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Cloning and Sequence of the *Salmonella typhimurium hemL* Gene and Identification of the Missing Enzyme in *hemL* Mutants as Glutamate-1-Semialdehyde Aminotransferase

THOMAS ELLIOTT,[1] YAEL J. AVISSAR,[2]† GI-EUN RHIE,[2] AND SAMUEL I. BEALE[2]*

Department of Microbiology, University of Alabama at Birmingham, Birmingham, Alabama 35294,[1] and Division of Biology and Medicine, Brown University, Providence, Rhode Island 02912[2]

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Salmonella typhimurium **forms the heme precursor δ-aminolevulinic acid (ALA) exclusively from glutamate via the five-carbon pathway, which also occurs in plants and some bacteria including *Escherichia coli*, rather than by ALA synthase-catalyzed condensation of glycine and succinyl-coenzyme A, which occurs in yeasts, fungi, animal cells, and some bacteria including *Bradyrhizobium japonicum* and *Rhodobacter capsulatus*. ALA-auxotrophic *hemL* mutant *S. typhimurium* cells are deficient in glutamate-1-semialdehyde (GSA) aminotransferase, the enzyme that catalyzes the last step of ALA synthesis via the five-carbon pathway. *hemL* cells transformed with a plasmid containing the *S. typhimurium hemL* gene did not require ALA for growth and had GSA aminotransferase activity. Growth in the presence of ALA did not appreciably affect the level of extractable GSA aminotransferase activity in wild-type cells or in *hemL* cells transformed with the *hemL* plasmid. These results indicate that GSA aminotransferase activity is required for in vivo ALA biosynthesis from glutamate. In contrast, extracts of both wild-type and *hemL* cells had γ,δ-dioxovalerate aminotransferase activity, which indicates that this reaction is not catalyzed by GSA aminotransferase and that the enzyme is not encoded by the *hemL* gene. The *S. typhimurium hemL* gene was sequenced and determined to contain an open reading frame of 426 codons encoding a 45.3-kDa polypeptide. The sequence of the *hemL* gene bears no recognizable similarity to the *hemA* gene of *S. typhimurium* or *E. coli*, which encodes glutamyl-tRNA reductase, or to the *hemA* genes of *B. japonicum* or *R. capsulatus*, which encode ALA synthase. The predicted *hemL* gene product does show greater than 50% identity to barley GSA aminotransferase over its entire length. Sequence similarity to other aminotransferases was also detected.**

The heme precursor, δ-aminolevulinic acid (ALA), is formed by two different biosynthetic routes: by condensation of glycine with succinyl-coenzyme A(CoA), catalyzed by ALA synthase (39); or from glutamate by a series of reactions including activation of glutamate by ligation to tRNA, reduction of the activated glutamate to yield glutamate-1-semialdehyde (GSA), and transamination of GSA to form ALA (Fig. 1). The ALA synthase route occurs in animal cells, yeasts, fungi, and certain bacteria including *Bradyrhizobium japonicum* (49) and *Rhodobacter capsulatus* (35). The five-carbon pathway from glutamate was first discovered in plants (5) but has also been found in a broad group of bacteria (3), including *Escherichia coli* (42, 52).

Two groups of ALA-requiring *Salmonella typhimurium* mutant strains were identified on the basis of separate locations of their mutated genes on the genetic map (21). One gene was designated *hemA* and the other *hemL*. The *S. typhimurium hemA* gene was cloned and sequenced (19) and found to be highly homologous to the *hemA* gene of *E. coli* (18, 43, 61). These *hemA* genes have no similarity to the *hemA* genes of *B. japonicum* and *R. capsulatus*, which encode ALA synthase (35, 49). In *E. coli, hemA* is required for expression of glutamyl-tRNA reductase, one of the steps of the five-carbon pathway (1). It has been determined that *hemA* encodes a structural component of that enzyme (2).

* Corresponding author.
† Present address: Department of Biology, Rhode Island College, Providence, RI 02908.

The *S. typhimurium hemL* gene has been characterized genetically, but the nature of its product has not been determined. It is thought that the *popC* gene of *E. coli* (55) is related to *hemL*, based on their similar map positions and phenotypes. In this report, we show that *S. typhimurium* forms ALA exclusively via the five-carbon pathway, report the nucleotide sequence of the *S. typhimurium hemL* gene, and identify the enzymatic deficiency responsible for the ALA requirement in *hemL* strains as GSA aminotransferase.

MATERIALS AND METHODS

Bacteria and bacteriophage. The *E. coli* and *S. typhimurium* strains used in this study are described in Table 1. The *S. typhimurium hemL* mutant strains used have been described previously (21). *E. coli* MH-1 (30) was used for the initial transformation and propagation of plasmids constructed in this study. *E. coli* TE1335 carries a derivative of the plasmid F'128 (*pro*$^+$ *lac*$^+$) with a P22 prophage integrated at the P22 attachment site of F'128. Its construction and use have been described previously (19). *E. coli recA* mutant strain CLT43 (56) was used for maxicell analysis of plasmid-encoded proteins.

Mu d-J refers to a transposition-defective mini-Mu phage, Mu dI-1734(*lac* Kan^r), constructed by Castilho et al. (13). This phage is deleted for all Mu functions except the transposon ends and the Mu *c* gene, and so lacks transposase.

Tn*10d*-Tet refers to a small transposition-defective deriv-

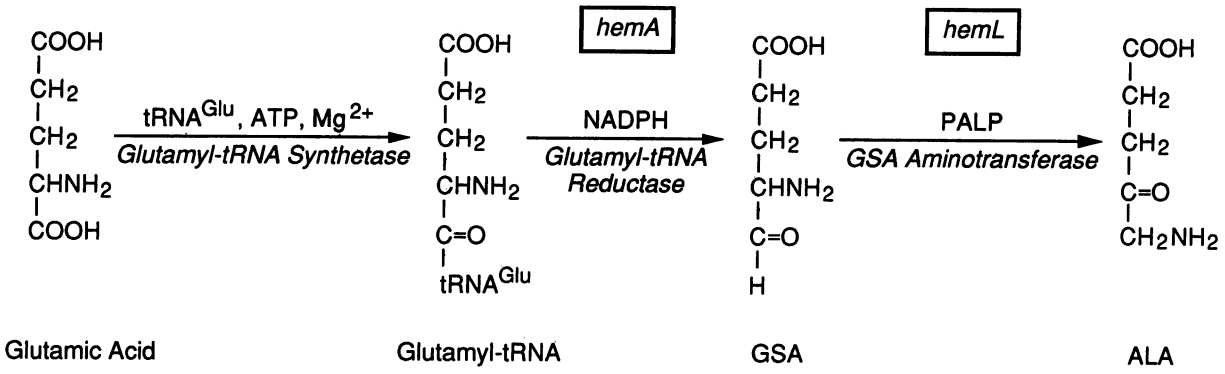

FIG. 1. Steps of ALA biosynthesis from glutamate via the five-carbon pathway. Also illustrated are the enzyme activities associated with the *hemA* and *hemL* genes. PALP, Pyridoxal phosphate.

ative of Tn*10*, Tn*10Δ16Δ17* Tet^r, constructed by Way et al. (63).

Cell culturing for determination of ALA biosynthetic route and aminotransferase activity. Strains were grown in E medium of Vogel and Bonner (62) supplemented with 0.5% (wt/vol) D-glucose. Cultures were grown in Erlenmeyer flasks on a rotary shaker (250 rpm) at 37°C.

Preparation of cell extracts. All procedures were done at 0 to 4°C. Cultures were harvested in the late exponential phase by centrifugation (4,000 × *g*, 15 min) and washed twice with extraction buffer [150 mM *N*-tris(hydroxymethyl)methylglycine (Tricine) (pH 7.9), 0.3 M glycerol, 20 mM $MgCl_2$, 5 mM dithiothreitol, 20 μM pyridoxal phosphate]. The cell pellet was weighed and suspended in 1.5 ml of extraction buffer per g of cells. Approximately one-third volume of glass powder (5-μm particle diameter) was added, and the suspension was sonicated with an MSE sonic disruptor for six 15-s periods with 30-s cooling intervals. The suspension was clarified by centrifugation (10,000 × *g*, 10 min). The extract was used directly or stored at −75°C until used for enzyme assays.

Assay of glutamate conversion to ALA. Incubations were done in 1.5-ml microcentrifuge tubes at 37°C for 1 h, in a total volume of 0.25 ml of extraction buffer supplemented with 10 mM potassium levulinate, 5 mM ATP, 2 mM NADPH, 200 μM glutamate containing 4 × 10^6 cpm as [1-^{14}C]glutamate (Dupont, NEN Research Products, Boston, Mass.), 1.25 mg of bovine serum albumin, 80 units of RNasin (Promega Biotec, Madison, Wis.), 0.6 A_{260} units of *E. coli* tRNA^{Glu} (Sigma Chemical Co., St. Louis, Mo.; added in 20 μl of RNA buffer containing 10 mM Tris hydrochloride [pH 7.5], 10 mM magnesium acetate, and 1 mM dithiothreitol), and 150 μl of cell extract.

