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# δ-Aminolevulinic Acid Dehydratase Deficiency Can Cause δ-Aminolevulinate Auxotrophy in *Escherichia coli*

GARY P. O'NEILL,[1] SIGRÍDUR THORBJARNARDÓTTIR,[2] UWE MICHELSEN,[1] SNAEBJÖRN PÁLSSON,[2] DIETER SÖLL,[1]* AND GUDMUNDUR EGGERTSSON[2]

*Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06511,[1] and Institute of Biology, University of Iceland, 108 Reykjavik, Iceland[2]*

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**Ethylmethane sulfonate-induced mutants of several *Escherichia coli* strains that required δ-aminolevulinic acid (ALA) for growth were isolated by penicillin enrichment or by selection for respiratory-defective strains resistant to the aminoglycoside antibiotic kanamycin. Three classes of mutants were obtained. Two-thirds of the strains were mutants in *hemA*. Representative of a third of the mutations was the *hem-201* mutation. This mutation was mapped to min 8.6 to 8.7. Complementation of the auxotrophic phenotype by wild-type DNA from the corresponding phage 8F10 allowed the isolation of the gene. DNA sequence analysis revealed that the *hem-201* gene encoded ALA dehydratase and was similar to a known *hemB* gene of *E. coli*. Complementation studies of *hem-201* and *hemB1* mutant strains with various *hem-201* gene subfragments showed that *hem-201* and the previously reported *hemB1* mutation are in the same gene and that no other gene is required to complement the *hem-201* mutant. ALA-forming activity from glutamate could not be detected by in vitro or in vivo assays. Extracts of *hem-201* cells had drastically reduced ALA dehydratase levels, while cells transformed with the plasmid-encoded wild-type gene possessed highly elevated enzyme levels. The ALA requirement for growth, the lack of any ALA-forming enzymatic activity, and greatly reduced ALA dehydratase activity of the *hem-201* strain suggest that a diffusible product of an enzyme in the heme biosynthetic pathway after ALA formation is involved in positive regulation of ALA biosynthesis. In contrast to the *hem-201* mutant, previously isolated *hemB* mutants were not ALA auxotrophs and had no detectable ALA dehydratase activity. Analysis of another class of ALA-requiring mutants showed that the auxotrophy of the *hem-205* mutant could be relieved by either methionine or cysteine and that the mutation maps in the *cysG* gene, which encodes uroporphyrinogen III methylase. The properties of these nonleaky ALA-requiring strains suggest that ALA is involved more extensively in *E. coli* intermediary metabolism than has been appreciated to date.**

In a wide variety of eubacteria, including *Escherichia coli*, and in the chloroplasts of higher plants and algae, the first committed precursor of tetrapyrrole biosynthesis is δ-aminolevulinic acid (ALA) derived from glutamate (3, 5, 22, 30; reviewed in references 7, 17, and 32). The transformation of glutamate into ALA, termed the $C_5$ pathway, can be reconstituted in vitro with purified Glu-tRNA synthetase, Glu-tRNA reductase, glutamate-1-semialdehyde (GSA) aminotransferase, $tRNA^{Glu}$, glutamate, ATP, and NADPH (9). Thus, three enzymes and $tRNA^{Glu}$ are sufficient for this conversion. However, our understanding of the exact mechanism of this biotransformation and its key role in the regulation of tetrapyrrole biosynthesis is still rather sketchy (reviewed in references 7 and 32).

