1059 cannot lysogenize, λ 112 was used as the helper phage for lysogenization (Maurer et al., 1980). The helper phage, λ 112, contains the *lacZ* gene and forms blue plaques in the presence of X-Gal. P22 *int3* HT12/4 (Schmeiger, 1972) was used for P22 generalized transduction.

Media. LB broth and agar, SM⁺ buffer, and λ plates are described in Davis et al. (1980). Thymine or thymidine was routinely added to cultures of ST640 and DB9191, as described in Davis et al. (1980). Drug concentrations were as follows: ampicillin, 75 μ g/mL in plates, 25 μ g/mL in liquid; tetracycline, 12 μ g/mL in plates. 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 soft agar.

Materials. The following materials were obtained from Sigma Chemical Corp.: thymine, thymidine, tetracycline hydrochloride, dithioerythritol (DTE), tris(hydroxymethyl)aminomethane base (Tris), ethylenediaminetetraacetic acid (EDTA), 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol (Bis-Tris)-propane, phosphoenol-

pyruvate (PEP), pyruvate kinase (PK), reduced nicotinamide adenine dinucleotide (NADH), and ATP. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was from U.S. Biochemicals.

All restriction enzymes, M13 mp18, and M13 mp19 were purchased from New England Biolabs; T4 DNA ligase and ligase 10 \times buffer were purchased from IBI; X-Gal, isopropyl thiogalactoside (IPTG), lactate dehydrogenase (LDH), and calf intestinal alkaline phosphatase (CIP) were from Boehringer Mannheim Biochemicals; agarose was from BRL; D-[¹⁴C]alanine (20 mCi/mmol), Klenow fragment, M13 (-20) primer, and [³⁵S]dCTP were from Amersham; all dNTPs and ddNTPs were purchased from Pharmacia. Other chemicals and solvents were of reagent grade.

Isolation of λ 1059 Recombinant Phage. Strain ST640 was lysogenized with λ 112 and checked for lysogenization by cross-streaking. The strain ST640(λ 112) was grown to late exponential phase in LB medium with maltose (0.2%) and 10 mM MgSO₄ and concentrated in 10 mM MgSO₄. The cells were infected at a multiplicity of infection (MOI) of 5 with the λ 1059 library, and after absorbing for 20 min at 37 °C, the cells were plated on LB agar at 42 °C. Large colonies were selected and purified by restreaking at 42 °C. Single colonies of the presumed double lysogens were stabbed onto a lawn of DB4548. The induced phage were picked as a plug, eluted in SM⁺ buffer, and plated on DB4548 on λ plates with X-Gal in the top agar. The helper phage, λ 112, forms turbid blue plaques under these conditions. Clear white plaques were chosen for further analysis. Plate lysates of these phage were grown on DB4548.

Complementation of these purified phage was tested in a spot test as follows. Strain ST640(λ 112) was grown as above, and 0.1 mL of the cells was incubated with 10 μ L of a phage lysate for 20 min at 37 °C. Five microliters of this incubation was spotted on LB plates at 42 °C.

DNA Preparation. Plasmid DNA was prepared as described in Maniatis et al. (1982); λ DNA was prepared as described in Davis et al. (1980).

Isolation and Manipulation of Insertions on Complementing λ Recombinant Phage. Insertions of the element *Tn10Δ16Δ17* on the recombinant λ phage were isolated as described by Maurer et al. (1984). Individual λ clones were replaquesed permissively on DB4548 to purify and were tested for the presence of the *Tn10Δ16Δ17* insertion by spotting on DB4548(λ 112) in LB with tetracycline. Complementation was tested on ST640(λ 112) in a spot test.

The insertions were transferred to the *S. typhimurium* chromosome with DB9191. This strain was grown to exponential phase in LB broth plus thymidine, maltose, MgSO₄, and ampicillin and concentrated 10-fold in SM⁺ buffer. For each transduction, 2 \times 10⁸ cells and from 2 \times 10⁸ to 2 \times 10⁹ λ *Tn10Δ16Δ17* phage were plated on LB plus thymidine and tetracycline. P22-generalized transduction was used to transfer the insertions to DB7000 (Davis et al., 1980).