ALA synthase assay. Incubations were done as above in extraction buffer supplemented with 8 mM ATP, 5 mM levulinate, 50 μM succinate, 50 μM glycine containing 4 × 10^6 cpm as [2-^{14}C]glycine (Dupont, NEN Research Products), 340 μM CoA, 240 μM pyridoxal phosphate, and 150 μl of cell extract.

Purification and analysis of labeled ALA. Incubations were terminated by the addition of 50 μl of 1 M citric acid and 250 μl of 10% (wt/vol) aqueous sodium dodecyl sulfate (SDS). The resulting mixtures were supplemented with 20 nmol of carrier ALA, heated to 95°C for 2 min, cooled rapidly to room temperature, and centrifuged in a microcentrifuge for 2 min to remove undissolved materials. The supernatant solu-

TABLE 1. Bacterial strains used in this study

Strain	Genotype	Source or reference
Escherichia coli		
CLT43	F$^-$ Δ(*argF-lac*)*U169 rpsL150 thiA1 relA1 deoC1 ptsF25 flbB5301 rbsR car-94 srl-300*::Tn*10 recA56*	56
MH-1	*araD139* Δ(*lac*)*X74 galU galK hsdR* (Str^r)	30
TE1335	*trp* Δ(*lac*)*X74* Str^r(F'128)(P22 HT*105/1 int-201 sieA44*)	19
W3110	F$^-$λ$^-$IN(*rrnD-rrnE*)*1*	C. Georgopoulos
Salmonella typhimurium		
LT-2	Wild type	J. Roth
TE162-1	*hemL331*::Mu d-J	21
TE162-5	*hemL335*::Mu d-J	21
TE274	*zae-1863*::Tn*10d*-Tet	21
TE395	*hemL331*::Mu d-J *recA1*	This study
TE3282	LT-2(pBR328)(Amp^r Cam^r Tet^r)	This study
TE3283	*hemL331*::Mu d-J *recA1*(pBR328)(Amp^r Cam^r Tet^r)	This study
TE3285	*hemL331*::Mu d-J *recA1*(pTE290)(Amp^r Cam^r *hemL*$^+$)	This study
TN1379	*leuBCD485*	34
TR5877	(SL4213 *gal*$^+$) *hsdL6 hsdSA29* (r_{LT}^- m_{LT}^+ r_S^- m_S^+) *metA22 metE551 ilv-452 trpB2 xyl-404 rpsL120* (Str^r) H1-b H2-e,n,x (Fels2$^-$) *nml*$^-$	B.A.D. Stocker
TR6612	*polA2 ara-9*	J. Roth

tions were applied to 250-μl columns of Dowex 50W-X8 (Na) contained in 1-ml plastic syringes. The columns were washed sequentially with 0.5 ml of water, 0.5 ml of 50 mM (Na^+) sodium citrate buffer (pH 3.0) containing 25% methanol (to remove the remaining SDS), 0.5 ml of 0.2 M (Na^+) sodium citrate buffer (pH 4.25), and 0.5 ml of distilled water. ALA was eluted with 1 ml of 0.5 M (Na^+) sodium phosphate buffer (pH 6.8) and heated to 95°C for 20 min in the presence of 40 μl of ethyl acetoacetate to form 1-methyl-2-carboxyethyl-3-propionic acid pyrrole (ALA-pyrrole) (48). The ALA-pyrrole solutions were adjusted to pH 8.5 with KOH, extracted twice with diethyl ether, and then adjusted to pH 2.5 with HCl, and the ALA-pyrrole was extracted three times with equal volumes of ether. The acidic ether extracts from each incubation were combined, back-extracted with 1 ml of water, transferred into a 7-ml plastic scintillation vial, and dried in air. The ALA-pyrrole was dissolved in 5 ml of Tritosol (24) scintillation liquid, and its radioactivity was determined with a Beckman scintillation counter.

Paper chromatography of ALA-pyrrole. ^{14}C-ALA-pyrrole obtained as described above was dissolved in diethyl ether and applied to a strip of Whatman 3MM paper and then chromatographed in the ascending direction with *n*-butanol–*n*-propanol–5% (wt/vol) aqueous NH_4OH (2:1:1, vol/vol/vol) (5). Each lane was cut into 1-cm segments which were immersed into 5.0 ml of Tritosol scintillation fluid, and the radioactivity was determined by liquid scintillation counting. Standard ALA-pyrrole was visualized after chromatography with Ehrlich spray reagent (200 mg of *p*-dimethylaminobenzaldehyde, 8 ml of ethanol, 2 ml of 12 N HCl) (5).

Succinyl-CoA synthetase assay. Succinyl-CoA synthetase was assayed by a published method (38) that was modified to bring the incubation conditions close to those used for the ALA synthase assay. Incubations were done in 1.5-ml microcentrifuge tubes at 37°C for 30 min in a total volume of 0.5 ml containing 800 mM NH_2OH (neutralized), 100 mM Tricine (pH 7.9), 100 mM disodium succinate, 5 mM ATP, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.4 mM CoA, and cell extract containing approximately 1 mg of protein. Incubation was started by the addition of cell extract and stopped by the addition of 0.5 ml of 5% (wt/vol) $FeCl_3$ in 0.1 N HCl, 0.5 ml of 3 N HCl, and 0.5 ml of 12% (wt/vol) trichloroacetic acid. After mixing, the suspension was clarified by centrifugation and the A_{540} was measured. The concentration of succinohydroxamic acid was calculated by using an absorption coefficient of 61.0 mM^{-1} (38).

GSA aminotransferase assay. Low-molecular-weight material was removed from cell extract by passage through a column of Sephadex G-25 previously equilibrated with assay buffer (50 mM Tricine [pH 7.9], 1 M glycerol, 15 mM $MgCl_2$, 1 mM dithiothreitol, 20 μM pyridoxal phosphate). Incubations were done at 30°C in 250 μl of reaction medium consisting of assay buffer supplemented with 5 mM levulinate, 5 μM GSA, and enzyme extract containing 10 to 20 mg of protein. The reaction was initiated by the addition of substrate (GSA) and terminated after 20 min with 25 μl of 1 M citric acid and 250 μl of 10% (wt/vol) SDS. The resulting mixture was heated at 95°C for 3 min and cooled rapidly to room temperature. The samples were applied to 0.25-ml columns of Dowex 50W-X8 (Na). The columns were washed sequentially with 0.5 ml of water, 0.5 ml of 50 mM (Na^+) sodium citrate buffer (pH 3.0) containing 20% (vol/vol) methanol, and 0.5 ml of water. ALA was eluted with 0.5 ml of 0.5 M (Na^+) sodium phosphate buffer (pH 6.8). The eluted ALA was supplemented with 20 μl of ethyl acetoacetate and converted to ALA-pyrrole by heating at 95°C for 15 min. The ALA-pyrrole was reacted with 520 μl of Ehrlich-Hg reagent (60), and the A_{553} was measured. The A_{553} of a control sample that was incubated with heat-denatured enzyme was subtracted from those of the other samples to determine net A_{553} values, and ALA concentration was calculated from a standard curve.

DOVA aminotransferase assay. Incubations and quantitation of product were identical to those for the GSA aminotransferase assay, except that in place of GSA, the incubation mixtures contained 100 mM glutamate and 2.6 mM γ,δ-dioxovalerate (DOVA). DOVA was prepared and quantitated as previously described (23).

Media and growth conditions for genetic studies. Nutrient broth (0.8% [wt/vol], Difco Laboratories) with 0.5% (wt/vol) NaCl added and LB broth (51) were used as rich media. For testing growth on glycerol as the sole carbon source, minimal NCE medium was used (7) (NCE medium is medium E of Vogel and Bonner [62] modified to eliminate citrate). Difco Bacto-Agar was added at a final concentration of 1.5% (wt/vol) for solid media. Supplementation with ALA was at 1 μM in minimal medium and 150 μM in rich medium. Antibiotics were added to final concentrations in rich medium as follows: sodium ampicillin, 30 μg ml^{-1}; kanamycin sulfate, 50 μg ml^{-1}; and chloramphenicol, 20 μg ml^{-1}. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; Bachem) was used at 20 μg ml^{-1}.

Genetic techniques. The high-frequency generalized transducing bacteriophage P22 mutant HT*105/1 int-201* (57) was used for all transductions. Phage P22 lysates of *S. typhimurium* strains were prepared as described by Davis et al. (16). Details of transduction methods were as previously described (20).

Plasmid constructions. Techniques for plasmid constructions followed standard methods (9, 46). DNA fragments were purified from excised gel slices with a GeneClean kit (Bio 101). Plasmid deletion mutants were constructed by unidirectional exonuclease III digestion (33).

Complementation and recombination analysis. Mutants of *S. typhimurium* defective in the *hemL* gene exhibit a slow-growth phenotype on NB or LB medium (they form small colonies), while they fail to grow on minimal NCE glycerol agar, provided the inoculum contains a sufficiently small number of cells (20; also see below). Both phenotypes are corrected by ALA addition at the appropriate concentration.