Analysis of the $C_5$ pathway in *E. coli* has so far relied on the isolation and characterization of mutant strains auxotrophic for ALA. Such strains have been isolated from several eubacterial species that are known to use the $C_5$ pathway, including *E. coli* (28, 34, 37, 39, 46), *Bacillus subtilis* (1, 19), and *Salmonella typhimurium* (13, 14, 38). The isolation of *hemA* mutants usually involves screening on media containing an aminoglycoside antibiotic such as neomycin (40). Slow-growing small-colony-variant (SCV) mutants or dwarf colonies grow on this selective medium (37, 40). The SCV mutant population has been shown to be enriched in respiratory-defective mutants, of which a small number are auxotrophic for ALA. The mutants are neither resistant to nor dependent on the aminoglycoside antibiotic, show a leaky growth requirement for ALA under nonfermentative growth conditions, and display wild-type growth characteristics under anaerobic conditions (14, 46). While the intriguing relationship between the resistance to aminoglycoside antibiotics and the requirement for hemin allows for the enrichment of ALA auxotrophs, it is not known whether this selection technique biases the mutant population or totally excludes certain types of ALA-requiring phenotypes. Mapping studies of *E. coli* ALA auxotrophs of the SCV type have defined two loci, *hemA* and *popC*, at min 27 and 4, respectively (6, 34, 39, 46). The *E. coli hemA* gene has been cloned by complementation of the original *hemA* strain by several groups (11, 22, 42), and homologous *hemA* genes have been isolated from *S. typhimurium* (13) and *B. subtilis* (33). Although direct biochemical evidence for the function of HemA has not been reported, reconstitution experiments with extracts of mutant strains implicates HemA at the level of Glu-$tRNA^{Glu}$ conversion to GSA by the Glu-tRNA reductase (3, 4). The original *E. coli popC* mutants have perished (27a), but the *hemL* locus of *S. typhimurium* appears to be analogous (13, 14). Recently an *E. coli* gene homologous to a barley gene encoding the GSA aminotransferase was cloned and physically mapped to a chromosomal position near *popC* (14a). However, in the absence of the original *popC* mutant, it is unclear whether this gene and *popC* are identical. The fact that a *hemA hemL* double mutant still was able to produce siroheme led to the suggestion that there are two pathways of ALA formation in *S. typhimurium* (14).

* Corresponding author.

TABLE 1. *E. coli* K-12 strains, plasmids, and phages[a]

| Strain, phage, or plasmid | Relevant genotype | Source or reference |
|---|---|---|
| Strains | | |
| W3110 | $F^-$ prototroph | A. J. Pittard |
| AB1157 | $F^-$ *thr-1 leuB6 proA2 lacY1 rpsL31* | CGSC collection |
| AB1976 | $F^-$ *rpsL8* (or *rpsL9*) *trp-3* | CGSC collection |
| SHSP1 | $F^-$ *hemB1* | CGSC collection |
| SJ2 | $F^-$ *lac*$^{\pm}$ *proC*$^{\pm}$ *hemB*$^{\pm}$ | CGSC collection |
| CA8000 | Hfr P01 prototroph | CGSC collection |
| JW380 | $F^-$ *zch-506*::Tn*10* | CGSC collection |
| GE319 | $F^-$ *cysG44* | Laboratory collection |
| GE1360 | $F^-$ *hem-201* | Mutant of W3110 |
| GE1361 | $F^-$ *lacY29 proC24* | Derived from GE1360 |
| GE1363 | $F^-$ *lacY29 proC24 hem-202* | Mutant of GE1361 |
| GE1369 | $F^-$ *lacY29 proC24 hem-205* (*cysG99*) | Mutant of GE1361 |
| Phage | | |
| P1 *kc* | | Laboratory collection |
| λ8F10, λ6E2 | | Y. Kohara (20) |
| Plasmids | | |
| pUC18 | $Ap^r$ | J. Messing (43) |
| pBluescript KS | $Ap^r$ | Stratagene Inc. |

[a] Abbreviations: $Ap^r$, ampicillin resistance; CGSC, Coli Genetic Stock Center, Yale University, New Haven, Conn.

Since the original *popC* mutant has been lost and no mutants have definitively been shown to result in loss of Glu-tRNA reductase or GSA aminotransferase activities, we carried out a mutant search in *E. coli* for additional ALA auxotrophs. The ALA auxotrophs isolated in the presence or absence of aminoglycoside antibiotics could be subdivided into three groups which mapped to three loci: the *hemA* locus and two new map positions, *hemB* and *cysG*, not previously associated with ALA auxotrophy. The ALA auxotrophy induced by these mutations is unusual because of their lethal, nonleaky phenotype and their occurrence in genes that encode enzymes that utilize ALA or ALA-derived products. The *cysG* gene in *E. coli* encodes an *S*-adenosylmethionine-dependent uroporphyrinogen III methylase required for biosynthesis of cobalamine and siroheme (10, 15, 25, 44). The product of the *hemB* gene is ALA dehydratase, which condenses ALA to porphobilinogen (12, 16, 23, 24). The *hemB* mutation analyzed here is markedly different from previously characterized mutants at this locus since it requires ALA for growth, contains no detectable ALA-forming activity, and retains about 10% of the wild-type levels of ALA dehydratase activity. In contrast, other *hemB* mutants (12, 24) have no requirement for exogenous ALA and have no detectable ALA dehydratase activity.