Plasmid Constructions. λ DNA from one of the complementing phage was digested with *Bam*HI. Vector DNA, pBR322, was digested separately with *Bam*HI and treated with CIP. Vector DNA and phage DNA were mixed together in equal amounts (w/w) at a final concentration of 60 μ g/mL and treated with T4 DNA ligase at 14 °C for 12 h. The DNA was used to transform ST640 to ampicillin resistance (Amp^r) at 30 °C. The transformants were resuspended in LB broth and spotted on LB with ampicillin at 42 °C. DNA from temperature-resistant Amp^r colonies was used to transform HB101 to Amp^r, and plasmid DNA was prepared from this

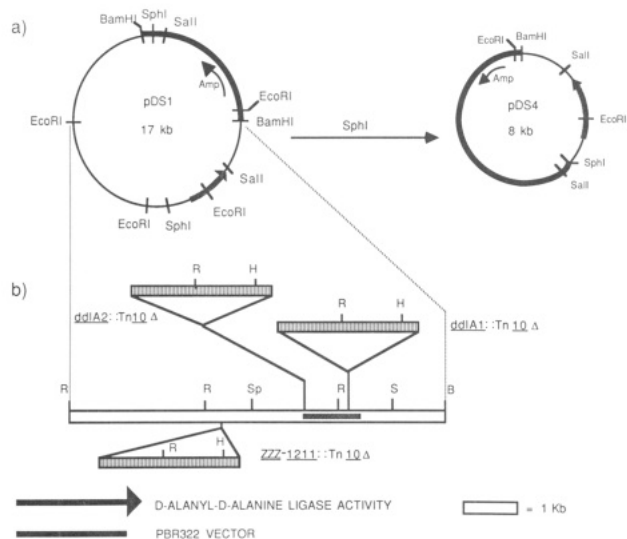


FIGURE 2: Restriction map and subcloning strategy of pDS1 and pDS4. (a) Plasmid pDS1 was digested with *SphI* and religated to form pDS4; the plasmid pDS5 was made by cutting pDS4 with *EcoRI* and religating at low DNA concentration. (b) Localization of the Tn10Δ16Δ17 insertions in pDS1. The three insertions, *ddlA1::Tn10Δ16Δ17*, *ddlA2::Tn10Δ16Δ17*, and *zzz-1211::Tn10Δ16Δ17*, were mapped on pDS1 by restriction enzyme analysis of λ recombinant phage bearing the insertions. Only the pertinent 7-kb *BamHI*-*EcoRI* region of pDS1 is shown here. The *BamHI*-*SphI* fragment containing *ddlA1::Tn10Δ16Δ17* and *ddlA2::Tn10Δ16Δ17* is the *S. typhimurium* insert in pDS4.

strain. These plasmids were designated pDS1 and pDS2.

A further subclone of the complementing activity was prepared by digesting pDS1 with *SphI* and religating the DNA with T4 DNA ligase at low DNA concentrations, 3 μg/mL, generating pDS4 (see Figure 2). The pDS4 plasmid was further digested with *EcoRI* and religated to give pDS5.

Enzymatic Assays. Three different assays for D-Ala-D-Ala ligase activity were utilized. The procedures are described as the following:

(1) **Formation of [¹⁴C]-D-Ala-D-Ala.** D-[¹⁴C]Alanine was purified on a 2-mL Dowex-50 column, concentrated on a Savant Speed-Vac concentrator without heat, and diluted with unlabeled D-Alanine (L-alanine free, as determined with D-amino acid oxidase). The specific activity was determined with D-amino acid oxidase, and contaminating L-alanine was determined with L-alanine dehydrogenase (from *Bacillus subtilis*), as described in Bergmeyer (1974). The final specific activity of the D-alanine was 5.4×10^5 dpm/μmol.

The cells were grown to a Klett value of 60 in LB medium. The cells (50 mL) were washed once with 50 mM Tris-HCl (pH 8) and resuspended in 1 mL of the same buffer. Toluene (20 μL) was added to the cells, and the suspension was vortexed for 30 s and then incubated on ice for 30 min. An aliquot of cells (20 μL) was added to an incubation mixture such that the final concentrations were 25 mM Tris-HCl (pH 8), 10 mM MgCl₂, 10 mM KCl, 10 mM ATP, and 10 mM D-[¹⁴C]alanine, in 0.1-mL total volume. The mixture was incubated at 37 °C for the desired length of time. The incubation was stopped by heating the mixture to 95–100 °C for 2 min, followed by cooling on ice (20 min). The precipitated protein was removed by centrifugation in a microcentrifuge for 10 min. The supernatant (20 μL) was streaked on Avicel thin-layer chromatography (TLC) plates (Analtech, Inc.) and developed once in *n*BuOH/AcOH/H₂O (12:3:5). Ninhydrin (in methanol) was used to visualize the standards, D-alanine and D-Ala-D-Ala. Regions corresponding to these compounds were scraped off the plate, and eluted in 3 mL of 1 M AcOH for

4 h. Two milliliters of the supernatant was counted in Liqiscent (National Diagnostics) in a Beckman scintillation counter.