Complementation was tested by introducing plasmids into strain TE395 *hemL331*::Mu d-J *recA1* by transduction, followed by testing growth phenotypes on NB medium and on minimal NCE glycerol agar in the absence or presence of ALA.

Recombination was tested as follows. Plasmids were introduced into *recA*$^+$ *hemL*::Mu d-J strains by transduction, selecting for Amp^r. For plasmids which cannot complement the *hemL* mutation, nearly all the Amp^r plasmid-carrying transductants exhibited a Hem$^-$ phenotype. Hem$^-$ Amp^r transductants were purified several times and then grown overnight in NB medium and plated on minimal NCE glycerol agar to select for Hem$^+$ recombinants.

DNA sequencing. DNA was sequenced by the dideoxy method as described previously (19), using alkaline-denatured CsCl-purified double-stranded plasmid DNA as the template. The T7 DNA polymerase (Sequenase) and sequencing kit were from U.S. Biochemical Corp.

The exonuclease III-generated plasmid deletion mutants were used as templates to obtain sequence information from one strand. To obtain the sequence of the complementary strand, appropriately spaced primers were synthesized. (The

TABLE 2. In vitro ALA formation from glutamate or glycine[a]

Source of label for incubation	Cell growth condition	Incubation time (min)	Label incorporation into ALA fraction (cpm)	Net ALA formation (pmol mg of protein^{-1})
[1-^{14}C]glutamate	−Cell extract	60	158	
[1-^{14}C]glutamate	−Levulinate	0	106	
[1-^{14}C]glutamate	−Levulinate	60	2,513	7.9
[1-^{14}C]glutamate	+Levulinate	0	156	
[1-^{14}C]glutamate	+Levulinate	60	5,486	24.7
[2-^{14}C]glycine	−Cell extract	60	1,059	
[2-^{14}C]glycine	−Levulinate	0	505	
[2-^{14}C]glycine	−Levulinate	60	477	
[2-^{14}C]glycine	+Levulinate	0	1,048	
[2-^{14}C]glycine	+Levulinate	60	1,033	

[a] Hem$^+$ strain LT-2 cells were grown to the late exponential phase in minimal glucose medium. Where indicated, 16 mM levulinate was added to the culture 2 h before harvest. Cells were extracted, incubations were done with the indicated source of label to measure ALA formation via the five-carbon and ALA synthase routes, ALA was extracted and purified, and its radioactivity was counted as described in the text. Incubation mixtures contained 3.8 mg (cells grown without levulinate) or 2.7 mg (cells grown with levulinate) of cell protein and 4×10^6 cpm of radioactivity.

sequences of these primers are available on request.) The sequence was determined on both strands. In some cases, pUC119 and pUC120 derivatives carrying fragments from the *hemL* gene were also used as templates for sequencing.

DNA sequences were edited and analyzed for open reading frames and restriction sites by using the DNA Strider program (47). Some analyses were performed by using the DNA Inspector IIe program (Textco, West Lebanon, N.H.). Both programs were run on a Macintosh SE computer (Apple Computer Inc., Cupertino, Calif.). Database searches were run against GenBank release 60.0 (June 1989) by using the University of Wisconsin Genetics Computer Group programs (17) run on a VAX 6000-410 computer.

Southern hybridization. Chromosomal DNA was purified from *E. coli* and *S. typhimurium* after lysis with lysozyme-EDTA-SDS and digestion with proteinase K as described previously (25). After phenol extraction and ethanol precipitation, the DNA was recovered by spooling on a glass rod, dried briefly, and then suspended in 10 mM Tris hydrochloride (pH 8.0)–0.1 mM EDTA. After restriction endonuclease digestion, DNA fragments were separated on agarose gels and transferred to Hybond N nylon membranes (Amersham) as described by the manufacturer (4-h transfers by the alkaline transfer protocol). Gel-purified fragments used as probes were labeled with [α-^{32}P]dATP (Amersham) by a random priming procedure (22). Prehybridization, hybridization, and washes were done in SSPE buffers as described by Maniatis et al. (46).

Phage lysates of appropriate clones from the Kohara phage λ library of *E. coli* (41) were prepared by gridding as described previously (32). Plaque lifts were made to Hybond N, and hybridization was performed as described above for Southern hybridizations.

Maxicell analysis of plasmid-encoded proteins. Plasmids to be analyzed were introduced into *E. coli* CLT43 by transformation. Maxicell preparation, protein labeling, electrophoresis, and detection were all performed exactly as previously described (19).

Other procedures. Protein concentration was determined by the method of Bradford (10) with bovine serum albumin as the standard. GSA was chemically prepared from 4-amino-5-hexenoic acid (a gift from Merrell Dow Pharmaceuticals, Inc., Cincinnati, Ohio) by the method of Gough et al. (26).

Nucleotide sequence accession number. The GenBank nucleotide sequence accession number is M36054.

RESULTS

ALA formation from glutamate by extracts of *S. typhimurium*. Initial experiments were designed to measure the incorporation of radioactivity into ALA from [1-^{14}C]glutamate and [2-^{14}C]glycine by extracts of *S. typhimurium*, using methodology adapted from previous experiments with extracts of photosynthetic bacteria and *E. coli* (2, 3). These were difficult to interpret because the rate of ALA synthesis by *S. typhimurium* extracts was very low. It was found that in vitro ALA-forming activity of the extracts could be increased considerably by adding levulinate to the culture medium shortly before harvesting the cells. With [1-^{14}C]glutamate as the source of label (under five-carbon pathway assay conditions), incubated samples produced significantly more radioactivity in the final acidic ether extract than unincubated control samples (Table 2). If the cells were grown in the presence of levulinate, the extracts produced increased amounts of radioactive ALA in the incubated samples, but the unincubated control samples were unchanged. With [2-^{14}C]glycine as the source of label (under ALA synthase assay conditions), the acidic ether extract from incubated samples contained no more radioactivity than that from unincubated control samples or from reaction mixtures incubated without cell extract. Extracts from levulinate-treated cells appeared to have increased radioactivity in the acidic ether extract of both the incubated and control samples when [2-^{14}C]glycine was the source of label. Background levels of radioactivity in the acidic ether extract derived from unincubated control samples and reaction mixtures incubated without cell extract were higher with [2-^{14}C]glycine than with [1-^{14}C]glutamate as the source of label.

Portions of the acidic ether extracts from the above incubations were further analyzed by paper chromatography. Significant amounts of radioactivity in the products from incubations with [1-^{14}C]glutamate comigrated with authentic ALA-pyrrole, whereas very little or no radioactivity comigrated with ALA-pyrrole in the products derived from the incubations with [2-^{14}C]glycine (Fig. 2). Cells grown in the absence of levulinate yielded significant amounts of radioactive ALA product from [1-^{14}C]glutamate as measured after paper chromatography. Addition of levulinate to the cultures increased the amount of radioactivity in the final chromatography product approximately threefold.

