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The Periplasmic Dipeptide Permease System Transports 5-Aminolevulinic Acid in *Escherichia coli*

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In a genetic screen designed to generate *Escherichia coli* strains completely devoid of the heme precursor 5-aminolevulinic acid (ALA), we isolated a class of mutants which were defective for exogenous ALA uptake. The mutations, designated *alu* (ALA uptake), mapped to the 80-min region of the *E. coli* chromosome. They were complemented by a recombinant plasmid containing the *dpp* operon, which encodes a dipeptide permease transport system. Alu mutants displayed a severe reduction in ALA import, as did a strain with a chromosomal insertion in the first gene of the *dpp* operon. A recognized substrate of Dpp transport, prolyl-glycine, effectively competed with ALA for uptake. *E. coli* strains defective in ALA biosynthesis (*hemA* or *hemL*) require exogenous ALA to achieve wild-type growth but show limited aerobic and anaerobic growth in the absence of ALA. The presence of an *alu* or *dpp* mutation in *hemA* or *hemL* strains abolishes growth in the absence of ALA and requires increased levels of ALA for normal growth. We conclude that the *alu* mutations are within the *dpp* operon and that the dipeptide transport system mediates uptake of the important metabolite ALA.

The universal first step in tetrapyrrole synthesis is the formation of 5-aminolevulinic acid (ALA). *Escherichia coli* synthesizes ALA from Glu-tRNA^{Glu} in the C_5 pathway through the sequential action of two enzymes (for a review, see reference 9). Glu-tRNA reductase, encoded by the *hemA* gene, catalyzes the reduction of the tRNA-activated glutamate to glutamate-1-semialdehyde, which is transaminated to ALA by glutamate-1-semialdehyde aminomutase, the product of the *hemL* gene.

hemA strains require ALA for growth on minimal medium with glycerol as the carbon source. However, they exhibit limited growth aerobically and grow well anaerobically on minimal glucose lacking ALA, indicating that siroheme is produced. This modified tetrapyrrole forms the prosthetic group of sulfite reductase, which is active in the assimilatory sulfate reduction required for cysteine and methionine formation. The growth of *hemA* mutants in the absence of ALA indicates that a small amount of siroheme, and thus ALA, is produced. In order to isolate mutations in a possible second pathway for ALA formation (6), we searched for mutants completely dependent on exogenous ALA for both aerobic and anaerobic growth.

MATERIALS AND METHODS

Bacteriological procedures. For growth of mutant strains requiring ALA, the media were supplemented with 25 μg of ALA per ml. Glycerol, glucose, or other carbon sources were routinely added at a final concentration of 2 to 4 mg/ml. Anaerobic environments were created with BBL GasPak systems. The bacterial strains used in this study are listed in Table 1.

Complementation of *alu* mutations. A recombinant cosmid library was prepared as previously described (15) from chromosomal DNA from *E. coli* EV61. Transformants into GE1391 were selected for complementation of the *alu* mutation on minimal glucose plates both aerobically and anaerobically.

DNA methods and chromosomal mapping. DNA analysis and chromosomal mapping were performed according to established protocols (3). An *E. coli* Gene Mapping Membrane containing DNAs transferred from 476 miniset clones (10) was obtained from Takara Biochemicals, Inc.

Assays for ALA uptake. Strains were grown overnight in minimal glucose medium with 10 μg of ALA per ml, diluted 1:100 in the same medium, and grown to early exponential phase. Cells were harvested at room temperature, washed twice, and then resuspended in minimal A salts buffer at a final A_{420} of ≈0.15. Cell suspensions were incubated for 1 h at 37°C to deplete endogenous ALA. The assays were initiated by the addition of [4-^{14}C]ALA (48.2 mCi/mmol; NEN Research Products) with either 0.1 or 1.0 μCi at a final ALA concentration of 2 μM or 1.0 mM, respectively. Samples (0.2 ml each) were removed at each time point and were added to an equal volume of buffer containing 2 mM unlabeled ALA, and then the cells were collected on Millipore filters (0.22-μm pore size). The filters were washed with 5 ml of buffer and dried before counting. Values at time zero were subtracted from each sample to correct for background.

RESULTS

Isolation of ALA uptake (*alu*) mutants. Strain GE1387 with the *hemA204* allele was used for the isolation of mutants which are absolutely dependent on ALA for aerobic and anaerobic growth. To obtain *alu* mutants, ethyl methanesulfonate treatment was followed by penicillin selection for strains unable to grow anaerobically without ALA. A total of 14 Alu mutants were collected. Transduction of the *hemA*$^+$ allele into mutants having the typical Alu phenotype (e.g., GE1390 and GE1820) showed that in a *hemA*$^+$ background these mutations have no visible effect on growth.

