JOURNAL OF BACTERIOLOGY, July 1997, p. 4583–4590 Vol. 179, No. 14
0021-9193/97/$04.00+0

Role of the *hemA* Gene Product and δ-Aminolevulinic Acid in Regulation of *Escherichia coli* Heme Synthesis

EVIE VERDERBER, LOUISE J. LUCAST, JEFFREY A. VAN DEHY, PAUL COZART, JEFFREY B. ETTER, AND ELAINE A. BEST*

Somatogen, Inc., Boulder, Colorado 80301-2857

Received 2 October 1996/Accepted 7 April 1997

We initiated these studies to help clarify the roles of heme, δ-aminolevulinic acid (ALA), *hemA*, and *hemM* in *Escherichia coli* heme synthesis. Using recombinant human hemoglobin (rHb1.1) as a tool for increasing *E. coli*'s heme requirements, we demonstrated that heme is a feedback inhibitor of heme synthesis. Cooverexpression of rHb1.1 and the *hemA*-encoded glutamyl-tRNA (GTR) reductase increased intracellular levels of ALA and heme and increased the rate of rHb1.1 formation. These results support the conclusion that heme synthesis is limited by ALA (S. Hino and A. Ishida, Enzyme 16:42–49, 1973; W. K. Philipp-Dormston and M. Doss, Enzyme 16:57–64, 1973) and that the *hemA*-encoded GTR reductase is a rate-limiting enzyme in the pathway (J.-M. Li, C. S. Russell, and S. D. Cosloy, Gene 82:2099–217, 1989). Increasing the copy number of *hemM*, whose product is believed to be required for efficient ALA formation (W. Chen, C. S. Russell, Y. Murooka, and S. D. Cosloy, J. Bacteriol. 176:2743–2746, 1994; M. Ikemi, K. Murakami, M. Hashimoto, and Y. Murooka, Gene 121:127–132, 1992), had no effect on either ALA pools or the rate of rHb1.1 accumulation. The *hemA*-encoded GTR reductase was found to be regulated by ALA. Some of our results differ from those reported by Hart and coworkers (R. A. Hart, P. T. Kallio, and J. E. Bailey, Appl. Environ. Microbiol. 60:2431–2437, 1994), who concluded that ALA formation is not the rate-limiting step in *E. coli* cells expressing *Vitreoscilla* hemoglobin.

In *Escherichia coli* and related bacteria, biosynthesis of heme (a generic term to denote either the ferrous or ferric form of protoporphyrin IX) occurs via a complex, branched pathway that involves up to 12 gene products (Fig. 1). The committed precursor in the heme pathway, δ-aminolevulinic acid (ALA), can be formed by either of two pathways. In *E. coli*, ALA is formed in three steps from the five-carbon skeleton of glutamate (C_5 pathway) (16, 20). In *Rhodobacter capsulatus* (11), ALA is formed by condensation of glycine and succinyl coenzyme A in a single enzymatic reaction (C_4 pathway) catalyzed by ALA synthase (15). Seven additional reactions, including assembly of eight ALA molecules into a cyclic tetrapyrrole, modification of the side chains, and incorporation of reduced iron into the molecule, are required to convert ALA to heme.

The roles of ALA, glutamyl-tRNA (GTR) reductase, and heme in *E. coli* heme pathway regulation have been investigated and debated by several groups. ALA-feed studies performed during the early 1970s supported the conclusion that heme synthesis is limited by the rate at which ALA is formed. Wild-type *E. coli* cells fed ALA accumulated increased levels of heme and porphyrins (10, 21). The regulatory role of ALA in heme synthesis was further supported by studies of heme synthesis in mammalian cells (7) and plant cells (2, 12).

A more recent study by Hart and coworkers (9) challenged the conclusion that ALA is rate limiting for heme synthesis. Hart and coworkers genetically engineered *E. coli* cells to produce high levels of a foreign hemoprotein, *Vitreoscilla* hemoglobin (vHb), and found that such cells accumulated at least 20-fold more heme than control cells, indicating that the vHb served as a "heme sink" (9). Contrary to what would be predicted from studies of *E. coli* cells with normal heme requirements, they found that *E. coli* cells expressing vHb failed to accumulate increased levels of heme when supplemented with high concentrations of ALA or when the copy numbers of certain heme biosynthetic enzymes (GTR reductase [*hemA* and *hemM*] or ALA dehydratase [*hemB*]) were greatly increased (9).

The structure and regulation of the *E. coli* GTR reductase has also been the subject of considerable debate. Jahn and coworkers purified two GTR reductase activities of different molecular masses (85 and 45 kDa) (14). Based on studies of the plant pathway (2, 12), Jahn and coworkers expected the *E. coli* GTR reductase to be inhibited by heme. Surprisingly, they found that neither the 85-kDa nor the 45-kDa protein was inhibited by heme in vitro (14). An *E. coli* gene specifying the 45-kDa enzyme (*hemA*) (26) but not the 85-kDa protein has been reported in the literature.

Two groups have suggested that the product of *hemM* is also involved in ALA formation (3, 13). The deduced *hemM* product is a 23-kDa protein whose amino acid sequence is unrelated to any enzyme known to play a role in ALA synthesis (13). The *hemM* gene is adjacent to and divergently transcribed from the *hemA* gene; the two genes are separated by 200 bp (13). Based on the results of some complementation experiments, Ikemi and coworkers proposed that the *hemM* product is the major GTR reductase in *E. coli* and that the *hemA*-encoded GTR reductase is involved in a minor ALA synthesis pathway (13). Unfortunately, these investigators never demonstrated that the *hemM*-encoded protein has GTR reductase activity, and the DNA fragments used in their complementation studies contained portions of adjacent genes and open reading frames, which, in some cases, resulted in growth of a heterogeneous mixture of colony sizes (13); these type of results are often suggestive of recombination rather than complementation.

Chen and coworkers (3) attempted to clarify Ikemi and coworkers' findings (13) by subcloning *hemA* and *hemM* on

* Corresponding author. Present address: Heska, 1825 Sharp Point Dr., Fort Collins, CO 80525.