To confirm that the inability of the extracts to catalyze

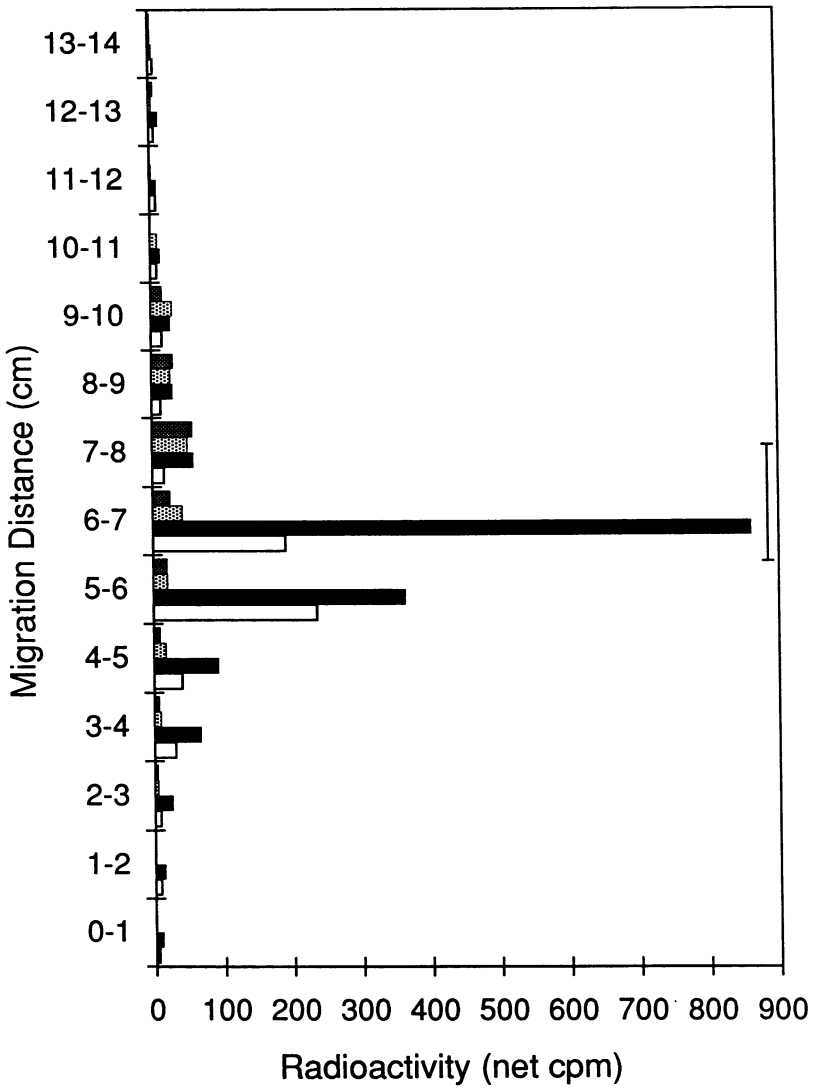

FIG. 2. Paper chromatography of radioactive ALA-pyrrole derivatives from samples listed in Table 2, generated during incubation of cell extracts with [1-^{14}C]glutamate and [2-^{14}C]glycine. Hem$^+$ strain LT-2 cells were grown in glucose-based minimal medium. Where indicated, 16 mM levulinate was added to the medium 2 h before harvest. Preparation and incubation of cell extracts, extraction, derivatization, purification of ALA, and paper chromatography were done as described in the text. White bars, Cells grown without levulinate, extract incubated for 60 min with [1-^{14}C]glutamate; black bars, cells grown with levulinate, extract incubated for 60 min with [1-^{14}C]glutamate; light stipple, cells grown without levulinate, extract incubated for 60 min with [2-^{14}C]glycine; dark stipple, cells grown with levulinate, extract incubated for 60 min with [2-^{14}C]glycine. Position of authentic ALA-pyrrole is indicated by brackets at the right margin.

ALA formation from glycine was due to the absence of ALA synthase activity, it was necessary to demonstrate that the extracts were capable of forming the ALA synthase substrate, succinyl-CoA, from the supplied succinate. Under reaction conditions similar to those used for the ALA synthase assay, 2.4 nmol of succinyl-CoA was formed per mg of protein during the 30-min incubation period. This rate is far in excess of the measured rates of ALA synthesis.

GSA aminotransferase activity in cell extracts. The parental *hemL*$^+$ strain, LT-2, yielded in vitro GSA aminotransferase activity (Table 3). Activity was absent in heat-denatured extract, and comparable amounts of activity were obtained from LT-2 cells grown in the presence or absence of added ALA. In contrast, extracts of *hemL* strain TE162-5 had little or no activity. Transformant strain TE3285, a *hemL* mutant which carries a plasmid with the *hemL* gene, had extractable GSA aminotransferase activity slightly higher than that of untransformed LT-2 cells, but extracts of strain TE3283, a *hemL* mutant with a control plasmid not containing the *hemL* gene, yielded little or no activity. The higher activity in extracts of strain TE3285 compared with LT-2 was not due to the presence of the plasmid per se or growth in medium supplemented with ampicillin, because strain LT-2 carrying a control plasmid (TE3282) yielded a level of activity similar to that of untransformed LT-2 cells. Slightly higher activity was obtained from strain TE3285 grown in the presence of ALA than in its absence.

DOVA aminotransferase activity in cell extracts. DOVA aminotransferase activity was measured in extracts of wild-type strain LT-2 and *hemL* strain TE162-5 cells. Both strains yielded comparable activity (Table 4). The measured net ALA-forming activity was greater than that of GSA ami-

TABLE 3. GSA aminotransferase activity[a]

Strain description	Addition to medium	ALA formation	
		Total nmol	Net nmol mg of protein^{-1}
Expt 1			
LT-2 (heat denatured)		0.45	
LT-2		2.97	1.59
LT-2	ALA	2.56	1.34
TE162-5 *hemL*	ALA	0.46	0.01
TE3283 *hemL*(pBR328)	Ampicillin	0.61	0.09
TE3283	Ampicillin + ALA	0.51	0.05
TE3285 *hemL*(pTE290) (*hemL*$^+$)	Ampicillin	3.53	1.95
TE3285	Ampicillin + ALA	3.28	2.50
Expt 2			
LT-2 (heat denatured)		0.75	
LT-2		3.07	1.34
TE3282 [LT-2(pBR328)]	Ampicillin	2.31	1.28

[a]Cells were grown in liquid minimal medium with glucose as the carbon source and containing the indicated additions. ALA and ampicillin were added at 20 μM and 15 μg ml^{-1}, respectively. Cells were harvested in the late exponential growth phase. Cell disruption, preparation of extracts, and assay for GSA aminotransferase activity were done as described in the text.

notransferase in incubations performed under similar conditions. However, no attempt was made to optimize the reaction conditions for either assay. Considerable ALA was formed from DOVA in the absence of cell extract, in agreement with a previous report (6). However, little ALA was formed in incubations containing heat-denatured cell extract.

Growth of strains in liquid medium with glycerol as the carbon source. Parental strain LT-2 (*hemL*$^+$) grew vigorously in liquid medium containing the nonfermentable carbon source glycerol, whereas strain TE162-5 (*hemL* mutant) grew very poorly unless the medium was supplemented with ALA (Table 5). Transformant strain TE3285, which carries a plasmid containing the *hemL* gene, grew in glycerol medium containing ampicillin, whereas strain TE3283, which carries a control plasmid without the *hemL* gene, grew very poorly unless the medium was supplemented with ALA. Both plasmids conferred resistance to ampicillin.

Although growth of the HemL$^-$ strains was poor in unsupplemented glycerol medium, very slow but steady growth did occur, with cultures reaching saturating cell density after approximately 60 h, compared with the much faster growth (saturating density in approximately 16 h) in medium containing ALA.

Growth of strains in liquid medium with glucose as the carbon source. Parental strain LT-2 (*hemL*$^+$) grew vigorously in liquid medium containing the fermentable carbon source D-glucose, whereas strain TE162-5 (*hemL* mutant) grew poorly unless the medium was supplemented with ALA (Table 5). Transformant strain TE3285, carrying a plasmid containing the *hemL* gene, grew in the glucose medium containing ampicillin. Strain TE3283, which carries a control plasmid without the *hemL* gene, also grew well in this medium, even without supplementation with ALA.

Growth of HemL$^-$ strains TE162-5 and TE3283 in glucose-containing medium without ALA was further investigated. With strain TE3283, it was found that the presence of ampicillin was required for good growth to occur. However, ampicillin could be replaced with cysteine at an equivalent molar concentration (Table 5). Strain TE162-5 grew nearly as well in cysteine-supplemented medium as in ALA-supplemented medium. Both HemL$^-$ strains grew very slowly, but steadily, in glucose medium without supplementations.

Cloning of *S. typhimurium hemL* gene. The *hemL* gene was isolated by screening for complementation of the Hem$^-$ phenotype of an *S. typhimurium hemL* mutant. As a source of the *hemL* gene, a library of clones was used which contains 7- to 10-kb *Sau*3A partial digestion products of *S. typhimurium* DNA from strain TN1379 (34). These fragments have been inserted in the *Bam*HI site of pBR328, and the resulting recombinant plasmids specify Amp^r and Cam^r. A phage P22 transducing lysate was grown on this pool of

TABLE 4. DOVA aminotransferase activity[a]

Strain and incubation conditions	ALA formation	
	Total nmol	Net nmol mg of protein^{-1}
No cell extract	3.01	
LT-2 (no DOVA added)	0.07	
LT-2 (heat denatured)	0.59	
LT-2	8.18	5.23
TE162-5 (no DOVA added)	0.06	
TE162-5 (heat denatured)	0.62	
TE162-5	8.48	5.62

[a]Cells were grown in liquid minimal medium with glucose as the carbon source. Wild-type (LT-2) and *hemL* (TE162-5) cells were harvested in the late exponential growth phase. Cell disruption, preparation of extracts, and assay for DOVA aminotransferase activity were done as described in the text.

TABLE 5. Growth in various media[a]

Carbon source	Addition(s) to medium	Growth of strain:			
		LT-2	TE162-5	TE3283	TE3285
Glycerol	None	+	−		
Glycerol	ALA		+		
Glycerol	Ampicillin			−	+
Glycerol	Ampicillin + ALA			+	
Glucose	None	+	−	−	+
Glucose	ALA	+	+	+	
Glucose	Ampicillin			+	+
Glucose	Ampicillin + ALA			+	+
Glucose	Cysteine		+	+	

[a]Cells were grown in liquid minimal medium with glycerol or glucose as the carbon source and containing the indicated additions. ALA, ampicillin, and cysteine were added at 20 μM, 15 μg ml^{-1}, and 5 μg ml^{-1}, respectively. Growth was scored positive (+) if overnight incubation with a small inoculum produced a stationary- or near-stationary-phase culture.