## MATERIALS AND METHODS

**Bacterial strains.** All bacterial strains, phages, and plasmids used are listed in Table 1. All bacterial strains used are derivatives of *E. coli*.

**Bacteriological procedures.** Bacterial cultures were routinely grown in L broth or on L-agar plates. When used for growing mutants requiring ALA, this medium was supplemented with ALA (25 μg/ml). When required for selection of ALA mutants, kanamycin was added to a final concentration of 5 μg/ml. The minimal medium used, unless otherwise stated, was medium M63 (29). Glucose or glycerol was added as a carbon source at a final concentration of 2 mg/ml. Transduction with phage P1 was carried out in L broth with $2.5 \times 10^{-3}$ M $CaCl_2$. Stationary-phase cells ($10^9$ to $2 \times 10^9$/ml) were mixed with approximately equal numbers of phage and incubated at 37°C for 10 to 15 min. The mixture was centrifuged twice and suspended in minimal salt solution before plating on selective media. Induction of mutations with ethyl methanesulfonate was done as described previously (29).

**Recombinant DNA procedures.** Procedures for growing phage λ and for isolation of DNA fragments were essentially as described by Maniatis et al. (26). DNA was sequenced by the dideoxy method (36).

**Enzyme assays.** Bacteria were grown aerobically to a density of $A_{600} = 0.8$ in M9 medium (100 ml) containing 0.4% glycerol, ALA (50 μg/ml), and ampicillin (50 μg/ml) and harvested by centrifugation. To exhaust the endogenous ALA supply, the cells were resuspended for 60 min at 37°C in M9 medium containing 0.4% glycerol but not supplemented with amino acids or ALA. The cells were then resuspended in 2 ml of the appropriate assay buffer for determining either ALA-forming activity or ALA dehydratase activity. The assay buffer for the determination of in vitro and in vivo ALA-forming activity contained 0.1 M Tricine hydroxide (pH 7.9), 0.3 M glycerol, 10 mM KCl, 5 mM dithiothreitol, and 2 mM phenylmethylsulfonyl fluoride. The assay buffer for the determination of ALA dehydratase activity contained 0.1 M potassium phosphate (pH 6.8) and 20 mM dithiothreitol. Aliquots of the cell suspensions in buffer A were assayed directly for in vivo ALA-forming activity as described below. The remaining cells were disrupted by treatment with lysozyme and sonication in the presence of glass beads (100-μm diameter; 1 g of beads per 1 g [wet weight] of cells). The cell extracts were clarified by centrifugation at 100,000 × *g* for 60 min and then dialyzed against assay buffer for 90 min. The in vitro ALA-forming activity was determined (30, 31) in 1.0 ml of buffer A supplemented with 15 mM $MgCl_2$, 1 mM NADPH, 2 mM ATP, 10 mM levulinate, 1.0 μCi of [$^{14}$C]glutamate (293 mCi/mmol), *E. coli* tRNA (5 $A_{260}$ units), and cell extract (10 mg of protein). Following incubation of the assay mixture at 37°C for 60 min, the reaction was stopped and the [$^{14}$C]ALA formed was purified and quantitated exactly as described previously (30, 31). The assay mixture (0.25 ml) for the in vivo conversion of [$^{14}$C]glutamate to [$^{14}$C]ALA by intact cells (2) contained $2.5 \times 10^8$ log-phase cells, 0.1 M Tricine hydroxide (pH 7.9), 10 mM KCl, 5 mM $MgCl_2$, 2 mM ATP,

50 mM levulinate, and 1.0 μCi of [$^{14}$C]glutamate (293 mCi/mmol). The assay mixture was incubated at 37°C for 30 min, and the [$^{14}$C]ALA formed was extracted, purified, and quantitated as described previously (2, 30, 31). For both the in vitro and in vivo assays, ALA was converted to ALA pyrrole and identified by thin-layer chromatography (30, 31).

