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# 5-AMINOLEVULINIC ACID BIOSYNTHESIS IN *ESCHERICHIA COLI*

**By**
**Wei Chen**

**A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York**

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# ABSTRACT

## 5-AMINOLEVULINIC ACID BIOSYNTHESIS IN *ESCHERICHIA COLI*

by

Wei Chen

Advisers: Dr. C. S. Russell & Dr. S. D. Cosloy

In *E. coli*, heme biosynthesis is regulated at the level of synthesis of its first committed precursor, 5-aminolevulinic acid (ALA). ALA is synthesized by the $C_5$ pathway. Glutamate is converted to glutamyl-tRNA by glutamyl-tRNA synthetase (GTS), the gene product of *gltX*. Glutamyl-tRNA is reduced to glutamyl-semialdehyde (GSA) by glutamyl-tRNA reductase (GTR), the gene product of *hemA*. GSA is converted to ALA by GSA aminotransferase, the gene product of *hemL*. This last step also takes place non-enzymatically. Because glutamyl-tRNA is used for protein synthesis as well, regulation of ALA synthesis is probably to be at the level of GTR synthesis or activity.

*hemM*, which is located 213 bp up stream from *hemA* and transcribed divergently, was assumed by some to be essential for ALA synthesis. *hemA* and *hemM* were cloned separately into multi-copy plasmids. *hemA* was required for ALA biosynthesis in two ALA-deficient mutant strains. Overexpression of *hemM* alone did not complement the ALA deficient mutants. More ALA was produced by strains harboring a plasmid with both *hemA* and *hemM* than by those with *hemA* alone. These results suggest that *hemA* alone is required for ALA biosynthesis but that expression of *hemM* or the function of the

extended upstream region of *hemA*, which is the complementary strand to part of *hemM*, is required for maximal ALA synthesis.

In order to study GTR, an overexpression strain was constructed. BL21($DE_3$), a protease-deficient strain which contains the T7 RNA polymerase gene in front of a *lac* promoter, was transformed with a pET14b-based vector, pWC01, harboring *hemA* in front of a T7 promoter, and *hemM* which is transcribed in the opposite direction. The transformed strain, WC1201, secreted ALA and porphyrins into the medium. Induction of expression of *hemA* by WC1201 was optimized for concentration of inducer (IPTG, 5 mM), temperature (37°C), and time of induction (2 h). GTR was observable as a 46 kDa band by Brilliant Blue G staining of SDS-PAGE gels. Sonicates of the induction mixture exhibited strong ALA synthesis activity. Most of the activity was in the supernatant of the sonicate indicating that GTR is a soluble enzyme. This activity was measurable without the use of radioactive substrate showing that a substantial amount of GTR had formed. Autoradiography on native gradient PAGE showed that GTR expressed *in vivo* by induction of WC1201 had a molecular weight of approximately 117 kDa. Gel filtration of the induced sonicate showed a peak of enzymatic activity at about 126 kDa. When pET14b- or pUC19-based plasmids harboring *hemA* and *hemM*, or importantly, a pUC19-based plasmid harboring only *hemA* but not *hemM*, were expressed in an *in vitro* transcription-translation system, native gradient PAGE showed a product with a molecular weight of approximately 175 kDa. When the 117 kDa and 175 kDa proteins were excised from their native gels respectively, and run on SDS PAGE, autoradiography showed bands at 46 kDa. These results suggest that the 117-126 kDa species may be a dimer of GTR

associated with glutamyl-tRNA or a complex of GTR, GTS and glutamyl-tRNA. GTS may be regulated by GTR. When GTR was overexpressed in WC1201, the level of GTS was 2.5 fold higher than in a strain with no insert on the plasmid. Uninduced WC1201 had 1.5 fold higher GTS than the same strain without the insert in the plasmid.