Genetic mapping of *alu* mutations. Approximate mapping of several *alu* mutations by conjugation showed that they are

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TABLE 1. *E. coli* K-12 strains

Strain	Genotype	Source or reference
E1847	*supE thi* Δ(*lac-proAB*) F′ *traD36 proA*$^+$ *proB*$^+$ *lacI*^{q}*ZΔM15 dppA30*::Kan	11
EV61	*thr-1 ara-14 leuB6* Δ(*gpt-proA*)*62 lacY1 tsx-33 supE44 galK2* Rac$^-$ *hisG4 rfbD1 mgl-51 rpsL31 kdgK51 xyl-5 mtl-1 argE3 thi-1 recB21 recC22 sbcB15 sbcC201 hemA*	15
EV149	Same as GE1387; *dppA30*::Kan	*dppA30*::Kan introduced into GE1387 by P1
EV176	Same as EV149; *hemA*$^+$	Ethyl methanesulfonate *hemA*$^+$ revertant of EV149
EV222	Same as GE1391; *recA srl*::Tn*10*	*recA srl*::Tn*10* introduced into GE1391 by P1
EV236	Same as GE1387; *recA srl*::Tn*10*	*recA srl*::Tn*10* introduced into GE1387 by P1
EV239	Same as GE1387; *hemA*$^+$ *recA srl*::Tn*10*	*hemA*$^+$ *recA srl*::Tn*10* introduced into GE1387 by P1
EV241	Same as EV149; *recA srl*::Tn*10*	*recA srl*::Tn*10* introduced into EV149 by P1
GE502	F$^-$ *ilvD145 his-85 trpR55 trpA9605 pro-48 tsx-84*	Laboratory collection
GE1358	F$^-$ *hemA204*	Mutant of W3110
GE1387	F$^-$ *ilvD145 his-85 trpR55 pro-48 tsx-84 hemA204 rpsL8* or *-9*	*trp*$^+$ *hemA204* and *rpsL8* or *-9* introduced into GE502 by P1
GE1390	Same as GE1387; *alu-6*	Mutant of GE1387
GE1391	Same as GE1387; *alu-7*	Mutant of GE1387
GE1393	Same as GE1387; *hemB208*	Mutant of GE1387
GE1820	Same as GE1387; *alu-20*	Mutant of GE1387
GE1825	F$^-$ *ilvD145 his-85 trpR55 trpA9605 pro-48 tsx-84 hemL102*::Tn*10 rpsl8* or *-9*	*hemL102*::Tn*10* and *rpsL8* or *-9* introduced into GE502 by P1
GE1832	Same as GE1820; *hemL102*::Tn*10*	*hemA*$^+$ and *hemL102*::Tn*10* introduced into GE1820 by P1
GE1836	Same as GE1387; *hemL102*::Tn*10*	*hemL102*::Tn*10* introduced into GE1387 by P1
GE1837	Same as GE1387; *hemB209*	Mutant of GE1387

located near min 80 on the chromosome. Further mapping was done by P1 transduction of the ability to use xylose as carbon source. Cotransduction at a frequency of 28 to 43% with *xylAB* was established for 6 of the 14 mutant strains. Mutations in two strains (GE1393 and GE1837) showed no cotransduction with *xylAB* and were determined by genetic mapping and complementation to occur in *hemB*. A representative *alu* mutation (in strain GE1820) was cotransduced with the *mtlA* gene at min 80.7 (4) at a frequency of 2%. These results indicate that the *alu* locus is located between min 79.5 and 80.0 on the chromosome. Six mutations which could not be mapped by P1 transduction were found by conjugation mapping to be in the region between min 74 and 80. These mutations may involve a deletion or rearrangement of the *alu* region, and the mutant strains behaved in a manner similar to that of strain GE1820 in the growth tests and complementation experiments described below.

Cosmid clones that complement *alu* mutations map to min 80. Transformants carrying cosmids complementing the *alu* mutation in GE1391 were selected on minimal-glucose plates. The analysis of DNA fragments from six transformants yielded a common 5.5-kbp *Eco*RI fragment (Fig. 1A) which hybridized strongly to Kohara (10) phage 9F6 and weakly to phage 1D1 on a membrane containing an ordered miniset of Kohara phage clones (data not shown). This locates the *Eco*RI fragment precisely to a region between 79.8 and 79.9 centisomes (Fig. 1A). DNA sequencing of the terminal regions of the *Eco*RI fragment revealed 380 nucleotides at the border which matched the *dppA* gene sequence (11) from bases 70 to 452 with 100% identity. The *dppA* gene is the first of several in an operon that encodes a dipeptide transport system (1, 2, 11). Further complementation experiments with subclones showed that the *dpp* operon DNA can complement the *alu* mutation.