ALA dehydratase activity in the supernatant fraction was determined by assaying the conversion of ALA to porphobilinogen and detection of the formed porphobilinogen by using Ehrlich reagent (16). Assay conditions were as follows: 0.2 ml of cell extract (1 mg of protein per ml), 0.8 ml of 0.1 M potassium phosphate (pH 6.8) containing 20 mM dithiothreitol, and 50 μg of ALA. The assay mixture was incubated for 60 min at 30°C, and the reaction was stopped by the addition of 1.0 ml of 10% (wt/vol) trichloroacetic acid containing 0.1 M $HgCl_2$. The terminated assay mixtures were centrifuged (12,000 × *g* for 10 min), and the precipitate was discarded. The supernatant (1.5 ml) was mixed with an equal volume of Ehrlich reagent (1 g of *p*-dimethylaminobenzaldehyde dissolved in 42 ml of glacial acetic acid and 8 ml of 70% perchloric acid). The Ehrlich salt of porphobilinogen yields a red compound quantitated at 555 nm with an extinction coefficient of 60,200 mol/liter (16).

**Nucleotide sequence accession number.** The sequence reported has been assigned GenBank accession number X17417.

## RESULTS AND DISCUSSION

**Isolation of mutants.** Mutants requiring ALA for growth (Ala$^-$) were isolated in strains W3110, AB1157, and GE1361 after treatment with ethyl methanesulfonate, penicillin selection in glycerol minimal medium, subsequent plating on glucose minimal agar supplemented with ALA (25 μg/ml), and replica plating on glycerol minimal medium without ALA. Ala$^-$ mutants were also obtained in strain GE1361 by picking slow-growing colonies after plating ethyl methanesulfonate-treated cells on L agar with kanamycin (40). A total of 14 mutants were obtained after penicillin selection, but 38 were obtained after kanamycin selection. All mutations are referred to as *hem* mutations, and their phenotype is referred to as Ala$^-$.

**Characterization of mutants.** Growth tests of mutants were routinely performed by suspending cells in a minimal salt solution and streaking them on glycerol minimal and L-agar plates with and without ALA (25 μg/ml). These tests permitted division of the mutants into three main phenotypic classes.

**(i) Class A.** Thirty-three mutants grew normally on L agar when supplemented with ALA but either did not grow or were growth retarded (leaky phenotype) on L agar without ALA supplementation. On glycerol minimal agar, all of these strains grew normally with 0.25 μg of ALA per ml. Mutants that had a leaky phenotype on L agar showed a similar degree of leakiness on glycerol minimal medium without ALA. All seven mutants obtained after penicillin selection showed a nonleaky phenotype, whereas most mutants obtained after kanamycin selection were leaky. The phenotype of this class of mutants (apart from the leakiness observed for many mutants in this study) corresponds to the phenotype of *hemA* mutants described in previous studies (28, 37, 39, 46).

**(ii) Class B.** Sixteen mutants were retarded in growth on L agar with or without ALA supplementation (25 μg/ml); representative mutant alleles are *hem-201* in strain GE1360 and *hem-202* in strain GE1363. The degree of growth retardation varied among these mutants but was not affected by addition of ALA to the medium. Mutants that showed the highest degree of growth retardation (e.g., GE1360) formed colonies with diameters of about 0.6 mm upon incubation for 2 days at 37°C. Mutants of this class failed to grow (e.g., GE1360) or grew to a limited extent (leaky mutants) when streaked on glycerol minimal medium without ALA. Normal growth was restored when this medium was supplemented with ALA at 2.5 μg/ml, but 0.25 μg/ml did not stimulate growth. On glycerol minimal medium with ALA, these mutants could be distinguished by their white color from wild type and from mutants of classes A and C. In cross-streaking tests on glycerol minimal medium without ALA, mutants of class A were clearly fed by mutants of class B. Mutants of classes A and B grew anaerobically on glucose minimal medium without ALA.

TABLE 2. Mapping of *hem-202* by P1 transduction[a]

| Selected marker | Unselected marker[b] | Cotransduction frequency (%) |
|---|---|---|
| *proC*$^+$ | *hem*$^+$ | 324/400 (81) |
| | *lacY*$^+$ | 56/400 (14) |
| *hem*$^+$ | *proC*$^+$ | 107/150 (71.3) |
| | *lacY*$^+$ | 32/150 (21.3) |

[a] The recipient strain was GE1363 (*lacY29 hem-202 proC24*); the P1 donor strain was SJ2 (*lacY*$^+$ *hem*$^+$ *proC*$^+$).

[b] Distribution of unselected markers: *hem*$^+$ *lacY*$^+$, 56; *hem*$^+$ *lacY*, 268; *hem lacY*$^+$, 0; *hem lacY*, 76.