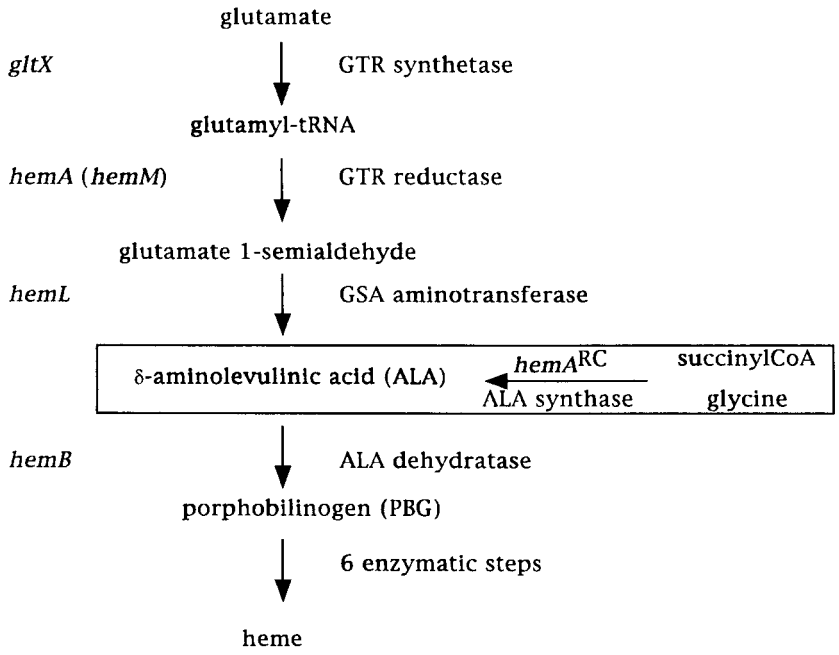

FIG. 1. Pathways for heme synthesis. Genes and enzymes relevant to this study are indicated. The $hemA_{RC}$ gene encodes the *Rhodobacter* ALA synthase involved in the C_4 pathway for ALA production. succinylCoA, succinyl coenzyme A.

separate plasmids. Unfortunately, the *hemA*- and *hemM*-containing plasmids used in Chen and coworkers' studies contained large portions of upstream and downstream genes. For example, their *hemA*-containing plasmid contained not only *hemA* but also the promoter and approximately half of the coding region of *hemM*, as well as a portion of an unrelated gene, *prfA* (3). Chen and coworkers observed that an *E. coli hemA* mutant containing multiple copies of a DNA fragment that included the complete *hemA* and *hemM* accumulated porphyrins faster, and to a higher level, than an isogenic mutant containing multiple copies of a DNA fragment that included all of *hemA* and a portion of *hemM* (3). Based on these and other results, they concluded that the products of both *hemA* and *hemM* are required for maximal production of ALA (3). Because Chen and coworkers' complementation studies were performed with very high copy number cloning vectors (pUC derivatives), the increased levels of ALA observed in their studies may have been due to titration of a regulatory factor rather than increased production of a rate-limiting enzyme (3, 16).

We initiated these studies to help clarify the roles of heme, ALA, *hemA*, and *hemM* in *E. coli* heme biosynthesis. Like Hart and coworkers (9), we performed our studies in *E. coli* cells expressing multiple copies of a heme sink, recombinant human hemoglobin (rHb1.1), so that cells would be spared from the toxic effects of high levels of free heme (27) that could accumulate as a result of our genetic manipulations. Moreover, to minimize the possibility of titration effects, which may have complicated other investigators' studies, we performed gene dosage experiments with medium-copy-number plasmids derived from p15A and used very discrete DNA fragments that included the genes of interest without flanking regions.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1. Although *E. coli* K-12 strains are typically impermeable to heme (23), the strains evaluated in this work are heme permeable. pSGE715 is a pUC-derived plasmid containing a synthetic, P_{tac}-controlled operon composed of two genetically fused alpha subunits and one beta subunit of human hemoglobin and the *lacI* gene for repression of the *tac* promoter. pSGE518 was derived from pRS415 (25) by replacement of the *Eco*RI-*Sma*I-*Bam*HI cloning region with an *Eco*RI-*Hin*dIII-*Eag*I-*Bgl*II-*Xho*I-*Hpa*I-*Bam*HI polylinker.

Genetic and recombinant DNA methods. P1*vir* transductions were performed according to standard methods (24). Multicopy *hemA-lacZ* operon fusions were constructed in pSGE518, transferred to λRS45 by homologous recombination, and then integrated as prophages into the chromosomal attachment site (*att*λ) according to published methods (25).

Chromosomal DNA for PCR amplification was obtained by standard methods (24). Oligonucleotides for PCR amplification and DNA sequencing were synthesized by using an Applied Biosystems model 392 DNA synthesizer (Applied Biosystems, Inc., Foster City, Calif.), using reagents obtained from Biogenex (San Ramon, Calif.) and methods recommended by the manufacturer.

All PCR amplification mixtures contained the following reagents: ~100 ng of template DNA, 20 to 50 pmol of each primer, 20 mM Tris-HCl, 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mM each of the four deoxyribonucleoside triphosphates, and 2.5 U of *Pfu* polymerase (Stratagene Cloning Systems, La Jolla, Calif.) in a 100-μl reaction volume. Thermocycling was performed with an Ericomp Twinblock system (Ericomp, San Diego, Calif.). Cycle conditions and primers are described below.

The *Bam*HI-*Hin*dIII fragment in pSGE1103 was obtained by PCR amplification using primers TG40 and TG224 and SGE1670 chromosomal DNA as the template. Cycle conditions were as follows: 1 cycle of 5 min at 95°C, 5 min at 65°C, and 1 min at 75°C; 35 cycles of 1 min at 95°C, 30 s at 65°C, and 30 s at 75°C; and 1 cycle of 10 min at 75°C.

All three *hemA* promoter fragments (Fig. 3) were amplified by using the following cycle conditions (program J): 1 cycle of 5 min at 95°C, 5 min at 50°C, and 1 min at 72°C; 28 cycles of 1 min at 94°C, 1 min at 50°C, and 30 s at 72°C; and 1 cycle of 10 min at 72°C. The *hemA* promoter fragment in pSGE863 was amplified by using primers EV50 and EV39. The *hemA* promoter fragment in pSGE864 was amplified by using two sets of primers: (i) EV45 and EV57 and (ii) EV53 and EV54. The use of two primer sets allowed removal of a small DNA segment containing the $hemA_1$ transcription start site described by Verkamp and Chelm (26).

The *hemA* gene fragment contained in pSGE494 was amplified by using program J and primers TG40 and TG41. The function of the *hemA* gene in pSGE494 was verified by complementation assay using an *E. coli hemA* mutant (data not shown).

A DNA fragment containing the coding sequence of $hemA_{RC}$ (the *Rhodobacter* gene that encodes ALA synthase [EC 2.3.1.37]) was obtained by PCR using chromosomal DNA isolated from *R. capsulatus* SB1003, primers EV98 and EV99, and program J (described above). In addition to confirming the DNA sequence of the $hemA_{RC}$ fragment in pSGE1110, we demonstrated that