(2) **Measurement of D-Alanine-Dependent P_i Release.** Enzymatic activity during enzyme purification was assayed by monitoring the D-alanine-dependent release of inorganic phosphate from ATP. The colorimetric method of Lanzetta et al. (1979) was used to detect nanomole amounts of P_i in the incubation mixture. The incubation mixture (50 μL) consisted of 25 mM HEPES, pH 7.8, 10 mM MgCl₂, 10 mM KCl, 2 mM ATP, and 10 mM D-alanine plus enzyme, which was added to 0.8 mL of the color reagent.

(3) **Spectrophotometric Assay for ADP Production.** The spectrophotometric assay used was essentially that of Neuhaus (1962a). The assay mixture contained the following components (final concentration): 100 mM HEPES (pH 7.8), 10 mM KCl, 10 mM MgCl₂, 5 mM ATP, 2.5 mM PEP, 0.1 mM NADH, 50 μg/mL lactate dehydrogenase (LDH), 0.14 mg/mL PK, enzyme (0.63 μg), and D-alanine (0.4–4 mM). The decrease in absorbance at 340 nm was measured.

Protein Determination. Protein was determined by the method of Lowry et al. (1951) or by the biuret method (Cooper, 1977).

Purification of D-Ala-D-Ala Ligase. The enzyme was purified from DB7000/pDS4. All steps were performed at 4 °C unless otherwise specified. The enzyme activity was followed with the D-alanine-dependent P_i assay. The standard column buffer consisted of 25 mM HEPES, 5 mM MgCl₂, 1 mM EDTA, and 1 mM DTE, pH 7.2.

The cells were grown at 37 °C to saturation in LB with ampicillin, collected by centrifugation, washed with 50 mM sodium phosphate, and frozen in liquid nitrogen. Forty grams of cells was used for purification.

The cells were thawed on ice and resuspended in lysis buffer (50 mM HEPES, 5 mM MgCl₂, 1 mM EDTA, 1 mM DTE, pH 7.2, and 20 mL/5 g of cells). The cells were incubated with 1 mg of lysozyme/g of cells for 30 min and subsequently broken by passage twice through a French press at 12 psi. The lysate was cleared by centrifugation at 10000 rpm for 30 min. Streptomycin sulfate solution (15% w/v) was added to a final concentration of 3% (w/v) with stirring, and the solution was incubated with stirring at 4 °C for an additional 30 min. The precipitate was removed by centrifugation at 10000 rpm for 30 min.

Solid ammonium sulfate was gradually added to the supernatant solution to a final concentration of 45% of saturation, and the solution was stirred for 30 min. The precipitate was removed by centrifugation at 10000 rpm for 30 min and was resuspended in 40 mL of the column buffer. After dialysis against three changes of the column buffer, any precipitated material was removed by centrifugation at 10000 rpm for 20 min. The cleared solution was loaded at 15 mL/h onto a DEAE-Sepharose C16B column (Pharmacia, 2.5 × 37 cm) equilibrated with the column buffer. The column was eluted with a 1100-mL gradient of 50–400 mM KCl in the column buffer at a flow rate of 30 mL/h. The activity eluted at 150 mM KCl. The active fractions were pooled and concentrated with an Amicon ultrafiltration cell with a YM5 membrane.

The pooled, concentrated fractions (8 mL) were loaded onto an Ultrogel AcA44 (LKB) column (2.5 × 90 cm) and eluted at 60 mL/h with the column buffer. The active fractions were pooled and concentrated by ultrafiltration as above.

The active material was then purified by fast protein liquid chromatography (FPLC) (Pharmacia) on a strongly anionic exchange column, Mono Q, at room temperature. The column

was equilibrated with 20 mM Bis-Tris-propane, 5 mM MgCl₂, and 1 mM DTE, pH 7.2. The sample was loaded onto the column and was eluted with a 0–350 mM gradient of KCl in the same buffer. The activity eluted at 105 mM KCl, and the active fractions were concentrated with a Centricon unit (Amicon).

N-Terminal Sequence Determination. The N-terminal sequence of the enzyme was determined by the Edman degradation procedure by William Lane at Harvard Microchemistry Facility, Harvard University, Cambridge, MA. Thirty-five residues were determined as shown in Figure 5.