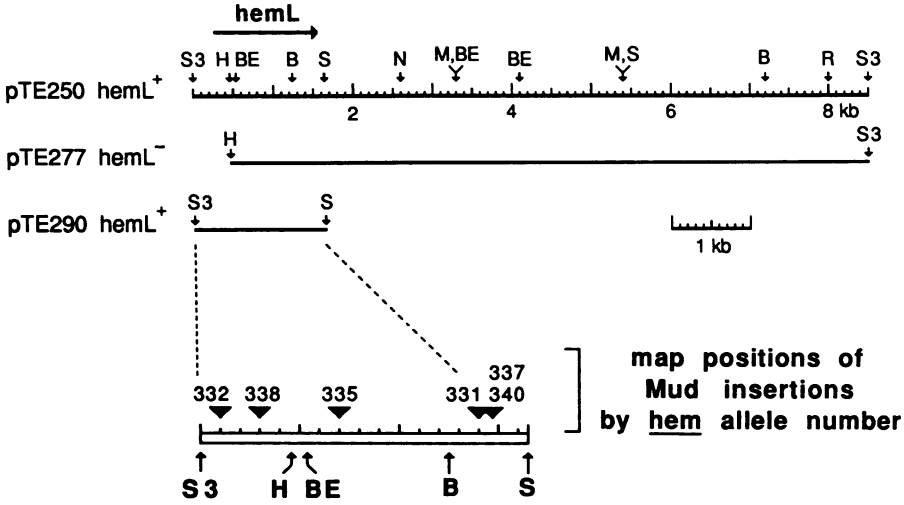

FIG. 3. Restriction maps of plasmids used in this study. Restriction sites are indicated as follows: B, *Bam*HI; BE, *Bst*EII; H, *Hin*dIII; M, *Mlu*I; N, *Nhe*I; R, *Eco*RI; S, *Sal*I; S3, *Sau*3A. The *hemL* open reading frame is indicated by the horizontal arrow. Also illustrated are the points of insertion of Mu d-J in various *hemL* mutant strains.

clones and used to transduce strain TE162-1 (*hemL331*::Mu d-J) to Amp^r on NB agar. Most of the Amp^r transductants were Hem$^-$ and formed small colonies, but approximately 0.1% of the Amp^r transductants had a Hem$^+$ (large colony) phenotype.

Nearly all these Hem$^+$ transductants were still Kan^r, indicating that the chromosomal *hemL*::Mu d-J insertion mutation was still present. For several such strains, the presence of a plasmid able to complement the *hemL* defect was confirmed by using phage P22 to transduce the plasmid into strain TE395 (*hemL331*::Mu d-J *recA1*). The Hem$^+$ and Amp^r phenotypes were completely linked in such crosses; all colonies inheriting Amp^r simultaneously became Hem$^+$.

Plasmid DNA was prepared from six Hem$^+$ strains and used to transform *E. coli* MH-1, selecting for Amp^r. Plasmids present in the transformants were analyzed by restriction enzyme digestion with *Sau*3A and other enzymes (data not shown). The digestion patterns indicated that three different plasmids were represented among the six isolates and that all the plasmids carried related DNA inserts. A restriction map was determined for one such plasmid, pTE250 (Fig. 3).

Deletions were made by first digesting pTE250 with *Mlu*I, which cuts at 3.3 and 5.4 kb of the map in Fig. 3, and *Sph*I, which cuts in the *tet* gene of pBR328. Since *Sph*I gives a 3′ overhang which is resistant to exonuclease III, while the *Mlu*I end is sensitive, the resulting deletions extend into the *hemL* gene from the 3′ end (the right side of the map in Fig. 3). In these plasmids, various lengths of DNA which all retain the 5′ end of the *hemL* gene were joined to the *Sph*I site of pBR328.

Two other deletion plasmids were used. The plasmids pTE277 and pTE290 were constructed by first digesting pTE250 with *Hin*dIII or *Sal*I (respectively), followed by religation. This results in loss of the DNA between the corresponding sites in pBR328 and the *S. typhimurium* insert. Maps of the inserts of these plasmids are also shown in Fig. 3.

It was important to show that these plasmids carry the *hemL* gene and not some other gene which complements at high copy number. The plasmid pTE250 was transduced into TR6612 (*polA2*), which is nonpermissive for ColE1 replication (27). Selection for Amp^r gave transductants that had pTE250 integrated at the *hemL* locus, as shown by linkage of the plasmid's Amp^r marker to a Tn*10d*-Tet near *hemL*. Additionally, in other experiments it was found that some deletion derivatives of pTE250 (e.g., pTE277, Fig. 3) had lost the ability to complement but could still recombine with *hemL* mutants.

Southern hybridization analysis of *hemL*::Mu d-J mutants. A physical map of the sites of Mu d-J transposon insertion within the *hemL* gene was constructed by Southern hybridization analysis. This was done primarily to test whether all the Mu d-J insertions map within the cloned DNA segment carried by pTE290, and secondarily to verify the genetic map determined previously (21). That genetic map summarizes the following information; (i) all Lac$^+$ *hemL*::Mu d-J mutants (those forming blue colonies on medium containing X-gal) carry Mu d-J in the same orientation in the chromosome, as assayed by a recombination test (36); (ii) the *hemL* gene is transcribed counterclockwise on the standard *S. typhimurium* genetic map; (iii) five intervals could be distinguished by deletion mapping. Each interval contains one Lac$^+$ Mu d-J except the most 3′ interval, which contains two; and (iv) the order of insertion sites for these Mu d-J insertions on the map, proceeding 5′ to 3′, is 332-338-335-331-337, 340. Insertion mutations are identified here and in Fig. 3 by their *hem* allele number. Insertions 337 and 340 are very close together and could not be separated by recombination.

The map resulting from these hybridization experiments is shown in Fig. 3. To summarize, (i) all *hemL*::Mu d-J insertion mutants map within the *Sau*3A-*Sal*I interval that was sequenced; (ii) the most 5′ Mu d-J maps within 100 to 200 bp 5′ N terminus of HemL, while the most 3′ Mu d-J maps near the C terminus; and (iii) the order of insertion sites predicted from the genetic map was confirmed in the physical map. Interpretation of the Southern hybridization data was simplified by considering only this set of Lac$^+$ Mu d-J insertions, each of which has been shown to have the same orientation in the chromosome. The direction of transcription of *hemL* relative to the physical map (Fig. 3) is suggested by the polarity of the open reading frame in the DNA sequence (see below). In addition, the DNA sequence

suggests that *fhuB* lies directly to the right of *hemL* on the physical map (Fig. 3; see below), and from the knowledge that these two genes are convergently transcribed, it can be inferred that transcription of *hemL* is from left to right.

The map positions determined by this method are only approximate. The data also do not allow us to distinguish simple insertions from insertions accompanied by small deletions or duplications of *hemL* DNA. However, placement of the insertions with respect to restriction sites which delimit probe sequences is unambiguous.

DNA sequence of *hemL* gene. The DNA sequence of *hemL* was determined by using deletion plasmids as templates for primer extension (Fig. 4). Given the sequence of one strand obtained by this method, oligonucleotide primers were constructed to sequence the complementary strand. The *hemL* gene is transcribed left to right relative to the map in Fig. 3. The region sequenced extends from the *Sau*3A site at the left end of the map (bp 0) to the *Sal*I site (bp 1653).

The sequence contains one long open reading frame with the polarity established for *hemL*. The *hemL* gene is the open reading frame which starts with an AUG at bp 231 and ends with a UAA at bp 1509. (Codons are numbered according to the position of the 5′ nucleotide in the sequence.) Translation of this open reading frame would result in synthesis of a polypeptide of 426 amino acids and a molecular weight of 45,273. Codon preference analysis showed that the *hemL* gene has a pattern of codon usage similar to that of weakly expressed *E. coli* genes.

The AUG codon at bp 231 was assigned as the N terminus of HemL because it is the first AUG codon in the long open reading frame encoding HemL, and it is preceded by what appears to be an excellent ribosome-binding site. Also underlined in Fig. 4 are two hexanucleotides with sequence and spacing similar to *E. coli* and *S. typhimurium* promoter elements, as well as a sequence at the 3′ end of *hemL* similar to rho-independent terminators. At present, it is not known whether the promoter(s) responsible for expression of *hemL* is included in the sequenced region, since it is possible that plasmid promoters contribute to the expression seen in complementation and maxicell experiments.

Other genes near *hemL*. Comparison of the *hemL* DNA sequence with sequences in the GenBank and EMBL databases revealed that the region downstream of the *Bam*HI site (from bp 1258 to 1509) is highly similar to that previously reported for a segment beyond the 3′ end of the *E. coli fhuB* gene (40). Thus, the *popC* gene (which is the *E. coli* equivalent of *hemL*) and the *fhuB* gene are adjacent and transcribed convergently. This conclusion is also consistent with previous genetic analysis. The *Eco*RI site 410 bp beyond the 3′ end of the *fhuB* gene (at the end of the sequence reported by Koester and Braun [40]) is at exactly the same position as the *Bam*HI site (bp 1258) of the *hemL* sequence.