TABLE 1. Bacterial strains, plasmids, oligonucleotides, and phages used

Strain, plasmid, phage, or oligonucleotide	Relevant description or genotype	Source
E. coli		
SGE1453	*gyrA96 endA hsdR17 relA1 supE44 recJ* pSGE715	This study
SGE1670	*gyrA96 endA hsdR17 relA1 supE44 recJ lacI*^{q1} *lacZ*::Tn5; source of chromosomal DNA for PCR amplification of wild-type *hem* genes and flanking regions	Lab collection
SGE1855	Δ(*gpt-lac*)5 *relA1 spoT1 thi-1*	Lab collection
SGE1857	SGE1855 with single-copy derivative of pSGE862	This study
SGE1858	SGE1855 with single-copy derivative of pSGE863	This study
SGE1859	SGE1855 with single-copy derivative of pSGE864	This study
SGE1856	Δ(*gpt-lac*)5 Tn*10*(Km) linked to *hemA relA1 spoT1 thi-1*	Lab collection
SGE1860	SGE1856 with single-copy derivative of pSGE862	This study
SGE1861	SGE1856 with single-copy derivative of pSGE863	This study
SGE1862	SGE1856 with single-copy derivative of pSGE864	This study
SGE2658	Derivative of SGE1453 containing pSGE1103	This study
SGE2664	Derivative of SGE1453 containing pSGE494	This study
SGE2680	Derivative of SGE1453 containing pSGE1104	This study
SGE2681	Derivative of SGE1453 containing pSGE1110	This study
R. capsulatus SB1003	Wild type, source of chromosomal DNA for PCR amplification of $hemA_{RC}$	C. Bauer
Plasmids		
pAlterEx2	Low-copy-number cloning vector with *tac* promoter, Tc^r	Promega
pACYC184	Low-copy-number cloning vector, Cm^r	Promega
pSGE518	Vector for constructing multicopy *lacZ* operon fusions, Ap^r	This study
pSGE1103	pACYC184 with 2.1-kb *Bam*HI-*Hin*dIII PCR fragment containing *hemAM* and the 200 bp that separate those two genes	This study
pSGE1104	pACYC184 with 0.8-kb *Bam*HI-*Hin*dIII PCR fragment containing *hemM* and the upstream 200 bp	
pSGE1110	pAlterEx2 with 1.2-kb *Bam*HI-*Bgl*II PCR fragment containing *R. capsulatus* $hemA_{RC}$ fused to P_{tac}	This study
pSGE715	High-copy-number plasmid for IPTG-controlled expression of rHb1.1, Tc^r	Lab collection
pSGE494	pACYC184 with 1.2-kb *Bam*HI-*Hin*dIII PCR fragment containing *hemA* coding sequence and the $hemA_1$ and $hemA_2$ promoters	This study
pSGE862	pRS518 with 0.5-kb *Hin*dIII-*Bgl*II fragment of pSGE494 containing $hemA_1$ and $hemA_2$ promoters fused to *lacZ*	This study
pSGE863	pRS518 containing 0.3-kb *Hin*dIII-*Bgl*II fragment containing $hemA_1$ promoter fused to *lacZ*	This study
pSGE864	pRS518 containing 0.4-kb *Hin*dIII-*Bgl*II fragment containing $hemA_2$ promoter fused to *lacZ*	This study
Oligonucleotides		
TG40	5′-CGGGAATACGGATCCAAATGCACCCTGTAAAAAAAGAAAATGATGTACTGC	This study
TG41	5′-CCCAATAATAAGCTTAAAATCGGGCAGGGGCATAGTGATGACAAGTCC	This study
TG224	5′-GGATATCCGAAGCTTCCACTGTGTCCGCATTATTTCAC	This study
EV53	5′-GAATCTAACGGCTTTCGGCAATTACTCCAAAAGGTTTCCCGCAGACATGACCCTTTTAGCACTCGGT	This study
EV54	5′-GGTCATGTCTGCGGGAAACCTTTTGGA	This study
EV113	5′-GCATGTGTCGGATCCGTCTGCGGGAAATAATAC	This study
TG222	5′-GCATGTGTCGAATTCGAAAGCCGTTAGATTCTG	This study
EV98	5′-GCGATACGGGATCCAAGGAGGAATTTACATATGGACTACAATCTCGCGCTC	This study
EV99	5′-GTGATCGAAAGATCTTCACGCACAGCGCGCCCAGAGCAG	This study
EV50	5′-GCTTTTACGCAGATCTTCTTCATTAAGATTGTG	This study
EV39	5′-CCAATGAATTCAAGCTTAATTACTCCAAAAGGGGGCGC	This study
EV45	5′-CCAATGAATTCAAGCTTAAAATCGGGCAGGGG	This study
EV57	5′-GTAGAGGCTTTTACGCAGATCTTCTTCATTAAGATTGTG	This study
Phages		
λRS45	Vector for transferring multicopy *lacZ* fusion to chromosome	R. W. Simons
P1*vir*	Generalized transducing phage	Lab stock

$HemA_{RC}$ allows an *E. coli* ALA auxotroph to grow without ALA supplementation (data not shown). Li and coworkers had previously shown that the *R. sphaeroides* ALA synthase could complement an *E. coli hemA* mutant for growth (16).

Primers and cycle conditions used for amplification of the *Bam*HI-*Hin*dIII fragment in pSGE1104 were as follows: EV113 and TG224; one cycle of 5 min at 95°C, 5 min at 60°C, and 3 min at 72°C, 33 cycles of 30 s at 95°C and 30 s at 60°C, and a final cycle of 8 min at 72°C.

Plasmid DNA for cloning and DNA sequencing was isolated by using a Wizard plasmid isolation kit (Promega, Madison, Wis.) according to the manufacturer's instructions. Restriction digests, gel electrophoresis, DNA ligations, and transformations were performed according to standard methods (22). PCR fragments and restriction fragments for ligation reactions were purified by using a Geneclean II kit (Bio 101, Vista, Calif.). Both strands of DNA fragments generated by PCR were sequenced by using the Prism DYE-terminator cycle sequencing system (Applied Biosystems) and an Applied Biosystems model 373 automated sequencer. DNA sequences were analyzed with programs contained in the MacVector (version 4.5.2) software package (International Biotechnologies Inc., New Haven, Conn.).

β-Galactosidase assays. Strains were grown overnight in 5 ml of M63 salts (19) supplemented with 0.4% glucose, 0.1% Casamino Acids, 50 μg of proline per ml, 40 μg of thiamine per ml, 1 mM $MgSO_4$, and appropriate antibiotics. Cells were diluted 1:50 into test tubes containing 5 ml of the same medium and grown at 37°C with shaking to an optical density at 600 nm (OD_{600}) of 0.4 to 0.8. Cultures were assayed for β-galactosidase as described by Miller (19).

Media and growth conditions. Shake-flask-scale experiments were performed in 50 ml of DM-1 (18) in 250-ml Erlenmeyer flasks. Cultures were grown at 30°C with shaking to an OD_{600} of ~0.6, induced with 300 μM isopropyl-β-D-thiogalactopyranoside (IPTG), and grown for an additional 4 h. At harvest, final culture densities (measured at OD_{600}) were between 2.8 and 4.0. ALA (Sigma Chemical Co., St. Louis, Mo.) was solubilized in water, filter sterilized, and added to cultures to yield solutions with final concentrations of 0.02, 0.10, 0.39, 1.56, 6.25,

25, and 100 μg/ml. Cell pellets for heme measurements were stored at −70°C until analysis.