DNA Sequence Determination of *ddlA*. The 1.1- and 1.4-kilobase (kb) *SalI*–*EcoRI* fragments (see Figure 2) were prepared by digestion of pDS4 with *EcoRI* and *SalI*. Fragments were separated by agarose gel electrophoresis (1.0%) and purified by subsequent electroelution into 7.5 M ammonium acetate utilizing an IBI analytical electroeluter. The DNA was precipitated with ethanol. These purified fragments were digested with *AluI*, *HaeIII*, or *NaeI* to generate blunt ends, or with *Sau3AI* which yields a four-base 5' overhang compatible with *BamHI*-cut sites. Single-enzyme digests were ligated with M13 mp18 or M13 mp19 (Messing & Viera, 1982). The ligations were transformed into the *E. coli* JM101 strain, and the ssDNA form was isolated ("M13 Cloning and Sequencing Handbook" by Amersham Corp., Arlington Heights, IL). Poly(ethylene glycol) was used at 8% final concentration to stimulate blunt-end ligations. With the use of the 17-base universal primer 5'-d[GTAAAACGACGGC-CAGT]-3' and deoxycytidine 5'-(α -[³⁵S]thiotriphosphate), the ssDNA was sequenced by the Sanger chain termination method (Sanger et al., 1977). For regions of apparent G,C stacking, the Sanger reaction was run with 7-deaza-dGTP (Thayer, 1985). This sequencing strategy using the above four enzymes afforded approximately 2000 bases of double-strand DNA sequence, including the 1092-base *ddlA* open reading frame (see Figure 4).

Computational Methods. Compilation of sequence overlap data was accomplished by using the Staden DBsystem programs (Staden, 1982a). Open reading frames, codon usage probabilities, and codon translation were plotted by using the ANALYSEQ program (Staden & McLachlan, 1982). A standard codon usage table constructed from the 6498 base pair (bp) coding region of the *S. typhimurium trp* operon was used to calculate coding probabilities (Crawford et al., 1980; Galakatos et al., 1986). Linear alignment and conservative homology comparisons between *E. coli ddl* and *S. typhimurium ddlA* gene sequences and translated protein sequences were performed by using the ALIGN program (Orcutt et al., 1984) and DIAGON program (Staden, 1982b), respectively.

RESULTS

Isolation of Complementing Clones. Strain ST640(λ 112) was used in a lysogenic selection for recombinant phage from a λ library of *S. typhimurium* DNA. The strain ST640 does not have a tight temperature-sensitive mutation, and survivors grow poorly at a frequency of 10⁻³–10⁻⁴ at 42 °C. Hence, the infection of ST640(λ 112) with the library was done at an MOI of 5, and only large colonies were purified for further study. Clear white plaques were isolated as described under Materials and Methods and retested on ST640(λ 112) in a spot test. Ten phage were isolated.

Restriction digests of the 10 complementing phage showed that 9 of the 10 had 3 *EcoRI* bands and a *SalI* band in common. The tenth phage has obviously undergone rearrangement to some extent, as the bands from the vector itself were not present; no simple explanation, such as the loss of a vector

restriction site, could explain the rearrangement.

Subcloning of the Complementing Activity from the Recombinant Phage Clones. Insertion of Tn10 Δ 16 Δ 17 was made in the recombinant phage by the method of Maurer et al. (1984). Two phage were isolated that have insertions in the *S. typhimurium* DNA insert and no longer complement the temperature-sensitive mutation in ST640(λ 112), as judged in a spot test. Restriction digests of the DNA isolated from these phage indicate that the insertions are in a 12-kb *BamHI* fragment (see Figure 2).

The complementing activity was isolated on pBR322 as described under Materials and Methods, yielding pDS1 and pDS2. These two plasmids have the same *BamHI* fragment in opposite orientations. The plasmid pDS1 was further subcloned to yield pDS4, as illustrated in Figure 2. Plasmid pDS4 still complements ST640. The plasmid pDS5, which resulted from a digest of pDS4 with *EcoRI* and religation with T4 DNA ligase, did not complement ST640, indicating that an *EcoRI* site may reside in the D-Ala-D-Ala ligase gene. This result has been substantiated by the DNA sequencing information.

Analysis of Tn10 Δ 16 Δ 17 Insertions in the Complementing Activity. A number of the Tn10 Δ 16 Δ 17 insertions in the insert of the recombinant phage were analyzed as follows. Two classes of insertions were found: the first class still complements ST640(λ 112) in a spot test, and these are presumed near-insertions (insertion zzz-1211::Tn10 Δ 16 Δ 17 is representative of this class); the second class no longer complements ST640(λ 112) in a spot test, and these are presumed insertions in the complementing activity (*ddlA1*::Tn10 Δ 16 Δ 17 and *ddlA2*::Tn10 Δ 16 Δ 17). Both classes of insertions can be transduced to the *S. typhimurium* chromosome by P22 transduction at the same frequency, yielding transductants that do not require any supplements for growth. The fact that the second class of insertions can be transduced to the chromosome without deleterious results indicates that the complementing activity isolated here is not essential for cell growth. If this gene were essential, it is expected that insertions in the gene could not be transduced to the chromosome to yield phenotypically wild-type transductants.