Alu mutants are defective for dipeptide transport. Since our results showed that *dpp* DNA complements the *alu* mutation, we tested 11 strains carrying *hemA alu* mutations for their ability to utilize the dipeptide prolyl-glycine (Pro-Gly). These *alu* mutant strains (e.g., GE1391) were unable to grow in minimal glucose medium with Pro-Gly, whereas the parent strain GE1387 grew well. To determine whether a mutation in *dppA* would result in an Alu$^-$ phenotype (strict ALA requirement aerobically and anaerobically), we constructed a *hemA dppA30*::Kan strain (EV149) by P1 transduction of a lysate prepared from strain E1847 (11), which has a chromosomal insertion in the *dppA* gene (Table 1). Kanamycin-resistant transductants of GE1387 were unable to utilize Pro-Gly and also exhibited an Alu$^-$ phenotype. The mutant phenotypes of strains GE1391 and EV149 were reversed by recombinant cosmid DNA.

To determine the minimal DNA fragment necessary for complementation of the *alu* mutation, we tested subclones containing various portions of the *dpp* region for their abilities to alleviate both the Alu$^-$ and the Dpp$^-$ phenotypes. Clear reversal of both mutant phenotypes was accomplished with a 6.5-kbp *Ssp*I subclone inserted in the *Eco*RV site of pBR322 (pAL40) (Fig. 1B). Included on this plasmid are DNA sequences 325 bp upstream and approximately 4.6 kbp downstream of the *dppA* gene coding region. Complementation occurred irrespective of the insert orientation. Partial complementation of the mutations with plasmids carrying sequences downstream in the *dpp* operon but not with *dppA* alone was observed (Fig. 1B).

ALA transport is decreased in *alu* mutants. We compared the concentration of ALA that could support growth of the *hemA* versus the *hemA alu* mutants in minimal medium. Strain GE1387 grew with 0.03 μM (0.005 μg/ml) ALA supplementation in minimal glucose medium, whereas GE1391 and other *alu* mutants required 100-fold higher ALA concentrations (3 μM) for equivalent growth (data not shown).

To examine whether ALA transport was reduced in *alu* and/or *dppA* mutants, we performed uptake assays with [^{14}C]ALA. The results obtained with a final concentration of