Fermentations were performed at 30°C in 15-liter fermentors (LSL Biolafitte, Inc., Princeton, N.J.). The seed inoculum for 15-liter fermentors was prepared in a two-stage process. The primary seed stage was 500 ml of DM59 medium in a 2.5-liter shake flask inoculated with 0.5 ml of a stock culture preserved in 20% (vol/vol) glycerol at −80°C. The salts used in DM59 medium were as follows: 33 mM KH_2PO_4, 46 mM K_2HPO_4, 13 mM NaH_2PO_4, 18 mM Na_2HPO_4, 19 mM $(NH_4)_2SO_4$, 5.4 mM K_3 citrate, 2.2 mM Na_3 citrate, 4.2 mM $MgSO_4$, and 7.2 mM H_3PO_4. Trace metals were added to DM59 salts to the final concentrations indicated: 0.91 mM $FeCl_3$, 0.14 mM $ZnCl_2$, 12 μM $CoCl_2$, 10 μM Na_2MoO_4, 0.13 mM $MnCl_2$, 0.41 mM $CaCl_2$, and 54 μM $CuSO_4$. Glucose was added to a final concentration of 1% (wt/vol); thiamine was added to a final concentration of 0.32 mg/ml. Antibiotics (Sigma) were added, when necessary, in the following concentrations: tetracycline, 15 μg/ml; chloramphenicol, 25 μg/ml; and ampicillin, 100 μg/ml.

The primary-seed-stage culture was grown at 30°C with shaking for 8 to 10 h or until the OD_{600} was 0.8 to 1.5, and 400 ml of culture was used to inoculate a secondary seed stage: a 2-liter Bioflo III fermentor (New Brunswick, Edison, N.J.) containing 1,600 ml of DM59 medium supplemented with 1% (wt/vol) glucose, trace metals (in concentrations described above), and appropriate antibiotics. The pH of the secondary-seed-stage culture was maintained at 6.8 by using a base feed of 15% (vol/vol) NH_4OH. Dissolved oxygen was maintained at 20% in 2-liter fermentors.

The secondary-stage-culture was grown to an OD_{600} of 5 to 10, and a 500-ml aliquot was used to inoculate the final stage culture: a 15-liter fermentor (LSL Biolafitte) containing 8 liters of DM59 medium. Glucose was added to 15-liter fermentors at a starting concentration of 2.0 g/liter and maintained thereafter at 2 to 10 g/liter. The pH and dissolved oxygen concentrations of 15-liter fermentors were maintained as described above. When cultures reached an OD_{600} of ~30, IPTG (Sigma) was added to a final concentration of 100 μM to induce rHb1.1 expression.

Heme, obtained as bovine hemin (Amresco, Solon, Ohio), was dissolved in 1 N NaOH to a final concentration of 50 mg/ml. Where specified, heme was added to fermentors at the time of IPTG induction, and at 3 and 6 h postinduction, as 10-, 13-, and 17-ml aliquots. Cell pellets were collected and stored at −70°C until analyzed.

ALA and PBG assays. One-milliliter fermentation samples were resuspended in 10 mM morpholineethanesulfonic acid (pH 6.0) and used for assays. Lysozyme, NaCl, and DNase were added to final concentrations of 500 μg/ml (lysozyme), 100 mM (NaCl), and 60 μg/ml (DNase), and samples were incubated on ice for 20 min and then at 37°C for 2 min. Proteinase K (Sigma) was added to a final concentration of 150 μg/ml, and samples were incubated for an additional 20 min on ice. The sample pH was lowered to 5.5 to 5.9 by addition of 10% acetic acid. Samples were freeze-thawed and then centrifuged for 10 min at 13,000 × *g* at 4°C. The supernatant was removed, and ALA and porphobilinogen (PBG) were separated by using a two-column chromatography system (ALA/PBG by Column Test Kit) obtained from Bio-Rad (Hercules, Calif.). ALA and PBG levels were quantified by their reactivity with Ehrlich's reagent, essentially as described in the protocol for the Bio-Rad test kit.

Heme extraction. Forty-milliliter samples of shake flask cultures were harvested by centrifugation at 13,000 × *g* for 5 min, and pellets were stored for 1 to 5 days at −20°C. The pellets were thawed on ice, resuspended in 1 ml of acetone–1 N HCl (9:1), and vortexed vigorously for 45 s. After a 1-h incubation on ice, the samples were vortexed again and centrifuged (13,000 × *g*) for 10 min. The supernatants were transferred to fresh tubes, and 25-μl aliquots were analyzed by reversed-phase high-performance liquid chromatography. Recovery of heme from spiked samples was greater than 90% under these conditions (data not shown).

Heme quantitation. Samples prepared by the method described above were injected onto a Hypersil ODS column (5 μm; 2.1 by 150 mm; Alltech Associates, Deerfield, Ill.) equilibrated with acetonitrile-methanol-H_2O (1:1:40) and 0.1% (vol/vol) trifluoroacetic acid. After injection, the solvent was changed to acetonitrile-methanol (1:1)–0.1% (vol/vol) trifluoroacetic acid, with a linear gradient over 4 min. Elution with the second buffer continued for 11 min at a flow rate of 0.5 ml/min. Elution was monitored at 404 nm with a Hewlett-Packard model 1090 diode array detector (Hewlett-Packard, Palo Alto, Calif.). Bovine hemin (Amresco) was dissolved in 0.1 N NaOH, quantified spectrophotometrically (6), and used as the standard for heme measurements. The heme values reported represent total, extractable heme present in the cell.

Hemoglobin assay. Samples (1 ml of fermentation broth at an OD_{600} of ~70) were pelleted and resuspended in 25 mM $Na_2B_4O_7$. Lysozyme and NaCl were added to final concentrations of 1 mM NaCl and 0.75 mg of lysozyme per ml, and the samples were incubated first at 4°C (30 to 40 min) and then at 37°C (3 min). DNase (60 μg/ml) was added, and the samples were incubated 15 min at room temperature. The samples were freeze-thawed, treated for 10 s with CO gas, and then diluted into a solution of 80 mM Tris Cl–2 M NaCl (pH 8.0). The crude lysates were heated at 65°C for 4 min and centrifuged at 13,000 × *g* for 2 min to remove cellular debris and contaminating proteins. The supernatant fraction containing partially purified hemoglobin was saved, and the hemoglobin was quantified by immobilized metal chelate chromatography using a Biocad perfusion chromatography workstation (PerSeptive Biosystems, Cambridge, Mass.). The capture column was charged with a solution of 20 mM zinc acetate–200 mM NaCl and equilibrated with 8 mM Tris-HCl–200 mM NaCl (pH 8.0). Samples were loaded onto the column in the same buffering system used for column equilibration, and the column was washed with 20 mM Tris-HCl–500 mM NaCl (pH 8.0). Hemoglobin was eluted from the column with 40 mM Tris-HCl–1 M NaCl–25 mM EDTA (pH 8.3). Hemoglobin elution was monitored at 412 nm.