*E. coli* RP523, a strain which cannot use ALA because there is a mutation in *hemB*, the structural gene for the next enzyme in the pathway, ALA dehydratase, accumulates ALA under aerobic and anaerobic growth conditions. The amount of ALA accumulation in the medium of RP523 decreased when hemin was present in the medium, suggesting regulation by hemin. This effect was concentration-dependent. It was found that the activity of ALA synthesis in a cell-free extract of WC1201 was also inhibited by hemin or hemin arginate, suggesting feedback inhibition of GTR by hemin. RP523 accumulated less ALA when it was grown on glucose-supplemented rich medium than when it was grown without glucose. The glucose effect was slightly reversed by cAMP.

$tRNA^{glu}$ enhances ALA synthesis in cell-free extracts from induced WC1201. $tRNA^{glu}$ also stimulates *in vitro* transcription and translation of two different *hemA* plasmids. LPL-1, a strain which contains *hemA* on one plasmid and the gene for $tRNA^{glu}$ on a compatible plasmid, produced 30-40% more ALA than the same host strain with the *hemA* plasmid alone. The stimulatory effects of $tRNA^{glu}$ may be attributed to (1) the stimulation of transcription or translation of *hemA* and (2) the production of more glutamyl-tRNA, the substrate for GTR.

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# ABBREVIATIONS

| | |
|---|---|
| ALA | 5-aminolevulinic acid |
| ALA S | 5-aminolevulinic acid synthetase |
| amp | ampicillin |
| ATP | adenosine triphosphate |
| bp | base pair |
| β-ME | β-mercaptoethanol |
| BSA | bovine serum albumin |
| cam | chloramphenicol |
| cAMP | cyclic adenosine monophosphate |
| copro | coproporphyrin |
| coprogen | coproporphyrinogen |
| CRP | cAMP receptor protein |
| DMSO | dimethyl sulfoxide |
| DTT | dithiothreitol |
| *E. coli* | *Escherichia coli* |
| EDTA | ethylenediaminetetraacetic acid |
| GSA | glutamic semialdehyde |
| GTS | glutamyl-tRNA synthetase |
| *gltX* | glutamyl-tRNA synthetase structural gene |
| glu | glutamate |
| GTR | glutamyl-tRNA reductase |
| *hemA* | glutamyl-tRNA reductase structural gene |
| *hemB* | ALA D dehydratase structural gene |
| kb | kilobase |
| kDa | kilodalton |
| LA | levulinic acid |
| NADPH | nicotinamide adenine dinucleotide phosphate (reduced form) |
| PAGE | polyacrylamide gel electrophoresis |

| | |
|---|---|
| PEG | polyethylene glycol |
| PDT | photodynamic therapy |
| PLP | pyridoxal-5-phosphate |
| PMSF | phenylmethylsulfonyl fluoride |
| proto | protoporphyrin |
| protogen | protoporphyrinogen |
| SDS | sodium dodecyl sulfate |
| TAE | Tris-acetate-EDTA |
| TBE | Tris-borate-EDTA |
| TCA | trichloroacetic acid |
| TE | Tris-EDTA |
| TEMED | N,N,N',N'-tetramethyl-ethylenediamine |
| tet | tetracycline |
| Tween | polyoxyethylene sorbitan monooleate |
| uro | uroporphyrin |
| urogen | uroporphyrinogen |
| X-gal | 5-bromo-4-chloro-3-indolyl-galactopyranoside |

# INTRODUCTION

## 1. Macrocyclic Tetrapyrroles

Macrocyclic tetrapyrroles play important roles in several biological processes including energy capture and utilization, oxygen transport and storage, enzymatic degradation of peroxides, oxidative metabolism of certain drugs and lipophilic chemicals, steroid biosynthesis, tryptophan metabolism and desaturation of fatty acids. Among the groups of biologically important macrocyclic tetrapyrroles in living organisms are hemes, chlorophylls, bacteriochlorophylls, siroheme, corrinoids and coenzyme$_{430}$ (Friedmann & Thauer, 1992) (Figure 1).