It seemed possible that the phenotypically wild-type transductants obtained from the noncomplementing Tn10 Δ 16 Δ 17 insertions, *ddlA1*::Tn10 Δ 16 Δ 17 and *ddlA2*::Tn10 Δ 16 Δ 17, could arise from chromosomal duplications in the transductants. Southern blot hybridizations were performed on strains DB7000, DB7960, DB7961, and DB7962 (data not shown; Daub, 1986). These results showed clearly that none of the phenotypically wild-type transductants carries chromosomal duplications. Hence, it can be concluded that the complementing activity isolated here is not essential for growth of *S. typhimurium*. Attempts to map these Tn10 Δ 16 Δ 17 insertions were inconclusive. Hfr mapping indicates that these insertions map between minute 97 and minute 2 on the *S. typhimurium* chromosome but show no linkage to *leu* (minute 2). In addition, enzymatic assays with the noncomplementing insertion strains DB7960 and DB7961, using the radioactive assay, showed no appreciable differences in enzyme activity between these cells and wild type (DB7000).

Backcrossing the ST640 Mutation into *E. coli* Wild-Type Background. The temperature-sensitive mutation in ST640 was generated by chemical mutagenesis and has been mapped by P1 and Hfr methods (Matsuzawa et al., 1969). Attempts were made to backcross the temperature-sensitive mutation from ST640 into an *E. coli* background. Briefly, the method used to isolate insertions near the temperature-sensitive mu-

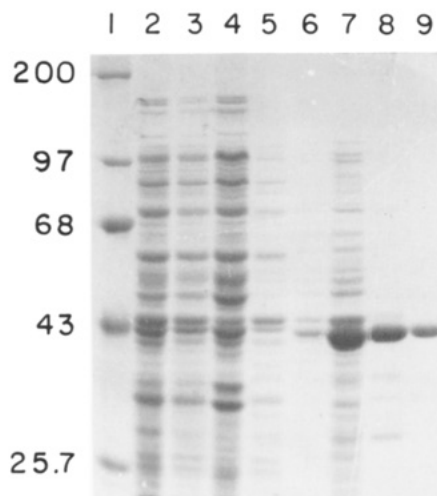


FIGURE 3: SDS-polyacrylamide gel analysis of enzyme purification. A 12.5% polyacrylamide gel was run to follow the course of purification of D-Ala-D-Ala ligase. Lane 1, molecular weight standards; lane 2, French press supernatant; lane 3, streptomycin sulfate supernatant; lane 4, ammonium sulfate pellet (0–45%); lane 5, ammonium sulfate supernatant; lane 6, DEAE-Sepharose C16B fractions, pooled; lane 7, DEAE-Sepharose C16B fractions, pooled and concentrated by Amicon filtration; lane 8, Ultrogel AcA 44 fractions, pooled and concentrated by Amicon filtration; lane 9, FPLC fractions, pooled and concentrated by a Centricon device.

tation in ST640 was developed by Denise Roberts and Nancy Kleckner (Roberts, 1986) and is an adaptation of the method of Low (1973). Insertions were found from the mating of an Hfr strain with ST640, which appeared to be linked to the temperature-sensitive mutation (Daub, 1986). Extensive attempts to use these insertions to move the mutation into an otherwise wild-type *E. coli* strain failed. The insertions are linked to *leu*. Hence, it is concluded that the temperature-sensitive mutation in ST640 is background dependent or the temperature-sensitive phenotype is the result of more than one mutation.

Purification of D-Ala-D-Ala Ligase Activity from *S. typhimurium*. The D-Ala-D-Ala ligase activity encoded on pDS4 was purified by a simple six-step procedure. The protein was purified 48-fold with an overall yield of 11%; see Table II for a purification summary. For reasons unknown, the largest loss of material occurred on the DEAE-Sepharose C16B column, a loss consisting of over 50% of the units. The anion-exchange column, however, provides good separation of the ligase enzyme from other cell proteins (Figure 3, lane 6). The total activity in the soluble fraction after the French press represents 1% of the total protein, on the basis of the final specific activity. Purification on an FPLC Mono Q column yielded a protein of about 42 000 molecular weight on sodium dodecyl sulfate (SDS)-polyacrylamide electrophoresis, essentially free from any major contaminants. The final specific activity, as determined by the P_i assay at pH 7.2, is $16.4 \mu\text{mol min}^{-1} \text{mg}^{-1}$, yielding a turnover number of 644 min^{-1} using 39 271 as the molecular weight. Figure 3 shows a 12.5% SDS-polyacrylamide gel of the steps in the purification.