The two sequences show 84% identity (212 bp in a 253-bp overlap), beginning at the *Bam*HI site (bp 1258) and continuing to the *hemL* termination codon (bp 1509). We suggest that this *E. coli* sequence is the 3′ end of the *E. coli popC* gene. There is one nucleotide present in the *S. typhimurium* sequence that is not found in the published *E. coli* sequence. Assuming that this represents an error in one of the sequences, the *S. typhimurium* and *E. coli* HemL protein fragments can be aligned nearly perfectly. The amino acid sequence predicted for *E. coli* HemL is more conserved than the nucleotide sequence, showing 95% identity to *S. typhimurium* HemL (79 amino acids in an 83-amino-acid overlap).

The restriction map of the *fhuACDB* region, based on the DNA sequence (12, 14, 15, 40) is entirely consistent with that of the *E. coli* physical map between 170 and 175 kb (41, 50). In addition, hybridization of probes derived from the *S. typhimurium hemL* gene to Southern blots of genomic digests from *E. coli* W3110, as well as to DNA of lambda phage from the Kohara library, establish that *hemL* lies in this region of the map (T. Elliott, unpublished data). The *hemL* *Bam*HI site (bp 1258), which in the *E. coli* sequence is an *Eco*RI site, lies at 175.86 kb of the Kohara map (coordinates of Medigue et al. [50]).

However, the sequence downstream of *S. typhimurium hemL* is not similar to the *E. coli fhuB* sequence. The two sequences diverge precisely at the *hemL* termination codon. Preliminary DNA sequence data for regions of pTE250 downstream of *hemL* near the *Nhe*I site (kb 2.6 of the map in Fig. 3) and the *Bam*HI site (kb 7.2) also fail to show similarity to the *E. coli fhuB* and *fhuACD* operons. This sequence divergence indicates the presence of a long DNA segment between the *fhuB* and *hemL* genes of *S. typhimurium* which is not present in *E. coli*.

The closest genes to *hemL* on the 5′ side, based on the current genetic map (4) and recent publications, are *optA* (renamed *dgt* [64]) and *htrA* (44, 45). By comparison with the Kohara map, about 5 kb of genes with unknown function (from 177 to 182 kb) lie between *hemL* and its closest known upstream neighbors. The *dgt* gene is transcribed clockwise (away from *hemL*) and the *htrA* gene lies directly downstream of *dgt*.

Similarity of HemL protein to barley GSA aminotransferase. The gene for barley GSA aminotransferase has recently been cloned and sequenced (28). The predicted amino acid sequence of HemL was compared with that of barley GSA aminotransferase (Fig. 5). There is more than 50% identity between the two sequences, extending over the entire length of the two proteins, except that barley GSA aminotransferase bears a 44-amino-acid N-terminal extension. The sequence similarity strongly suggests that the *S. typhimurium hemL* gene encodes GSA aminotransferase.

Similarity of HemL protein to other aminotransferases. GenBank and EMBL databases (release 60.0) were searched for sequences which might encode proteins similar to HemL. The search was performed with the TFASTA program (54) of the University of Wisconsin Genetics Computer Group (17). As expected, the open reading frame at the end of *fhuB* which we propose encodes *E. coli popC* was the highest-scoring sequence. Another class of proteins with sequence similarity to HemL include members of a family of ornithine aminotransferases of humans, rats, and yeasts (e.g., see reference 37). Also included in this family is the *E. coli* gene *bioA*, which encodes 7,8-diaminopelargonic acid aminotransferase (53). These similarities are discussed below.

Maxicell analysis. The proteins synthesized from plasmids bearing the *hemL* gene were analyzed with maxicells (59). Two different *hemL* plasmids were tested. The first, pTE290, is the smallest deletion derivative of pTE250 which retains the ability to complement *hemL* mutants. It includes the DNA between the *Sau*3A site (bp 0 of Fig. 3) and the *Sal*I site (bp 1658) inserted between the *Bam*HI and *Sal*I sites of pBR328. The same *hemL* DNA segment, together with 146 bp of DNA from pBR328 extending upstream to an *Nhe*I site in the *tet* gene, was inserted into pUC120 to form pTE393. In pTE393, the *hemL* gene is in the orientation in which it might be transcribed from the *lac* promoter.

The results of the maxicell analysis are shown in Fig. 6. One strongly labeled protein with an apparent molecular

```
                                                GATCTCTGCG CCGTAAACGC ACAATTTGCT GAGCTAAAAA GGTGGAAGTG    50

TCTGTTTTCA TCAGAAGATC ATTAATTGGT AATACAAATC TAAAGTCGGC ATTCTACTCG CCAACGCCGC GAAAATCCCC TGCAAATCGT   140

ATTGTTTCAA ATGCGTAAAA CTTTCATAAC CTTCTCTGAA TAACTTAGAA TAGCGGGTAT TCACTTTTTC TACGAACCAG GAATCTTTCC   230

ATG AGT AAG TCT GAA AAT CTC TAT AGC GCG GCC CGC GAG CTG ATC CCC GGC GGC GTG AAC TCC CCT GTT CGC GCC   305
Met Ser Lys Ser Glu Asn Leu Tyr Ser Ala Ala Arg Glu Leu Ile Pro Gly Gly Val Asn Ser Pro Val Arg Ala    25

TTC ACT GGC GTG GGC GGC ACC CCG CTG TTT ATC GAA AAA GCG GAC GGC GCT TAT CTT TAT GAT GTC GAT GGC AAA   380
Phe Thr Gly Val Gly Gly Thr Pro Leu Phe Ile Glu Lys Ala Asp Gly Ala Tyr Leu Tyr Asp Val Asp Gly Lys    50

GCG TAT ATC GAC TAT GTC GGT TCC TGG GGA CCA ATG GTA CTG GGG CAT AAC CAT CCG GCT ATC CGC AAT GCG GTG   455
Ala Tyr Ile Asp Tyr Val Gly Ser Trp Gly Pro Met Val Leu Gly His Asn His Pro Ala Ile Arg Asn Ala Val    75

                                Hind III                                                        Bst EII
ATC GAA GCT GCG GAG CGC GGT TTA AGC TTC GGC GCG CCA ACC GAA ATG GAA GTG AAA ATG GCG GAA CTG GTC ACC   530
Ile Glu Ala Ala Glu Arg Gly Leu Ser Phe Gly Ala Pro Thr Glu Met Glu Val Lys Met Ala Glu Leu Val Thr   100

AAC CTG GTG CCG ACC ATG GAC ATG GTG CGC ATG GTG AAC TCC GGC ACC GAA GCG ACG ATG AGC GCT ATT CGC CTG   605
Asn Leu Val Pro Thr Met Asp Met Val Arg Met Val Asn Ser Gly Thr Glu Ala Thr Met Ser Ala Ile Arg Leu   125

GCG CGT GGT TTT ACT GGC CGC GAT AAG ATT ATC AAA TTC GAA GGC TGC TAC CAC GGC CAC GCA GAC TGT CTG CTG   680
Ala Arg Gly Phe Thr Gly Arg Asp Lys Ile Ile Lys Phe Glu Gly Cys Tyr His Gly His Ala Asp Cys Leu Leu   150

GTC AAA GCC GGT TCT GGC GCG CTG ACG CTC GGT CAG CCG AAC TCG CCG GGC GTG CCG GCA GAT TTC GCG AAA CAT   755
Val Lys Ala Gly Ser Gly Ala Leu Thr Leu Gly Gln Pro Asn Ser Pro Gly Val Pro Ala Asp Phe Ala Lys His   175

ACG CTG ACC TGC ACT TAT AAC GAT CTG ACG TCA GTG CGC GCG GCA TTT GAA CAA TAT CCG CAG GAA ATC GCC AGT   830
Thr Leu Thr Cys Thr Tyr Asn Asp Leu Thr Ser Val Arg Ala Ala Phe Glu Gln Tyr Pro Gln Glu Ile Ala Ser   200

ATC ATC GTC GAA CCC GTG GCG GGC AAT ATG AAC TGC GTC CCG CCG CTG CCG GAA TTT CTG CCA GGT CTG CGC GCC   905
Ile Ile Val Glu Pro Val Ala Gly Asn Met Asn Cys Val Pro Pro Leu Pro Glu Phe Leu Pro Gly Leu Arg Ala   225

TTG TGC GAT GAG TTC GGC GCG CTG CTG ATT ATC GAC GAA GTA ATG ACC GGT TTT CGC GTA GCG CTG GCC GGA GCC   980
Leu Cys Asp Glu Phe Gly Ala Leu Leu Ile Ile Asp Glu Val Met Thr Gly Phe Arg Val Ala Leu Ala Gly Ala   250

CAG GAT TAC TAC GGC GTC GTG CCG GAC CTG ACC TGT CTG GGT AAA ATC ATC GGC GGC GGG ATG CCG GTA GGC GCG 1055
Gln Asp Tyr Tyr Gly Val Val Pro Asp Leu Thr Cys Leu Gly Lys Ile Ile Gly Gly Gly Met Pro Val Gly Ala   275

TTT GGC GGT CGT CGC GAT GTA ATG GAT GCG CTG GCG CCG ACG GGC CCG GTT TAC CAG GCG GGC ACC CTT TCC GGC 1130
Phe Gly Gly Arg Arg Asp Val Met Asp Ala Leu Ala Pro Thr Gly Pro Val Tyr Gln Ala Gly Thr Leu Ser Gly   300

AAC CCG ATT GCG ATG GCG GCC GGT TTC GCC TGC CTG AAT GAA GTC GCC CAG CCC GGC ATT CAT GAA ACG CTG GAT 1205
Asn Pro Ile Ala Met Ala Ala Gly Phe Ala Cys Leu Asn Glu Val Ala Gln Pro Gly Ile His Glu Thr Leu Asp   325

                                                                Bam HI
GAG CTC ACC ACC CGC CTG GCG GAA GGG CTG TGC GAA GCG GCG CAG GAG GCG GGG ATC CCA CTG GTC GTC AAC CAT 1280
Glu Leu Thr Thr Arg Leu Ala Glu Gly Leu Cys Glu Ala Ala Gln Glu Ala Gly Ile Pro Leu Val Val Asn His   350

GTC GGC GGC ATG TTC GGG ATT TTC TTC ACC GAC GCT GAG AGC GTA ACC TGC TAT CAG GAC GTG ATG GCG TGC GAC 1355
Val Gly Gly Met Phe Gly Ile Phe Phe Thr Asp Ala Glu Ser Val Thr Cys Tyr Gln Asp Val Met Ala Cys Asp   375

GTG GAA CGC TTT AAG CGT TTC TTC CAC CTG ATG CTG GAG GAA GGC GTA TAT CTG GCG CCA TCG GCG TTT GAG GCG 1430
Val Glu Arg Phe Lys Arg Phe Phe His Leu Met Leu Glu Glu Gly Val Tyr Leu Ala Pro Ser Ala Phe Glu Ala   400

GGC TTT ATG TCG GTC GCA CAC AGC ATG GAC GAC ATT AAT AAT ACT ATT GAC GCC GCG CGT CGG GTG TTT GCG AAA 1505
Gly Phe Met Ser Val Ala His Ser Met Asp Asp Ile Asn Asn Thr Ile Asp Ala Ala Arg Arg Val Phe Ala Lys   425

CTG TAA     AAGAGAACG TCCAGAAACA ACGTAGGCCG GGTAAGGCGA AGCCGCCACC CGGTTTTTTT TTGGTTCATA CCAGGTTAGC 1590
Leu STOP                                                                                            426
                                                                                  Sal I
GTGAATCTTC GCCAAACACC CAGCCCCCCT AAAGCTCATC AATAATATAC TGGAAGCTAC AGGTCGAC                          1658
```