**(iii) Class C.** One mutant of GE1361, strain GE1369 with mutant allele *hem-205* obtained after penicillin selection grew normally on L agar with or without ALA supplementation. Growth on glycerol or glucose minimal medium was dependent on the supplementation by either ALA, methionine, or cystine. On glycerol or glucose minimal agar without ALA, methionine, or cystine, this mutant had a nonleaky phenotype but reverted at a frequency of about $10^{-5}$.

**Genetic mapping.** Fifteen mutations of class A obtained in W3110, AB1157, and GE1361 were mapped by transduction with phage P1. All of these mutations showed cotransduction with *zch-506*::Tn*10* present at min 27.25 in strain JW380. Six of the mutations were also tested for cotransduction with the *trp-3* mutation present in strain GE1976. Cotransduction frequencies of 8 to 10% were found. It is concluded that these 15 mutations are in the *hemA* gene, which is located at 26.7 min (6, 39).

Mutations of class B were mapped by conjugation and P1 transduction. In interrupted mating experiments with strain CA8000, the *hem-200* mutation present in a derivative of AB1157 was located near min 9 on the map. This result was confirmed by P1 transduction, which showed 20 to 25% cotransduction between *lacY1* and *hem-200*. Further mapping of *hem-200*, *hem-201*, *hem-202*, and 11 other mutations of phenotype B showed their close linkage to the *proC* locus. For the fine genetic mapping, we used the *hem-202* allele; for the biochemical studies, the *hem-201* allele was used. Results of mapping experiments with *hem-202* are shown in Table 2. Cotransduction between *hem-202* and *proC24* was about 80% when selection is made for *proC*$^+$ but near 70% when selection was made for *hem*$^+$. A cotransduction frequency of 21% was found for *lacY1* and *hem-202*. Distribution of unselected markers showed the gene order *lacY-hem-proC*. A cotransduction frequency of 0.8 is estimated to correspond to a map distance of about 0.2 min (27, 45). With

the *proC* gene being located at min 8.8 to 8.9 (6), our data put *hem-202* at min 8.6 to 8.7.

The *hem-205* mutation of class C (originally present in strain GE1369) was by conjugation found to be closely linked to the *rpsL* locus. Further mapping was done by P1 transduction. First, *hem-205* was shown to be cotransducible at a frequency of 15% with the *rpsL* gene (at min 73.4). Second, strain GE319 carrying the *cysG44* mutation (at min 74) and requiring cystine for growth (not responding to ALA or methionine) was used as the recipient for P1 phage grown on GE1369. Selection was made for transductants on minimal medium supplemented with methionine or ALA. No transductants were obtained on medium with ALA. Transductants obtained on medium with methionine (50 tested) grew with methionine or cystine, but ALA could not support their growth. It is concluded that in these transductants *cysG44* has been replaced by *hem-205* and that *hem-205* is in or very close to the *cysG* gene. Clearly, the ability to respond to ALA is lost by transfer of *hem-205* into GE319. This ability was unaffected after transduction of *hem-205* from a streptomycin-resistant (*rpsL8* or *-9*) derivative of GE1369 into GE1361, showing that it is solely due to the *hem-205* mutation. The reason for this difference between GE319 and GE1369 is not known.

**ALA formation from glutamate in the *hem-201* mutant.** When grown aerobically, the *hem-201* mutant GE1360 displayed a strict dependence for aerobic growth on the addition of exogenous ALA, suggesting that the defect was in one of the components of the $C_5$ pathway. The ability of GE1360 cells and extracts to transform [$^{14}$C]glutamate into ALA was analyzed by using the in vivo and in vitro assays for ALA formation described in Materials and Methods (2, 30, 31). Cells and cell extracts were prepared from log-phase cultures of GE1360 grown in M9 medium (29) supplemented with 0.4% glycerol and 50 μg of ALA per ml and assayed by using the in vivo and in vitro ALA-forming tests. Conversion of [$^{14}$C]glutamate into ALA could not be detected in strain GE1360 in vivo (<0.01 pmol of ALA formed in 30 min per $2.5 \times 10^8$ cells) or in cell extracts (10 mg of protein formed <0.01 pmol of ALA in 60 min at 37°C), while it was easily detected in wild-type W3110 cells (78 pmol of ALA formed in 30 min per $2.5 \times 10^8$ cells) and in cell extracts (18 pmol of ALA formed in 60 min at 37°C). These results suggested that the cause of the ALA auxotrophy is a defect in ALA-forming activity.