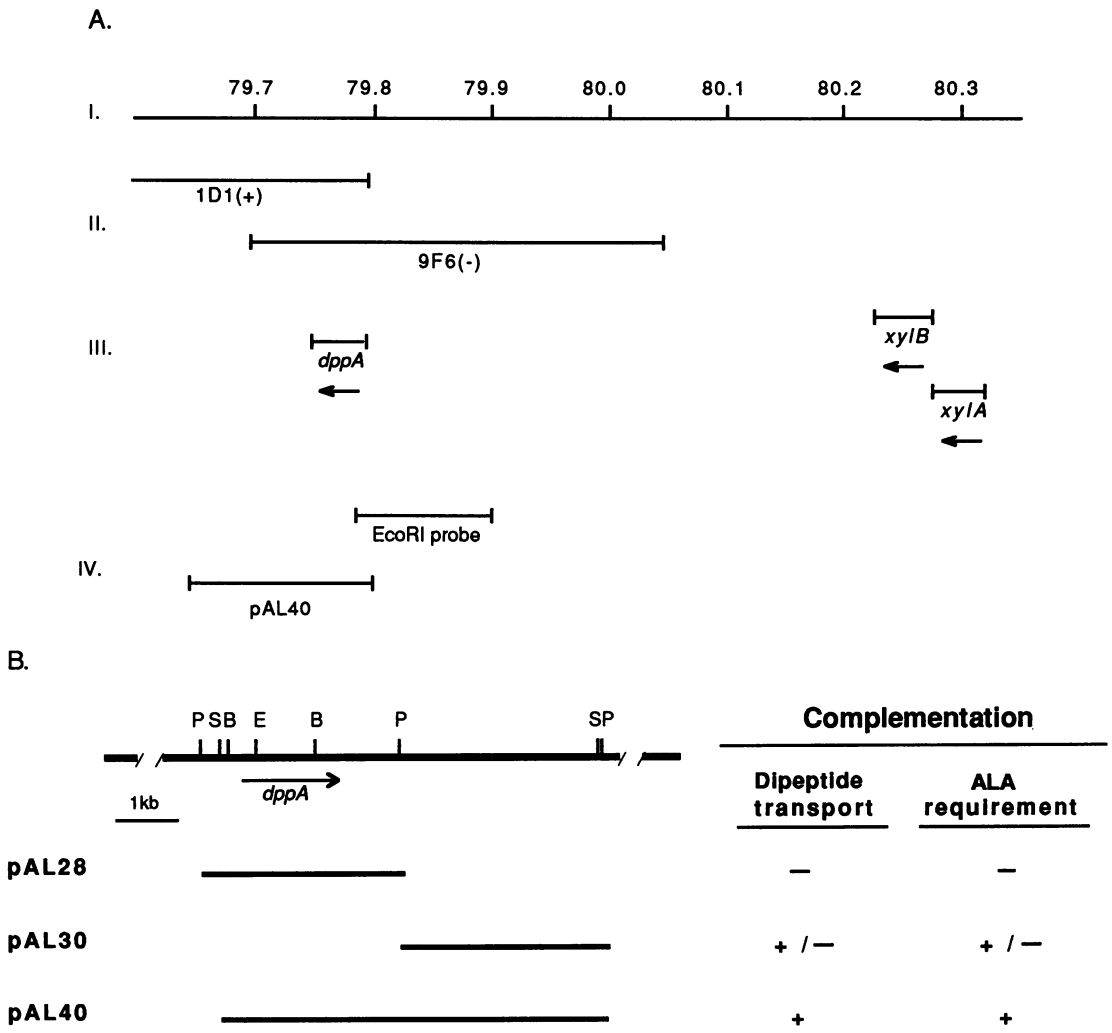

FIG. 1. (A) A schematic representation of the *E. coli* chromosome, based on version 6 of the integrated restriction map (13, 14) (I). The coordinates given denote centisomes (13) derived from the kilobase scale. The Kohara miniset clones (II) and the *dppA* and *xylAB* genes (III) are depicted. The arrows indicate the orientations of DNA sequences and genes. The DNA insert contained in pAL40 is labeled in line IV, along with the *Eco*RI fragment used to probe the Kohara phage miniset membrane. (B) Complementation of the *alu* mutation by subcloned DNA from the 80-min region of the *E. coli* chromosome. The chromosomal region flanking *dppA* is drawn schematically, and DNA restriction enzyme sites are indicated. The coding region of the *dppA* gene is represented by a solid arrow. The DNA fragments contained on subclones are depicted directly below their corresponding genomic DNA, and their capacities for complementation are indicated at the right side of the diagram. B, *Bam*HI; E, *Eco*RI; P, *Pst*I; S, *Ssp*I.

2 μM [^{14}C]ALA are presented in Fig. 2. Strains with mutations in *alu* (EV222) or *dppA* (EV241) accumulated severely reduced amounts of ALA compared with their parent strain EV236, consistent with the observation that these mutants grew very poorly on 2 μM ALA (data not shown). The presence of plasmid pAL40 in strains EV222 (Fig. 2), EV236, and EV241 (data not shown) enabled ALA uptake greater than that of strain EV236, presumably because of the presence of multiple copies of the *dpp* operon. The mutants accumulated [^{14}C]ALA that was provided at a 1 mM final concentration, but additional experiments to determine the uptake threshold were not done. In order to test whether dipeptides and ALA compete for the same transport machinery, Pro-Gly was added to an EV236 cell suspension at a concentration of 100 μM 10 min prior to the addition of [^{14}C]ALA. This abolished ALA uptake (Fig. 2), suggesting that the dipeptide competes with ALA for transport.

Another test of the role of the *dpp* mutations was based on the observation that *hemA* mutant or *hemA*$^+$ *E. coli* strains accumulate intermediate porphyrin compounds when grown in the presence of excess ALA (25 μg/ml), as evidenced by fluorescence of the colonies and surrounding medium under UV irradiation. Neither the *hemA alu* mutants, the *hemA dppA30*::Kan mutant, nor a *hemA*$^+$ *dppA30*::Kan strain (EV176) visibly accumulated porphyrins under these conditions, reinforcing the evidence that the *dpp* locus mediates ALA accumulation. Plasmid pAL40 reversed this phenotype.