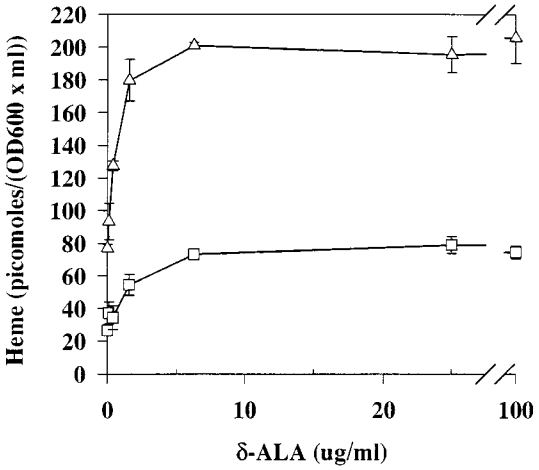

FIG. 2. Effect of ALA supplementation and rHb1.1 production on heme pools. Cultures of SGE1453 in which rHb1.1 expression is induced are represented by triangles; isogenic, uninduced control cultures are indicated by squares. Each data point represents the average of three independent trials. Experimental variation (standard deviation) is shown by error bars.

RESULTS

Role of heme in pathway regulation. Plasmid pSGE715 is a high-copy-number plasmid for IPTG-controlled expression of the alpha and beta subunits of human hemoglobin. Following IPTG induction, *E. coli* strains containing pSGE715 produce large quantities of fully functional tetrameric hemoglobin (rHb1.1). Because each hemoglobin tetramer has the capacity to bind four heme groups, induction of rHb1.1 is expected to rapidly deplete cellular pools of heme. Accordingly, if heme is a repressor of *E. coli* heme synthesis, removal of heme by a heme sink is expected to activate heme synthesis. Our results are similar to those of Hart and coworkers (9) and consistent with the hypothesis that heme is a negative regulator of the heme pathway. Prior to induction of rHb1.1, shake flask cultures of SGE1453 contain approximately 25 pmol of heme/OD_{600}/ml (Fig. 2). Four hours following induction of rHb1.1, cultures of SGE1453 have approximately 75 pmol of heme/OD_{600}/ml (Fig. 2).

Effect of exogenous ALA on heme synthesis. We examined the effect of ALA levels on heme synthesis by feeding shake flask cultures various quantities of ALA and then monitoring cellular heme levels 4 h after induction of rHb1.1. Maximal heme accumulation occurred with 6.25 μg of ALA per ml; higher levels of ALA resulted in no additional increase in heme pools (Fig. 2). These results imply that ALA formation limits heme synthesis. In our studies, ALA supplementation resulted in a threefold increase in cellular heme (Fig. 2). The effects of ALA supplementation and the heme sink were approximately additive. Cultures expressing rHb1.1 which were supplemented with 6.25 μg of ALA per ml contained approximately sevenfold more heme than an uninduced, unsupplemented control.

Role of GTR reductase in heme synthesis. Like Hart and coworkers (9), we were interested to know if increasing ALA pools by genetically manipulating enzymes involved in ALA

TABLE 2. Effects of *hemA*, *hemM*, and $hemA_{RC}$ on intracellular levels of ALA, PBG, and heme in fermentation cultures

Strain	Relevant characteristics	Level (pmol/OD_{600}/ml)[a]				
		ALA (−IPTG)	ALA (+IPTG)	PBG (−IPTG)	PBG (+IPTG)	Heme (+IPTG)
SGE1453	Control	ND	<25	ND	ND	101 ± 11
SGE2664	Multicopy *hemA*	1,017 ± 102	1,690 ± 140	277 ± 45	87 ± 5	164 ± 19
SGE2658	Multicopy *hemA* + *hemM*	795 ± 121	1,735 ± 324	457 ± 69	68 ± 9	211 ± 13
SGE2658[b]	Multicopy *hemA* + *hemM*	NT	2,208 ± 221	NT	52 ± 6	NT
SGE2680	Multicopy *hemM*	ND	ND	ND	ND	90 ± 7
SGE2681	Multicopy $hemA_{RC}$	ND	391 ± 148	<25	873 ± 45	165 ± 13

[a] ALA and PBG assays in the absence of IPTG were measured at induction (OD_{600}, ~30). ALA and PBG values for SGE2680 represent the averages of two experiments. All other ALA and PBG values represent the averages of at least four trials. Variation for ALA and PBG measurements is given as the standard error. ALA and PBG assays in the presence of IPTG were performed on cells 8 h after induction of the heme sink. Samples for heme measurements were collected 8 h after induction of the heme sink. Four to eight replicate fermentations were performed for each strain reported. Heme measurements reflect total extractable heme found in the cell. Variation is reported as the standard error. ND, not detectable; NT, not tested.

[b] Heme was added to fermentors as described in Materials and Methods. Values are the averages of eight fermentation trials.

formation, especially the GTR reductase, would have a greater effect on cellular heme content than supplementation with exogenous ALA. Accordingly, plasmids for overexpression of the *hemA* and *hemM* genes from their native promoters were constructed and evaluated in fermentation cultures. In contrast to other investigators (3, 9, 13), we used medium-copy-number plasmids and were careful to exclude extraneous flanking sequences that might complicate our evaluations. The *hemA* (pSGE494) and *hemM* (pSGE1104) plasmids used in this work contained the complete coding sequences of the relevant gene and 200 bp of upstream DNA. The *hemA hemM* plasmid (pSGE1103) included the complete *hemA* and *hemM* coding regions and the 200 bp of DNA that normally separate those two genes.

In our studies, cells expressing a heme sink and containing multiple copies of either *hemA* alone or *hemA* and *hemM* together accumulated large intracellular pools of ALA relative to a control (Table 2). Because ALA accumulated before and after induction of the heme sink, we conclude that multiple copies of *hemA* (SGE2664) or *hemA* plus *hemM* (SGE2658) result in constitutive expression of GTR reductase and accumulation of ALA (Table 2). Control strains (SGE1453) had undetectable levels of ALA prior to induction of the heme sink and only low levels of ALA postinduction. No significant differences in the ALA pools were found when cells containing multiple copies of *hemA* (SGE2664) and multiple copies of *hemA* plus *hemM* (SGE2658) were compared (Table 2). ALA pools in SGE2680, which expresses *hemM* and rHb1.1, were similar to those in a control strain (SGE1453) both pre- and postinduction (Table 2). Pools of other pathway intermediates (uroporphyrinogen III, coproporphyrinogen III, and protoporphyrin IX) were indistinguishable in strains SGE1453, SGE2644, SGE2658, and SGE2680 (data not shown).

Purification of HemM has not yet been reported, and no antibodies or enzyme assays are available for monitoring expression of the protein. We were therefore unable to verify HemM function by enzyme assay or Western analysis. We can, however, provide indirect evidence to support expression of HemM from the *hemM* plasmids evaluated in this study. We fused a DNA fragment containing the 200-bp region upstream of the *hemM* start codon and the first 18 codons of the *hemM* coding sequence to codon 9 of the *lacZ* gene in pMLB1034 (28) to create a *hemM-lacZ* protein fusion. A wild-type strain containing this *hemM-lacZ* fusion produced 6,118 ± 492 Miller units of β-galactosidase activity, while a control strain containing the pMLB1034 vector produced less than 1 U of activity.