**Cloning and DNA sequence of the *hem-201* gene.** The nonleaky Ala$^-$ phenotype of strain GE1360, its lack of ALA-forming activity, and the mapping of *hem-201* to a locus other than *popC* or *hemA* indicated that we had identified a new locus involved in ALA formation. To further characterize this locus, the *hem-201* gene was cloned by complementation. DNA from two recombinant λ phages, 8F10 and 6E2, covering min 8.5 to 9.3 of the *E. coli* chromosome (20), was inserted into pUC18 and shown to rescue the GE1360 strain to ALA prototrophy. Phage 8F10 contained a 16.6-kb insert. Subcloning experiments localized the *hem-201*-complementing activity to a 7.5-kb *Eco*RI-*Eco*RI fragment that was progressively subcloned to a 3.1-kb *Bam*HI-*Bam*HI fragment and then a 2.5-kb *Bam*HI-*Bst*XI subfragment (Fig. 1). These subclones, but not subclones pUC18ALAH1.7 and pUC18ALAH1.4 (Fig. 1), still rescued *hem-201* from auxotrophy. These data indicate that the *Hin*dIII site used to prepare pUC18ALAH1.7 and pUC18-ALAH1.4 is located within the region conferring *hem-201*-complementing activity. Nucleotide sequence analysis of the 2.5-kb *Bam*HI-*Bst*XI subfragment revealed that the previously characterized *hemB* gene was present on this fragment (12, 23). The *hemB* gene had previously been mapped at min 8.1 (6).

The ATG initiation codon of the *hemB* gene on the 2.5-kb *Bam*HI-*Bst*XI *hem*$^+$ fragment was located 629 nucleotides from the *Bam*HI site. The ATG initiation codon of the *hemB* gene reported by Li et al. (23) was located 458 nucleotides from the *Bam*HI site. Thus, our sequence of *hemB*, in comparison with that of Li et al. (23), contains an additional 171 nucleotides between the *Bam*HI site and the ATG initiation codon of *hemB* (Fig. 2). Further examination of the *hemB* sequence by Li et al. (23) shows that the first 26 nucleotides of their sequence are identical to the *Sph*I-*Pst*I-*Xba*I-*Bam*HI multiple cloning site of the sequencing vectors M13mp18 and -19 (43). The published *hemB* sequence (23) extends another 1,882 nucleotides from the *Bam*HI site and is identical to our sequence of the *hem*$^+$ *Bam*HI-*Bst*XI fragment. Mapping of restriction sites predicted from the nucleotide sequence (23) to occur in the *hemB* gene for *Nru*I, *Sal*I, *Pst*I, and *Hin*dIII also map to the exact positions in the *hem*$^+$ 2.5-kb *Bam*HI-*Bst*XI fragment. From these results we conclude that the 2.5-kb *Bam*HI-*Bst*XI *hem*$^+$ fragment derived from phage 8F10 (20) is essentially identical to the *hemB* gene of Li et al. (23) except that through an insertion of 171 nucleotides the *Bam*HI site is located upstream from the previously reported *Bam*HI site. Whether this is due to strain differences is unclear. Our sequence analysis of the 5′ and 3′ flanks of the *hemB* gene on the 2.5-kb *Bam*HI-*Bst*XI *hem*$^+$ fragment did not reveal any open reading frames greater than 50 amino acids. This suggests that the ability of the 2.5-kb *Bam*HI-*Bst*XI *hem*$^+$ fragment to rescue the *hem-201* mutant strain from ALA auxotrophy must be due to the expression of the *hemB* gene product. This is an unexpected finding since no *hemB* mutants are known to affect ALA biosynthesis. We have confirmed that strain SHSP1 carrying the *hemB1* mutation is unable to grow on minimal media with ALA.