Neuhaus (1962a) purified D-Ala-D-Ala ligase from *Streptococcus faecalis* 200-fold from a wild-type strain to a specific activity of $5.5 \mu\text{mol min}^{-1} \text{mg}^{-1}$, with an overall yield of 23%. The activity eluted at about 250 mM KCl on an ionic exchange column, as opposed to 150 mM for the *S. typhimurium* enzyme. The molecular weight of the *S. faecalis* protein was not determined, and thus a k_{cat} value could not be calculated. The *S. faecalis* enzyme is stable to heating to 63 °C for 5 min; however, the *S. typhimurium* enzyme was completely inac-

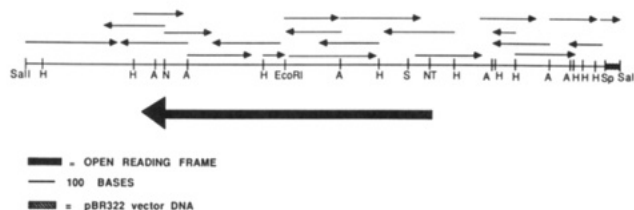
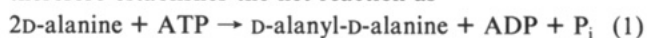


FIGURE 4: DNA sequencing strategy. The entire 2.5-kb *Sall*-*Sall* region from pDS4 is shown. The lengths of the arrows are drawn to scale. The restriction sites are read as A = *AluI*, H = *HaeIII*, N = *NaeI*, S = *Sau3AI*, and Sp = *SphI*. The DNA was sequenced by the method of Sanger, using pDS4 as starting material.

tivated under these conditions.

The enzyme from *S. typhimurium* can be assayed by all three methods outlined under Materials and Methods, and therefore establishes the net reaction as



The enzyme catalyzes the formation of [^{14}C]-D-Ala-D-Ala from D- [^{14}C]alanine, as determined with partially purified fractions, and the specific activity obtained from this method is the same as that obtained with the P_i assay on these same fractions (data not shown). The purified protein also catalyzes D-alanine-dependent release of P_i from ATP, as determined by the P_i assay. In addition, the reaction generates ADP, as is seen in the coupled spectrophotometric assay. All components in the coupled assay are necessary, and none of the concentrations used was found limiting (data not shown). This is the first homogeneous D-Ala-D-Ala ligase reported with a molecular weight value, and hence the first k_{cat} value to be determined for this enzyme.

The enzyme which precedes D-Ala-D-Ala ligase in the cell wall pentapeptide biosynthesis is alanine racemase. These laboratories have reported turnover numbers for the *S. typhimurium* racemase gene products *dadB* and *alr* as (5.6×10^4) – $(6.0 \times 10^4) \text{ min}^{-1}$ and 860 min^{-1} , respectively, in the L-alanine to D-alanine direction (Wasserman et al., 1984; Esaki & Walsh, 1986). Thus, the turnover numbers for the *ddlA* and *alr* gene products are of the same order of magnitude, and it is the *alr* gene product which is proposed to be the biosynthetic enzyme. The biosynthetic enzyme following the D-Ala-D-Ala ligase, the D-Ala-D-Ala adding enzyme, has not been purified and characterized in *S. typhimurium* or *E. coli*.

Gene Sequence and Amino Acid Composition. The 1092-base open reading frame encodes 346 amino acids, including the 35-residue N-terminal sequence obtained by Edman degradation of the purified enzyme (see Figure 5). The predicted molecular weight of the enzyme is 39 271, within 8% of the value given by SDS-polyacrylamide gel electrophoresis. The codon usage probability, as seen in Figure 6, is greater than 50% in reading frame 1 and suggests not only that the correct reading frame is being translated but also that the proper C-terminus was located.