We found that cells expressing rHb1.1 and either HemA (SGE2664) or HemA plus HemM (SGE2658) contained more heme (Table 2) and accumulated rHb1.1 at a faster rate than a control strain (Table 3). We do not believe that multicopy *hemA-hemM* has a greater effect on rHb1.1 accumulation than multicopy *hemA*. When HemM and rHb1.1 were expressed in the same cell (SGE2680), the rate of rHb1.1 accumulation was no faster than in a strain expressing only rHb1.1 (SGE1453). Based on these data, we conclude that ALA formation is a rate-limiting step and that the *hemA*-encoded GTR reductase is a rate-limiting enzyme in the *E. coli* heme pathway.

Feeding exogenous heme to cells overexpressing *hemA* plus *hemM* and rHb1.1 (SGE2658) had no effect on intracellular ALA levels (Table 2). Because cultures of an isogenic strain, SGE1453, produce rHb1.1 at a higher rate when supplemented with heme (Table 3), we believe that SGE2658 is capable of taking up exogenous heme, and we conclude that ALA formation in *E. coli* is not repressed by heme.

Effects of exogenous ALA and heme on *hemA* expression. Using the method of S1 nuclease protection, Verkamp and Chelm identified two potential transcription start sites, −38 and −131, and two σ^{70} consensus promoters ($hemA_1$ and $hemA_2$) for the *hemA* gene in aerobically growing *E. coli* cells (26). The $hemA_1$ and $hemA_2$ promoters were proposed to lie at nucleotides −45 to −70 and −145 to −173, respectively, relative to the *hemA* start codon (26). A divergent transcript which started 83 nucleotides upstream of the $hemA_2$ promoter on the opposite strand was also detected (26). This divergent

TABLE 3. Effects of *hemA*, *hemM*, and $hemA_{RC}$ on rates of rHb1.1 accumulation

Strain	Relevant characteristics	Avg rHb1.1 (+IPTG) (mg/OD_{600}/liter/h) ± SE[a]
SGE1453	Control	0.28 ± 0.05
SGE1453[b]	Control	0.95 ± 0.08
SGE2664	Multicopy *hemA*	0.45 ± 0.05
SGE2658	Multicopy *hemA* + *hemM*	0.59 ± 0.03
SGE2680	Multicopy *hemM*	0.19 ± 0.03
SGE2681	Multicopy $hemA_{RC}$	0.34 ± 0.04

[a] Four to eight replicate fermentations were performed for each strain reported. rHb1.1 measurements were made during the 8-h period following induction. Slopes and variation were obtained from linear regression lines with correlation coefficients ranging from 0.72 to 0.97.

[b] Heme was added to cultures as described in Materials and Methods. Results are the averages of four fermentation trials.

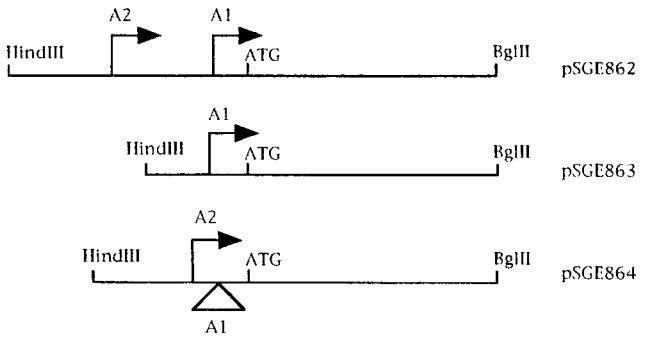

FIG. 3. Features of the DNA fragments used to identify promoters of *hemA*. The start of the *hemA* coding sequence is indicated by the ATG codon. Arrows mark the transcription initiation sites identified by Verkamp and Chelm (26). The triangle below the pSGE864 DNA fragment represents the 72-bp DNA segment containing the A1 transcription start site that was deleted in the PCR amplification.

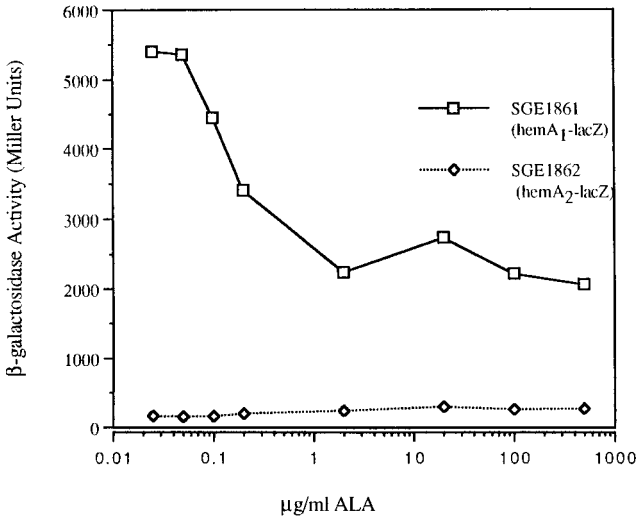

FIG. 4. Effect of ALA concentration on *hemA-lacZ* expression. Cells were grown and assayed as described in Materials and Methods. Each data point represents the average of four independent trials. Variation for each of the data points reported was 10% or less.

transcript was shown to encode a 23-kDa protein, *hemM*, and a role for HemM in ALA synthesis was proposed (3, 13).

To assess the biological relevance of the two potential *hemA* promoters, we fused 244 nucleotides of the *hemA* coding sequence and 158 to 230 nucleotides of sequence 5′ of the *hemA* start codon to the *E. coli lacZ* gene and then transferred those fusions to the *E. coli* chromosome (Fig. 3). Three different *hemA-lacZ* fusions were constructed. One fusion (SGE1858) included the $hemA_1$ promoter, one (SGE1859) included the $hemA_2$ promoter, and one (SGE1857) included both the $hemA_1$ and $hemA_2$ promoters. Because a *hemA* mutant requires ALA supplementation for growth, our initial analyses of these fusions were performed in a $hemA^+$ host. β-Galactosidase activity assays indicated that both the $hemA_1$ and $hemA_2$ promoters are biologically active and that the $hemA_1$ promoter is the stronger promoter (Table 4). Similar results were obtained when the three *hemA-lacZ* fusions were integrated into the genome of a *hemA* mutant and grown with 0.2 μg of ALA per ml (data not shown). A control plasmid containing the 244 nucleotides of *hemA* coding sequence fused to *lacZ* produced approximately 10% of the β-galactosidase activity produced by a similar plasmid containing the weaker of the two *hemA* promoters ($hemA_2$) fused to *lacZ* (data not shown). Our results are similar to Choi and coworkers' findings about the importance of the *hemA* P1 promoter in high level expression of the *Salmonella typhimurium hemA* (4).