**ALA dehydratase levels in various strains.** To confirm that the *hem-201* mutation is in *hemB*, the gene for ALA dehydratase, and also to confirm that the 2.5-kb *Bam*HI-*Bst*XI *hem*$^+$ fragment encoded ALA dehydratase, we assayed for ALA dehydratase activity in the *hem-201* mutant, GE1360, and in GE1360 containing a plasmid carrying the 2.5-kb *Bam*HI-*Bst*XI *hem*$^+$ fragment. The results clearly showed that GE1360 contained drastically reduced but detectable levels of ALA dehydratase activity and that plasmids carrying the 2.5-kb *Bam*HI-*Bst*XI *hem*$^+$ fragment conferred highly elevated levels of ALA dehydratase to the mutant strains (Table 3). The *hemB1* mutant strain (SHSP1) could also be transformed to prototrophy by plasmids carrying various subclones of the *hem-201* gene (Fig. 1). Complementation by pUC18ALA*Bam*3 was demonstrated for *hem-202* and other *hem* mutations that were mapped at this locus.

**Conclusions.** Our search for new genetic loci to characterize ALA formation in *E. coli* has yielded ALA auxotrophs resulting from mutations in genes not previously associated with the regulation of ALA formation. One class of mutant strains does not have detectable levels of ALA-forming activity and has greatly reduced levels of ALA dehydratase. The mutation was mapped to the *hemB* locus, which encodes ALA dehydratase (EC 4.2.1.24), an enzyme in the heme biosynthetic pathway which catalyzes the condensation of two ALA molecules to form porphobilinogen. Various *E. coli hemB* mutants and the corresponding *hemB* gene have been characterized by several groups (12, 23, 28); none of the known *hemB* mutant *E. coli* strains has been reported to

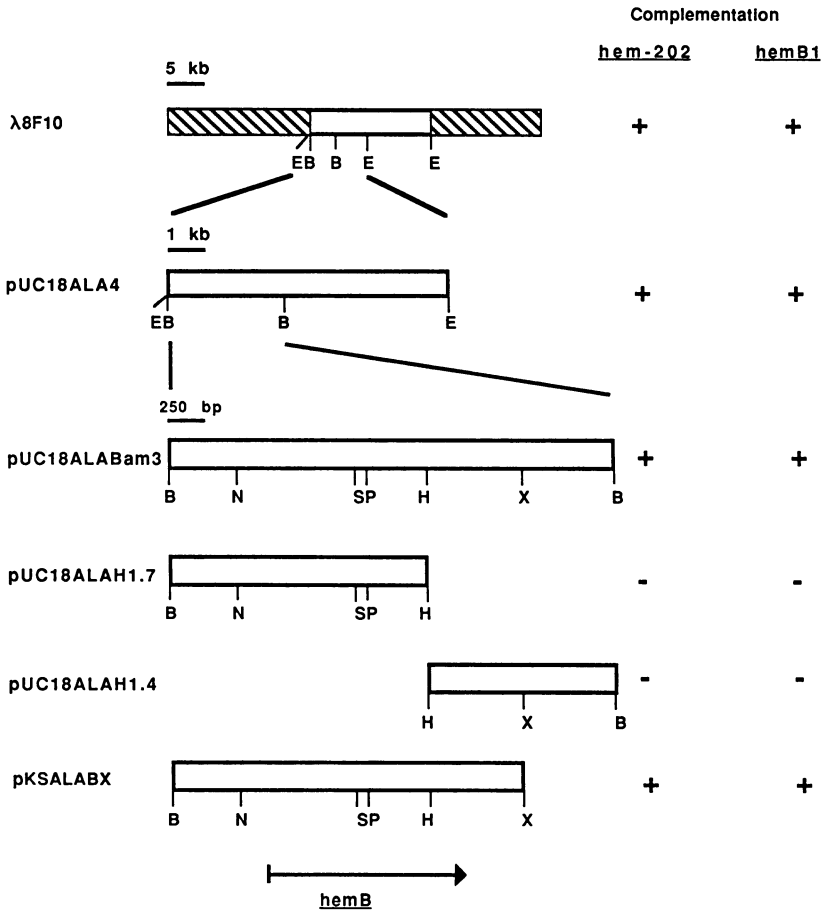


FIG. 1. Physical map of phage λ8F10 and the subclones in plasmids pUC18 and pBluescript used to localize the *hemB* gene. λEMBL140 DNA and insert DNA are represented by hatched and open boxes, respectively. Restriction sites: E, *Eco*RI; H, *Hin*dIII; N, *Nru*I; P, *Pst*I; S, *Sal*I; X, *Bst*XI.