FIG. 4. DNA sequence of the 1.65-kb *Sau*3A-*Sal*I fragment. The sequence is oriented in the direction of transcription of the *hemL* gene, which has coordinates from bp 231 to bp 1511. The deduced amino acid sequence is also shown. Underlined sequences are discussed in the text.

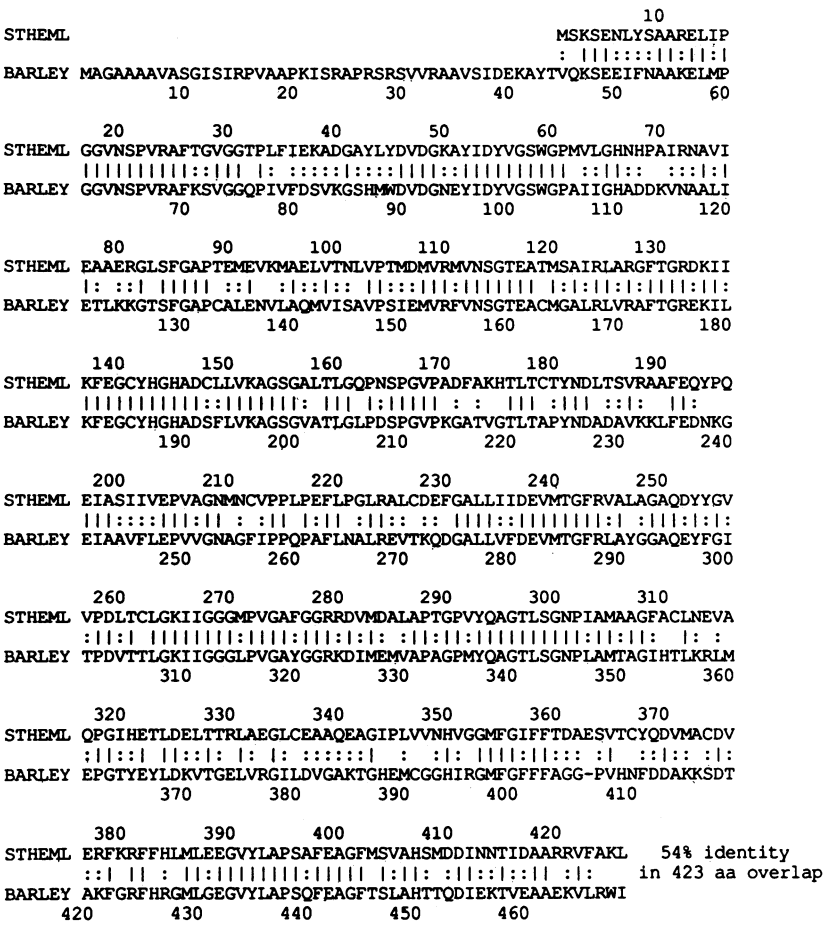

FIG. 5. Comparison of the amino acid sequences of the *S. typhimurium* HemL protein and barley GSA aminotransferase. This output was generated by the TFASTA program (17). A vertical line represents identity, while a colon represents a conservative substitution. The two proteins are homologous over their entire lengths, with a total identity greater than 50%.

mass of 40 kDa is synthesized in cells bearing plasmids with the *hemL* gene (lanes 2 and 3) but not in cells bearing pUC120 (lane 1). This size corresponds reasonably well with a size of 45.3 kDa predicted from the DNA sequence of the *hemL* gene, although the labeled protein is slightly smaller than expected. Labeled proteins of the correct apparent molecular mass of the precursor and mature forms of β-lactamase (predicted sizes 31.5 and 28.9 kDa) are seen with all three plasmids. The plasmid pTE290 also carries the *cat* gene encoding chloramphenicol acetyltransferase (predicted size, 25.6 kDa), and a labeled protein is seen at the correct apparent molecular weight in lane 2 of Fig. 6.

The signal from [^{35}S]methionine-labeled HemL protein in Fig. 6 is quite strong. This is not the result of an unusually high incidence of methionine in the protein but rather reflects its abundance relative to other labeled proteins. In addition, both HemL and chloramphenicol acetyltransferase could be identified among abundant cellular proteins visible in the Coomassie blue-stained gel the autoradiograph of which is shown in Fig. 6 (data not shown). Since it is not known which promoter(s) is responsible for transcription of the *hemL* gene on these plasmids, the relevance of this high expression in maxicells to a more natural in vivo situation is not clear.

DISCUSSION

Of the two ALA-forming pathways, the route from glutamate appears to be widely distributed in nature, whereas the ALA synthase route occurs only in nonphototrophic eucaryotes and a few bacterial groups. In comparison with the ALA synthase route, relatively little is known about the enzymes catalyzing the steps of the five-carbon pathway and the regulation of this process. The recent discovery that the five-carbon route operates in enteric bacteria such as *E. coli* (1, 42, 52) as well as in gram-positive organisms such as *Bacillus subtilis* (52) has provided a new opportunity for studying this pathway at the molecular genetic level. In this report, the operation of the five-carbon pathway has been shown to occur in a second enterobacterial species, *S. typhimurium*, and a new class of ALA-requiring mutants has been characterized at the enzyme and genetic levels.