The effects of exogenous ALA and heme on *hemA* expression were evaluated in *hemA* mutants containing single-copy *hemA-lacZ* fusions. We found that the $hemA_1$ but not the $hemA_2$ promoter is regulated in response to the intracellular level of ALA. Expression of a $hemA_1$-*lacZ* (SGE1861) fusion was activated approximately twofold when the concentration of ALA was decreased (Fig. 4), while expression of a $hemA_2$-*lacZ* (SGE1862) fusion was unaffected by the ALA concentration (Fig. 4). Addition of exogenous heme (40 μg/ml) to cultures of SGE1861 ($hemA_1$-*lacZ*) grown in a glucose minimal medium containing ALA (either 0.2 or 0.05 μg/ml) did not reduce expression of any of the *hemA-lacZ* fusions tested, suggesting that *hemA* gene expression may not be regulated by heme (data not shown).

TABLE 4. Relative strengths of *hemA* promoters

Strain[a]	Relevant characteristics	Avg β-galactosidase activity (Miller units) ± SE[b]
SGE1857	$hemA_1A_2$-*lacZ*	2,022 (± 216)
SGE1858	$hemA_1$-*lacZ*	3,049 (± 351)
SGE1859	$hemA_2$-*lacZ*	333 (± 28)

[a] Cells were grown and assayed as described in Materials and Methods.
[b] Average of four independent experiments. A control strain containing an integrated copy of pSGE518 produced less than 10 Miller units of β-galactosidase.

Overexpression of ALA synthase. In contrast to *E. coli*, *Rhodobacter* synthesizes ALA by the C_4 or Shemin pathway (Fig. 1) (15). *E. coli hemA* mutants, which require ALA supplementation for growth, can be complemented by $hemA_{RC}$ (11, 16). Thus, *E. coli* can produce heme by using enzymes from either the C_4 or C_5 pathway. We placed $hemA_{RC}$ under control of the *tac* promoter to create an inducible system for studying the effect of high-level expression of ALA synthase on heme pathway regulation.

ALA and PBG pools in cells expressing $hemA_{RC}$ (SGE2681) were significantly higher than in a control strain (SGE1453) following induction of $HemA_{RC}$ and the heme sink (Table 2). Although strains expressing $HemA_{RC}$ and the heme sink (SGE2681) produced more heme, the rates of rHb1.1 accumulation for strains SGE1453 and SGE2681 were indistinguishable (Table 2). Interestingly, we found that the ratios of ALA and PBG in cells expressing *hemA* (SGE2644), *hemA* plus *hemM* (SGE2658), and $hemA_{RC}$ (SGE2681) were considerably different. Strains SGE2644 (*hemA*) and SGE2658 (*hemA hemM*) accumulated very large pools of ALA and smaller pools of PBG, while SGE2681 ($hemA_{RC}$) accumulated large pools of both ALA and PBG (Table 2). An SGE1453 control strain failed to accumulate PBG either before or after induction, and ALA pools were low or undetectable pre- and postinduction.

DISCUSSION

Results described in this work support the hypothesis that ALA formation is a rate-limiting step in *E. coli* heme biosynthesis (10, 17, 21). Based on studies similar to ours, Hart and coworkers concluded that ALA synthesis is not a rate-limiting step (9). Hart and coworkers observed that *E. coli* cells expressing high levels of a heme sink (vHb) were impaired in

their abilities to accumulate heme when supplemented with ALA. Hart and coworkers used 10 to 100 mM ALA in their studies; we used ALA at a concentrations of 0.025 to 0.5 mM (Fig. 2). Based on the results of Harris and coworkers (8), who observed that pseudomonads fed concentrations of ALA in excess of 2 mM were impaired in growth and porphyrin production, we suggest that Hart and coworkers may have used an excessively high concentration of ALA.

In contrast to our studies, Hart and coworkers observed no increase in heme accumulation when they overexpressed a heme sink and provided increased copies of *hemA* and *hemM* (9). The differences in our results may be related to differences in gene dosage and/or to the amounts of ALA that accumulated in cells. Hart and coworkers expressed *hemA* and *hemM*, and the heme sink, from a single, very high copy number plasmid (9). In our studies, the *hemA* gene and the *hemA* and *hemM* genes were expressed separately from the heme sink, on medium-copy-number plasmids.

Although ALA measurements were never monitored, Hart and coworkers assumed that the intracellular ALA level was sufficiently high to create a new pathway limitation at the *hemB*-catalyzed step (9). In follow-up experiments in which *hemA*, *hemM*, *hemB*, and the heme sink were expressed from a single high-copy-number plasmid, they observed a significant decrease in heme accumulation. Unfortunately, no measurements of ALA or PBG were performed, and so the effects of the additional gene copies could not be evaluated thoroughly (9). We suspect that the decrease in heme accumulation may be due, in part, to the increased metabolic burden imposed by their expression plasmid (1).

Our results do not support the hypothesis that both *hemA* and *hemM* are required for maximal accumulation of ALA (3). We observed an enhancement in ALA accumulation when cells were provided with multiple copies of *hemA* but no further enhancement when both *hemA* and *hemM* were provided. We note that, in contrast to the studies of Chen and coworkers (3), our *hemA hemM* plasmid was of moderate copy number, and the cloned DNA fragment lacked any sequences beyond the natural stop codons present in *hemA* and *hemM*. Chen and coworkers' plasmid contained a portion of *prfA* and an uncharacterized open reading frame, either of which may have contributed to their results (3). Moreover, it remains possible that Chen and coworkers' results were due to titration of a regulatory factor by a sequence present on their very high copy number plasmid (3).

Like Chen and coworkers, we found that a *hemM*-containing plasmid was unable to complement a *hemA* mutant (data not shown) and that wild-type cells containing multiple copies of *hemM* did not accumulate larger amounts of ALA (3). Our results do not support Ikemi and coworkers' conclusion that *hemM* encodes a GTR reductase (13).

We found that intracellular ALA levels in cultures containing multiple copies of *hemA* plus *hemM* were not reduced by exogenously supplied heme. Based on results presented earlier in the text, we feel confident that the exogenous heme was taken up by cells. When considered together with Jahn and coworkers' in vitro results (14), these data suggest that the *E. coli* GTR reductase may be insensitive to heme levels and thus regulated differently from other GTR reductase enzymes (2, 12).

Two genetic systems for increasing ALA pools, *hemA* and $hemA_{RC}$, were evaluated in this study. Although both systems allowed for an increase in cellular heme content, the two systems had different effects on rHb1.1 accumulation. The strong *tac* promoter that drives the $hemA_{RC}$ gene in pSGE1110 is probably much more efficient than either the $hemA_1$ or $hemA_2$ promoter, neither of which has ideal −10 or −35 consensus elements (26). It is possible that the level of $HemA_{RC}$ protein produced by pSGE1110 is so high that the cell's capacity to produce rHb1.1 is compromised, even though the cell accumulates more heme. In support of this hypothesis, we note that others have observed a decrease in protein synthetic capacity in cells expressing genes from very strong promoters (5).