Effect of *alu* mutations on the *hemL* phenotype. In the absence of ALA, *hemL* mutants are very leaky on both glucose and glycerol media (5, 7, 8). This leakiness is also

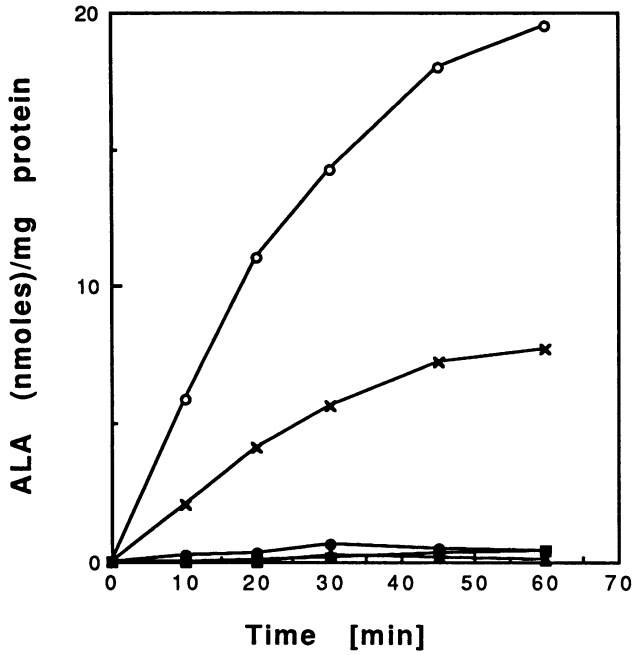

FIG. 2. Accumulation of [^{14}C]ALA by various strains of *E. coli* during uptake assays. x, EV236 (*hemA alu*$^+$); ●, EV222 (*hemA alu*); ▲, EV241 (*hemA dppA30*::Kan); ○, EV222/pAL40; ■, EV236 plus Pro-Gly.

apparent in strain GE1825, which is isogenic with GE1387, except for the fact that it has a Tn*10* insertion, *hemL102*::Tn*10* (7), in the *hemL* gene and is *hemA*$^+$. A double-mutant GE1836 that carries both *hemA204* and *hemL102*::Tn*10* had a growth phenotype similar to that of a *hemA* strain. In order to test the effect of *alu* mutations on the *hemL* phenotype, the *hemL* allele of GE1825 was introduced by P1 transduction into a *hemA*$^+$ derivative of the *alu-30* mutant GE1820. The resulting strain, GE1832, had a nonleaky *hemL* phenotype when grown aerobically on glycerol or glucose medium or anaerobically on glucose medium. Thus, the *alu* mutation makes both *hemA* and *hemL* mutants tight ALA auxotrophs.

Arginine stimulates growth of *hemA* mutants. In the course of this work, we observed an unexpected phenomenon. Arginine (25 μg/ml) stimulates the growth of *hemA* strains aerobically on glucose and glycerol minimal media. Mutations that abolished the stimulatory effect of arginine were selected, and these were identical to the *alu* mutations described above (data not shown). The basis of arginine rescue has not been characterized (see Discussion), but arginine did not affect [^{14}C]ALA accumulation by *E. coli* EV236 in uptake assays (data not shown).

DISCUSSION

Recently, it was shown (16) that *Salmonella typhimurium* mutations (*alu*) exist which show similarity to our *E. coli alu* mutations with respect to an increased ALA requirement (16) and to their map location in the *dpp* operon. It therefore seems likely that the *alu* loci of *E. coli* and *S. typhimurium* are equivalent.

We initiated this study expecting to reveal additional genes involved in the biosynthetic pathways of ALA formation. On the basis of genetic and biochemical evidence, an alternative route of ALA formation in *E. coli* and *S. typhimurium* was suggested (6). Instead, our studies revealed that ALA is actively taken up in *E. coli* by the dipeptide permease transport system. Since the *dpp* transport system shows great flexibility in accommodating structurally varied dipeptides (12), it may not be surprising that *E. coli* also uses this system for the transport of a nonpeptide substrate, the important porphyrin precursor ALA.

An intriguing question is how arginine can substitute for ALA in relieving the ALA auxotrophy in *hemA* mutants. There is no common intermediate in arginine and ALA biosynthesis. The two most similar intermediates, glutamate-1-semialdehyde in ALA synthesis and glutamate-5-semialdehyde, which is formed from two independent reactions catalyzed by the products of the *argE* or *proA* gene products in proline biosynthesis, are unlikely to be chemically (spontaneously) interconverted. The noted sequence similarity of acetylornithine aminotransferase (the *argD* gene product) and glutamate-1-semialdehyde aminomutase (see reference 5) is an indication of the known similarity of aminotransferases (7).

The observations in this study underscore the fact that there are still blank areas in the metabolic chart of an organism as well studied as *E. coli*.

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