Operation of the five-carbon pathway and ALA synthase in extracts of *S. typhimurium* were assessed separately by measurement of the incorporation into ALA of label from [1-^{14}C]glutamate and [2-^{14}C]glycine, respectively. Although C-2 through C-5 of glutamate can be incorporated into ALA via either pathway, C-1 is incorporated only via the five-carbon route (58). Because the ALA-forming activity of *S.*

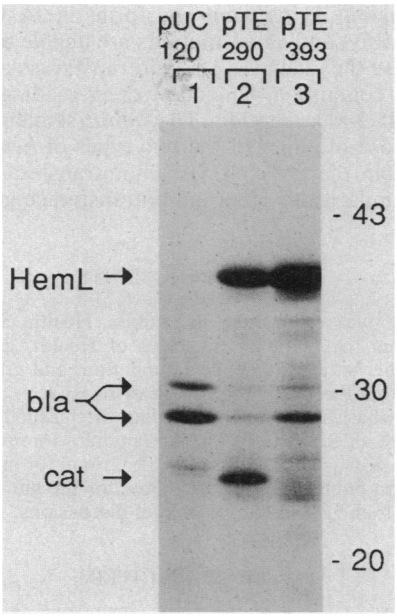

FIG. 6. Maxicell analysis. Polypeptides encoded by various plasmids were labeled in maxicells and separated by SDS-polyacrylamide gel electrophoresis. Plasmids are indicated above the individual lanes, and their maps are shown in Fig. 3. The sizes of the proteins were determined by comparison with unlabeled protein standards which were run in the same gel; the positions of these standards and their apparent molecular masses (in kilodaltons) are indicated at the right of the figure.

typhimurium extracts is low, it was difficult to measure in vitro incorporation of label into ALA from either glutamate or glycine. Activity could be increased by adding levulinate to the growth medium 2 h before cell harvest. Levulinate probably causes the induction of higher levels of ALA-forming enzymes by inhibiting the transformation of ALA to tetrapyrrole end products. Because of the low ALA-forming activity of the cell extracts, it was difficult to reliably distinguish actual ALA formation from carryover of label from substrates and other metabolites into the final ALA-pyrrole whose radioactivity was measured. Therefore, it was necessary to analyze the product further by paper chromatography. In paper chromatography, radioactivity in the ALA-pyrrole fraction derivative from the incubation with [1-^{14}C]glutamate comigrated with ALA-pyrrole derived from authentic ALA. Growth in the presence of levulinate increased, but was not necessary for detection of, incorporation of label from [1-^{14}C]glutamate into the final product. In contrast, radioactivity in the ALA-pyrrole fraction derived from incubation with [2-^{14}C]glycine did not comigrate with that from authentic ALA, regardless of whether the extract was obtained from cells grown in the presence or absence of levulinate. Although ALA synthase activity was not detected, cell extracts were capable of generating the ALA synthase substrate, succinyl-CoA, from succinate supplied in the incubation. Therefore, it appears that although the rate of ALA synthesis in extracts of *S. typhimurium* is very low, the ALA is synthesized exclusively from glutamate via the five-carbon pathway, and ALA synthase activity is absent.

The *S. typhimurium hemL* gene was cloned by complementation of a *hemL*::Mu d-J insertion mutant. Both genetic and hybridization tests confirmed that the cloned DNA encodes the *hemL* gene. A detailed analysis by Southern hybridization showed a close correspondence between sites of Mu d insertion and the genetic map determined previously. Together, these data indicate that all Mu d-J insertions in the region which confer a Hem$^-$ phenotype map within the *hemL* gene. Since no polar insertion mutants were found, it seems likely that the *hemL* gene has its own promoter. However, the possibility that insertions in an upstream gene are lethal cannot be excluded.

GSA aminotransferase activity was found in extracts of Hem$^+$ parental cells, but little or no activity was present in extracts of *hemL* mutant cells. When a plasmid-borne *hemL* gene was introduced, GSA aminotransferase activity was restored to the *hemL* mutant. The enzyme activity in the *hemL* cells carrying this multicopy plasmid was less than twofold higher than in wild-type cells. The lack of a substantial gene dosage effect suggests that expression or activity of GSA aminotransferase may be limited by some other factor. Neither the presence of a control plasmid without the *hemL* gene nor growth in medium containing ampicillin affected the level of in vitro GSA aminotransferase activity. Growth of the cells in medium containing 20 μM ALA affected the level of in vitro GSA aminotransferase activity only slightly. These results suggest that expression of the *hemL* gene is not repressed by high levels of exogenous ALA.

It was initially puzzling that *hemL* strain TE3283, which carries a control plasmid without the *hemL* gene, was able to grow in glucose-based minimal medium without ALA, if ampicillin was also provided in the medium. Later, it was found that ampicillin could be replaced by cysteine and that in glucose-based medium, cysteine could also replace ALA and allow growth of *hemL* strain TE162-5, which does not contain a plasmid. The most likely explanation for these observations is that in minimal medium containing glucose (a fermentable carbon source) and lacking a reduced sulfur source, respiratory cytochromes (and therefore cytochrome hemes) are not necessary for growth, but ALA formation is still needed for the synthesis of siroheme, the prosthetic group of sulfite reductase. In medium supplemented with cysteine as the reduced sulfur source, ALA formation is not necessary because neither siroheme nor cytochrome hemes are required for growth on glucose. Finally, in Amp^r HemL$^-$ strains growing on glucose in the presence of ampicillin, the S-containing antibiotic can satisfy the requirement for reduced sulfur. Growth in minimal medium containing the nonfermentable carbon source glycerol requires respiratory hemes in all cases.

The above results provide strong evidence that GSA aminotransferase, which catalyzes ALA formation in vitro, is required for ALA biosynthesis from glutamate in vivo. This is an important consideration, because another enzyme that was previously reported to catalyze ALA formation in vitro, DOVA aminotransferase, has also been proposed to participate in ALA biosynthesis in vivo (11). However, the latter reaction is probably catalyzed by glyoxylate aminotransferase, which can accept DOVA as an artificial substrate in vitro but has not been demonstrated to participate in ALA formation in vivo (23). Extracts of both wild-type and *hemL S. typhimurium* cells contained DOVA aminotransferase activity. This indicates that DOVA aminotransferase is not encoded by the *hemL* gene and that the reaction is not catalyzed by GSA aminotransferase. Moreover, these results, together with the fact that purified, homogeneous GSA aminotransferase catalyzes the conversion of GSA to ALA in the absence of an amino donor other than GSA (29), indicate that DOVA aminotransferase has no

role in ALA biosynthesis. In contrast, the concomitant disappearance of GSA aminotransferase activity and induction of ALA auxotrophy in *hemL* mutants, and their concomitant reversal in the transformants, strongly links this enzyme with ALA biosynthesis.

The DNA sequence of the *hemL* gene was determined. Analysis of the sequence allows us to predict that the HemL protein is encoded by a 426-codon open reading frame and has a molecular mass of 45.3 kDa. Maxicell analysis showed that plasmids carrying the *hemL* gene program synthesis of a polypeptide of the appropriate size. Comparison of the *S. typhimurium hemL* DNA sequence with that from the 3′ end of *E. coli fhuB* indicates that these two genes are adjacent and convergently transcribed and confirms our previous suggestion that *S. typhimurium hemL* is equivalent to *E. coli popC*. In view of the clearly established role of *hemL* in ALA and heme biosynthesis, it may be appropriate to rename the *popC* locus.

The predicted amino acid sequence of HemL was compared with that predicted from the recently determined sequence of the gene for barley GSA aminotransferase (28). The two sequences show more than 50% identity. We believe that this sequence similarity reflects an underlying functional homology between the two proteins. The highly conserved sequence homology is particularly striking in view of the evolutionary distance separating enterobacteria and barley. Since there is immunological evidence which firmly links the barley gene product with purified GSA aminotransferase, *hemL* seems very likely to encode *S. typhimurium* GSA aminotransferase.

A search of translated forms of the available DNA sequence databases revealed several other proteins with sequences similar to HemL. These proteins are all aminotransferases with related substrates. The family includes the ornithine aminotransferases of humans, rats, and yeasts (e.g., see reference 37), the *E. coli bioA* gene product (7,8-diaminopelargonic acid aminotransferase [53]), and the *E. coli argD* gene product (acetylornithine aminotransferase [31]).

The regions of similarity in these sequences extend for relatively long distances. For example, *E. coli* acetylornithine aminotransferase and HemL were found to contain 30% identical amino acids in a 332-amino-acid overlap. Pairwise tests of the other family members gave similar results. Furthermore, the most highly conserved regions were found in all the genes. Similarity between the *E. coli bioA* gene product and the ornithine aminotransferases was reported and discussed previously (53). Here, we merely note that HemL seems to be a member of this family of enzymes.

The long sequence similarity suggests a relationship among these proteins, and this idea is supported by the structural similarities among their substrates or products, which are glutamate semialdehydes (except in the case of *bioA*). We have concluded that the HemL protein is GSA aminotransferase. The other members of this family bind glutamate-5-semialdehyde as either substrate or product. (For ArgD, the substrate in vivo is N-acetylated.) *E. coli* also makes a second, cryptic, catabolic enzyme which is very closely related to the ArgD protein (8), whose gene sequence has not been reported. It seems possible that the existence of related aminotransferases could explain the apparent leaky phenotype of *hemL* compared with *hemA* mutants, as well as the existence of mutations which suppress the growth defect of *hemL* deletion and insertion mutants (T. Elliott, unpublished data).

In conclusion, *S. typhimurium* forms ALA via the five-carbon pathway, and *hemL* mutants are unable to synthesize ALA because they lack GSA aminotransferase activity. The *hemL* gene contains a 426-codon open reading frame that encodes a 45.3-kDa peptide. The *hemL* sequence does not resemble those of either of the two types of *hemA* gene but does resemble the barley GSA aminotransferase gene and has some similarity to other aminotransferase genes.

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