Harris and coworkers (8) found that supplementation of *Pseudomonas* cultures with large quantities of ALA increased heme pathway flux and led to excretion of high levels of porphyrins, with uroporphyrinogen I, a nonenzymatically formed pathway intermediate which is physiologically nonrelevant, appearing as the predominant product (8). In some strains, a significant amount of this incorrect isomer could be further processed. Based on these findings, Harris and coworkers proposed that high levels of ALA saturate the enzymatic machinery that normally converts four PBG molecules to the physiologically relevant uroporphyrinogen III (8). It is possible that IPTG induction of $hemA_{RC}$ provides a sufficiently high quantity of ALA to overload the enzymatic machinery that converts PBG to heme, for example, by allowing formation of incorrect isomers which cannot be further processed or incorporated into rHb1.1. It is possible that Hart and coworkers' experimental design also allowed for the production of incorrect isomers (9). Our analytical methods do not allow us to discriminate among the various porphyrin isomers.

ACKNOWLEDGMENTS

We are grateful to members of the Somatogen Fermentation Research Group, the Somatogen Molecular Biology Group, and the Somatogen Analytical Services Division for outstanding technical assistance. We thank members of the DNA sequencing facility at Eli Lilly Research Laboratories for helping us verify the DNA sequences of several of the PCR clones described in this work. We thank our colleagues Dan Doherty and Bob Sclafani, for critiquing the manuscript and Debbie Abbott-Brown for encouragement.

REFERENCES

1. **Bentley, W. E., N. Mirjalili, D. C. Andersen, R. H. Davis, and D. S. Kompala.** 1990. Plasmid encoded protein: the principal factor in the "metabolic burden" associated with recombinant bacteria. Biotechnol. Bioeng. **35:**668–681.
2. **Castelfranco, P. A., and S. I. Beale.** 1983. Chlorophyll biosynthesis: recent advances and areas of current interest. Annu. Rev. Plant Physiol. **34:**241–278.
3. **Chen, W., C. S. Russell, Y. Murooka, and S. C. Cosloy.** 1994. 5-Aminolevulinic acid synthesis in *Escherichia coli* requires expression of *hemA*. J. Bacteriol. **176:**2743–2746.
4. **Choi, P., L. Wang, C. D. Archer, and T. Elliott.** 1996. Transcription of the glutamyl-tRNA reductase (*hemA*) gene in *Salmonella typhimurium* and *Escherichia coli*: role of the *hemA* P1 promoter and the *arcA* gene product. J. Bacteriol. **178:**638–646.
5. **Dong, H., L. Nilsson, and C. G. Kurland.** 1995. Gratuitous overexpression of genes in *Escherichia coli* leads to growth inhibition and ribosome destruction. J. Bacteriol. **177:**1497–1504.
6. **Falk, J. E.** 1964. Absorption spectra, p. 231–246. *In* F. E. Falk (ed.), Porphyrins and metalloporphyrins. Elsevier Publishing Company, New York, N.Y.
7. **Granick, S.** 1966. The induction *in vitro* of the synthesis of delta aminolevulinic acid synthetase in chemical porphyria: a response to certain drugs, sex hormones, and foreign chemicals. J. Biol. Chem. **241:**1359–1375.
8. **Harris, W. F., III, R. S. Burkhalter, W. Lin, and R. Timkovich.** 1993. Enhancement of bacterial porphyrin biosynthesis by exogenous aminolevulinic acid and isomer specificity of their products. Bioorg. Chem. **21:**209–220.
9. **Hart, R. A., P. T. Kallio, and J. E. Bailey.** 1994. Effect of biosynthetic manipulation of heme on insolubility of *Vitreoscilla* hemoglobin in *Escherichia coli*. Appl. Environ. Microbiol. **60:**2431–2437.
10. **Hino, S., and A. Ishida.** 1973. Effect of oxygen on heme and cytochrome content in some facultative bacteria. Enzyme **16:**42–29.
11. **Hornberger, U., R. Liebetanz, H.-V. Tichy, and G. Drews.** 1990. Cloning and sequencing of the *hemA* gene of *Rhodobacter capsulatus* and isolation of a δ-aminolevulinic acid-dependent mutant strain. Mol. Gen. Genet. **221:**371–378.
12. **Huang, D.-D., and W.-Y. Wang.** 1986. Chlorophyll biosynthesis in *Chlamy-*

domonas starts with the formation of glutamyl-tRNA. J. Biol. Chem. **261:** 13451–13455.
13. **Ikemi, M., K. Muakami, M. Hashimoto, and Y. Murooka.** 1992. Cloning and characterization of genes involved in the biosynthesis of δ-aminolevulinic acid in *Escherichia coli*. Gene **121:**127–132.
14. **Jahn, D., U. Michelsen, and D. Soll.** 1991. Two glutamyl-tRNA reductase activities in *Escherichia coli*. J. Biol. Chem. **266:**2542–2548.
15. **Kikuchi, G., A. Kumar, P. Talmage, and D. Shemin.** 1958. The enzymatic synthesis of δ-aminolevulinic acid. J. Biol. Chem. **233:**1214–1219.
16. **Li, J. M., O. Brathwaite, S. D. Cosloy, and C. S. Russell.** 1989. 5-Aminolevulinic acid synthesis in *Escherichia coli*. J. Bacteriol. **171:**2547–2552.
17. **Li, J. M., C. S. Russell, and S. D. Cosloy.** 1989. Cloning and structure of the *hemA* gene of *Escherichia coli*. Gene **82:**209–217.
18. **Looker, D., A. J. Mathews, J. O. Neway, and G. L. Stetler.** 1994. Expression of recombinant human hemoglobin in *Escherichia coli*. Methods Enzymol. **231:**364–374.
19. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **O'Neill, G. P., M.-W. Chen, and D. Soll.** 1989. δ-Aminolevulinic acid biosynthesis in *Escherichia coli* and *Bacillus subtilis* involves formation of glutamyl-tRNA. FEMS Microbiol. Lett. **60:**255–260.
21. **Philipp-Dormston, W. K., and M. Doss.** 1973. Comparisons of porphyrin and heme biosynthesis in various heterotrophic bacteria. Enzyme **16:**57–64.
22. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
23. **Sasarman, A., G. S. M. Surdeanu, T. Horodniceanu, V. Greceanu, and A. Dumitrescu.** 1968. Hemin-deficient mutants of *Escherichia coli* K-12. J. Bacteriol. **96:**570–572.
24. **Silhavy, T. J., M. L. Berman, and L. W. Enquist.** 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. **Simon, R. W., F. Houman, and N. Kleckner.** 1987. Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. Gene **53:**85–96.
26. **Verkamp, E., and B. K. Chelm.** 1989. Isolation, nucleotide sequence, and primary characterization of the *Escherichia coli* K-12 *hemA* gene. J. Bacteriol. **171:**4728–4735.
27. **Vincent, S. H.** 1989. Oxidative effects of heme and porphyrins on proteins and lipids. Semin. Hematol. **26:**105–113.
28. **Weinstock, G. M., M. L. Berman, and T. J. Silhavy.** 1983. Chimeric genetics with β-galactosidase, p. 26–64. *In* T. S. Papas, M. Rosenberg, and J. G. Chirikjian (ed.), Gene amplification and analysis. Elsevier/North Holland Publishing Co., New York, N.Y.