show ALA auxotrophy. Because *hemB* mutations were not expected to display ALA auxotrophy, previous selection schemes for *hemB* mutants (e.g., reference 24) have incorporated a screen that eliminates ALA-requiring mutants. In contrast, our mutant search concentrated on ALA$^-$ mutants. Obviously such mutants were discarded in earlier investigations. It should be noted that the use of aminoglycoside antibiotics as a selection scheme to enrich for respiration-defective mutants may result in a biased mutant population. Indeed, in our selections for ALA auxotrophs using kanamycin, only leaky ALA auxotrophs were obtained, whereas nonleaky ALA$^-$ phenotypes and the *hem-201* mutant were obtained only by using penicillin enrichment techniques. It is not clear why the newly isolated *hemB* mutants display an ALA requirement for aerobic growth. Possibly the product of ALA dehydratase, porphobilinogen, or another product of the heme biosynthetic pathway is

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  1      GGATCCCAATCAAGTGCTGTAACGGGATTATCGATATAAGTCCAG  45  8F10

 46 GTATCATAGTTTGCTCGATTAGCCTGAGAGGTGATAGCCAGAGTGCAAGC  95  8F10

 96 CAATGCTAATTGTGATACTACAAGTTTCTTTTTCCAGGAGTGCATTTGTC 145  8F10

146 ATCCCTCCTCAGGGACGTAATAAGTTGATC                     175  8F10
  1 TG-ATG---GCA--T--ACTCT--AG----                    30  pHU1016
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FIG. 2. DNA sequence comparison of the 5′-flanking regions of the *hem-201*-complementing fragment and the previously reported *hemB* gene (23). The sequence of the *hem-201*-complementing fragment begins with nucleotide 1 at a *Bam*HI restriction site and is designated 8F10 to identify its source as λEMBL140(8F10) from the Kohara library (20). The sequence of *hemB* by Li et al. (23) also begins at a *Bam*HI site and is designated pHU1016 to identify its source as the phasmid pHU1016 (24). Only the mismatches between 8F10 and pHU1016 are shown. The nucleotide sequences of 8F10 and pHU1016 after positions 171 and 26, respectively, are identical for the next 1,800 nucleotides.

TABLE 3. ALA-dehydratase levels in *E. coli* strains containing cloned *hem-201*-complementing fragments

| Bacterial strain | Plasmid | Synthesis of porphobilinogen in cell extracts[a] | |
|---|---|---|---|
| | | Inactivated | Experimental |
| W3110 | pKS | 0.001 | 0.082 |
| GE1360 | pKS | 0.002 | 0.009 |
| GE1360 | pKSalaBX | 0.004 | 2.27 |

[a] Micromoles of porphobilinogen synthesized per milligram of protein of total cell extract.

required for derepression or activation of one of the $C_5$ enzymes in *E. coli*. For example, it is generally accepted that heme is a potent feedback repressor of ALA biosynthesis (reviewed in reference 7). Studies on the regulation of expression of ALA synthase in yeast cells, have shown that its regulation is a composite of activation and repression (18). In related work, it was shown that the product of the *rhm-1* locus is required for normal levels of ALA-forming activity in vivo (8). Furthermore, the product of ALA dehydratase, porphobilinogen, is required for the induction of porphobilinogen deaminase activity in *E. coli* (41). Our genetic and biochemical data do not distinguish whether the newly characterized *hem-201* mutation lies within the structural gene or in a regulatory sequence. The nature of the published *hemB* mutations has not been determined (12, 24). It is noted that a relatively high concentration of ALA is needed for aerobic growth of the new *hemB* mutants. Under such conditions, the mutants must have enough dehydratase activity to permit growth, which is completely inhibited in the absence of ALA. Possibly, ALA is needed to activate transcription of the *hemB* gene. In the mutants, this might lead to the production of a partially active enzyme. Studies to characterize the *hem-201* mutation should give insight into the complex regulation of ALA and heme biosynthesis.

Mutations of the *cysG* gene which respond to ALA have not been described before. On the other hand, a response to methionine has been reported for certain mutations of *cysG* and other *cys* genes (21, 35). It has been suggested that such mutations affect regulation of *cys* gene function (35). While *hem-205* maps to the *cysG* locus, we did not establish the relative activity of the *cysG* gene product. The response of *hem-205* to ALA suggests that ALA may play a role in controlling the expression of the *cysG* gene.

A further search for ALA auxotrophs may reveal additional loci and provide further information on the regulation of ALA synthesis and its involvement in the intermediary metabolism of *E. coli*.

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