About 750 bp of DNA sequence upstream of the *ddlA* gene was determined. In this region, no significant open reading frame (ORF) was found on the same DNA strand as *ddlA*, but an ORF of at least 721 bp was found on the opposite strand (this ORF could extend beyond the *SphI* site). Luktenhaus and Wu (1980) determined that in *E. coli* the *murC* gene maps directly upstream of *ddl* and is translated in the same direction, and Robinson et al. (1986) suggest that the end of *murC* closely flanks the start of the *ddl* gene. Hence, it appears that the *ddlA* gene from *S. typhimurium* is in a different transcriptional unit than the *ddl* gene in *E. coli*. It is not known whether or not the ORF found upstream of *ddlA* is translated.

```

-98      -90          -70          -50          -30          -10          10
CGGTCTCAGATCCCATAAACATTTATGGCAGAAAGCCGCGAAACCCGCGTTTGTCTGGTAATGTATACGCTTTGCATTTCGATGGAAATGGGTTTTTAAGATGGCGAAATTCGGGTAGGA
M A K L R V G
...A..K..L..R..V..G..

22      30          50          70          90          110          130
ATAGTATTTGGCGGTAAGTCGGCGGAAATGAAAGTGTCTTTGCAATCAGCGAAAAATATCGTTGATGCGATCGATAAAACCCGCTTCGACGTGGTGTCTTTAGGGATAGATAAGCGGGGA
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 A G
I..V..F..G..G..K..S..A..E..A..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..A..G..

142     150          170          190          210          230          250
CAATGGCATGTTAACGATGCGGAAATTTATTTGCAGAAATGCAGACGATCCGGCACATATCGCGCTACGCTCTTCGGCAATCACTCTGGCCAGGTGCGCTGGCAAAACACCAACCAATTG
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

262     270          290          310          330          350          370
ATTAACGCTCAGAAACGGGCGAGCCGCTACCGACGGTAGATGTTATTTTTCCTATCGTCCACGGTACGCTGGCGGAAGACGGCTCAATTGCAAGGGATGCTGCGCGTCCGCAAACTTACCGTTT
I N A 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

382     390          410          430          450          470          490
GTCGGTTCAGACGTGCTGAGCTCCGCGAGCCCTGCATGGAATAAGACGTCGCGCAAAACGACTGCTACGCGATCGCGGACTGAATATCGCCCGCTTATTACACTCACCGCAACGAAATCGTCA
V G S D V L S S 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

502     510          530          550          570          590          610
GCGTTTAGTTTTGCCAGGTTGAATCCCGTCTGGGACTGCCGCTGTCTGTTAAAGCCCGCAATCAGGGCTCTTCGGTCCGGCTCAGTAAAGTCCGTAACGAAGCGCAATATCAGCAGGCG
A F S F A E V E S 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

622     630          650          670          690          710          730
GTCGCGCTGGCTTTCGAATTCGATCATAAAGTGGTGGTGGAGCAAGGGATCAAAGGGCGGAGATCGAATGCGCCGATATTAGGTAACGATAACCCAGGCCAGTACCTGCGCGGAAATC
V A L A F E F D H K V V V E 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

742     750          770          790          810          830          850
GTACTGAACAGCGAAATTTTACGCCCTACGATACTAAGTATATGATGATAATGGCGCAAGTTGTCGTGCTCGCAGATCCCTTCGAGGTGAATGACAAGATCCGCGCGATCGCGATC
V L N S E F Y A Y D T K Y I D D N G A Q V V V P A Q I P S E V N D K I R A I A I

862     870          890          910          930          950          970
CAGGCTTATCAGACGCTGGGATGCGCGGGTATGGCGCGTGTGATGTTCTTAAACGGCAGCAATGAGGTCGTGATTAACGAGATCAATACGCTGCCAGGTTTTACCAATATCAGTATG
Q A Y Q T L G C A G M A R V D V F L T A D N E V V I N E I N T L P G P T N I S M

982     990          1010         1030         1050         1070         1090
TATCAAACCTCTGGCAGGCGAGCGGATGGGCTATACCGATTTGATCAGCCGCTGATGAGCTGGCGCTGGAAGCCATACGGCGAATAACGCCCTTAAAAACCGATGTAATAG 1098
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 N N A L K T T M * *

```

FIGURE 5: *ddlA* gene, predicted protein, and N-terminus sequence determined from purified protein. The *ddlA* gene sequence, given on the first line, is numbered so that the A in the ATG start site is in position 1. The map position on the *S. typhimurium* chromosome is not known at this time. Ninety-eight nucleotides upstream from the start site are shown here. The predicted protein sequence on line two and the N-terminus protein sequence on line three differ by one residue at nucleotide positions 49–51. Otherwise, the predicted protein and sequenced enzyme N-terminus match completely.

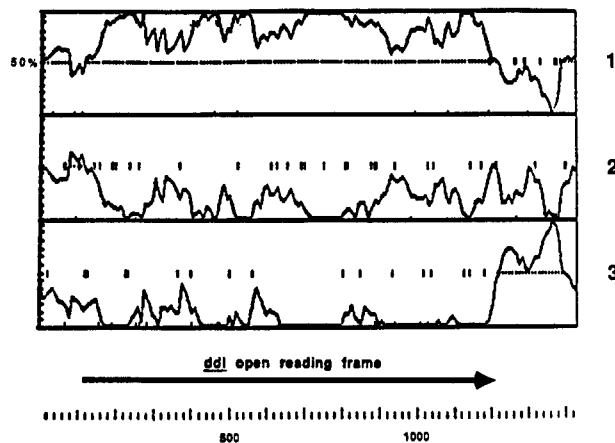


FIGURE 6: Codon usage plot for the *ddlA* open reading frame (Staden & Mclachlan, 1982). Three reading frames are shown, with the ordinate representing 0–100% codon usage probability for each reading frame box. Reading frame 1 depicts the codon usage of the *ddlA* gene (window size = 25). The reading frame shown here begins 105 bases upstream from the *ddlA* start site. The arrow depicts the start and stop of the *ddlA* gene, and the abscissa is marked with a base pair ruler.

Comparison of *E. coli* *ddl* and *S. typhimurium* *ddlA* Gene Products. The recent report of the *E. coli* K-12 *ddl* gene (Robinson et al., 1986) allows comparisons to be made for *ddl* and *ddlA*, the two gene sequences which experimentally complement the ST640 mutant. Whereas the *E. coli* gene of 918 nucleotides encodes a 32 840 molecular weight protein product, the *ddlA* gene of 1092 bases encodes a 39 271 molecular weight protein. Not only do these proteins differ substantially in size but also identical amino acid residue homology is only 39% (see Figure 7). Although conservative

protein homology appears to be >39% (see Figure 8), a large absence in homology is readily apparent in the enzyme's N-terminus.

D-Ala-D-Ala Ligase ATP Binding Site. Two highly conserved regions believed to be involved in the nucleotide binding fold have been identified in kinase and other ATP/ADP-requiring enzymes (Walker et al., 1982; Finch & Emmerson, 1984; Millar et al., 1986). The *ddlA* product appears to contain one of these conserved homologies at residues 7–21, G-I-V-F-G-G-K-S-A-E-H-E-V-S-L, which is similar to the consensus sequence G-X₄-G-K-T-X₆-I/V given in the reference above. This region, however, is not fully conserved in the *E. coli* Ddl sequence, especially since the crucial lysine residue at the seventh position of the consensus sequence is missing. The lysine may interact with the α -phosphate in adenylate kinase and is completely conserved in a large number of ATP-utilizing enzymes (Walker et al., 1982; Finch & Emmerson, 1984; Millar et al., 1986; Higgins et al., 1986).

The question is then raised if another conserved region between the two ligases exists which contributes to ATP binding. For example, as seen in Figure 7, the segment L-G-L-P-X₂-V-K-P-X₃-G-S-S-V-G-X-S-K-V at positions 183–203 is highly conserved. Whether or not these conserved residues play a common functional role in ATP binding or another role in ligase activity remains to be determined.

DISCUSSION

The enzyme D-Ala-D-Ala ligase from *S. typhimurium* has been isolated from an overproducing strain of *S. typhimurium* bearing pDS4. A temperature-sensitive mutant of *E. coli* was used to isolate recombinant λ clones that complement the mutation and code for D-Ala-D-Ala ligase activity. It has been confirmed by DNA sequencing that the isolated enzyme is

Table II: Purification of D-Ala-D-Ala Ligase Activity from DB7000/pDS4

step	weight ^a (mg)	net ^b act.	sp ^c act.	x-fold purifica- tion
French press	3773	467	0.344	
streptomycin sulfate	2995	582	0.336	
ammonium sulfate ppt	1323	465	0.528	1.5
DEAE-Sepharose	48.6	179	3.7	10.7
Ultragel	11.0	99	9.0	26
FPLC Mono Q	4.0	65.7	16.4	48

^aDetermined by the biuret method. ^bMilliunits per milligram; activity determined in the presence and absence of D-alanine. ^cDetermined via a P₁ release assay.

DdlA versus 33 000 molecular weight for Ddl; however, the enzyme from *E. coli* has not been purified. The nucleic acid homology of 50% and identical protein sequence homology of 39% for D-Ala-D-Ala ligase from *E. coli* and *S. typhimurium* suggest that the genes may have evolved from a common ancestor, although, this level of homology is still less than anticipated for true interspecies D-Ala-D-Ala ligase homologues between *E. coli* and *S. typhimurium*.

We raise the prospect of more than one D-Ala-D-Ala ligase gene in *S. typhimurium*. The gene isolated in this study is not essential for growth of *S. typhimurium* under the experimental conditions described here, despite the expectation that D-Ala-D-Ala is a crucial cell wall building block. Attempts made to backcross the temperature-sensitive mutation in ST640 were consistent with, but did not prove, the hypothesis that *E. coli* also has two genes coding for D-Ala-D-Ala ligase activity.

We have earlier reported the presence of two genes for alanine racemase, *dadB* and *alr*, in *S. typhimurium* (Wasserman et al., 1983, 1984; Galakatos et al., 1986). Alanine racemase catalyzes the metabolic step preceding the formation of D-Ala-D-Ala in the D-alanine branch of the peptidoglycan assembly pathway. Current studies include the search for a second D-Ala-D-Ala ligase gene in *S. typhimurium*. If a second gene is found, then the relationship between the two enzymes in cell wall physiology will be examined, similar to the studies done on alanine racemase in *S. typhimurium*.

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Registry No. DNA (*Salmonella typhimurium* gene *ddlA*), 113180-29-3; D-Ala:D-Ala ligase (*Salmonella typhimurium* reduced), 113180-30-6; D-Ala:D-Ala ligase, 9023-63-6.

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