*E. coli* synthesizes hemes (Figure 2) and siroheme. It uses heme as the prosthetic group of cytochromes for the transfer of electrons and several enzymes for mineral nutrition and oxidative catalysis (Beale, 1996). It uses siroheme as the prosthetic group of sulfite and nitrite reductase for reduction of sulfite and nitrite (Ingledew & Poole, 1984).

### 1.1. The structure of heme molecule

The basic structure of heme is the protoporphyrin ring. Four pyrroles joined by four methine (-CH=) bridges to form a square planar structure with an iron atom in the center (Figure 2). There are two forms iron protoporphyrin depending on the valence of the iron. Heme is ferro-protoporphyrin and hemin is ferri-protoporphyrin. Free heme in solution is rapidly oxidized to hemin.

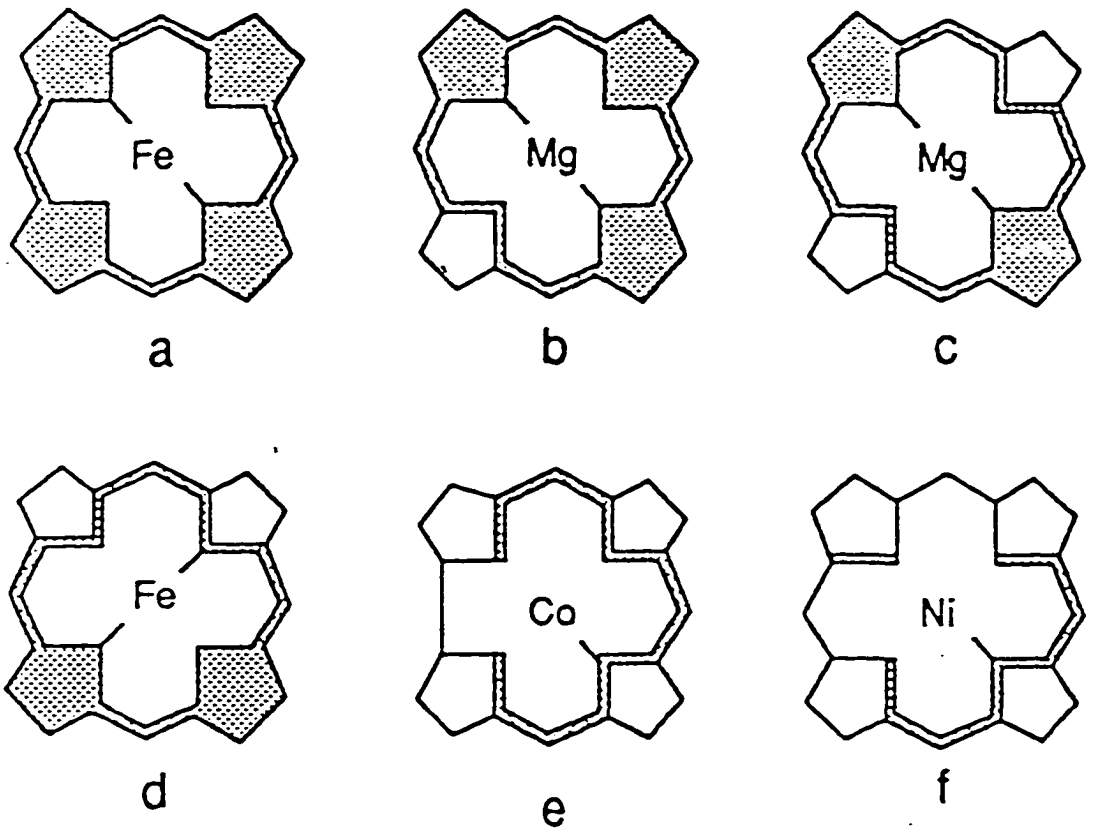



Figure 1. Structure of various tetrapyrroles (a) hemes, (b) chlorophylls and bacteriochlorophylls c, d,and e, (c) bacteriochlorophylls a, b, and g, (d) siroheme,(e) corrinoids, and (f) coenzyme $F_{430}$ (adapted from Friedmann and Thauer, 1992).

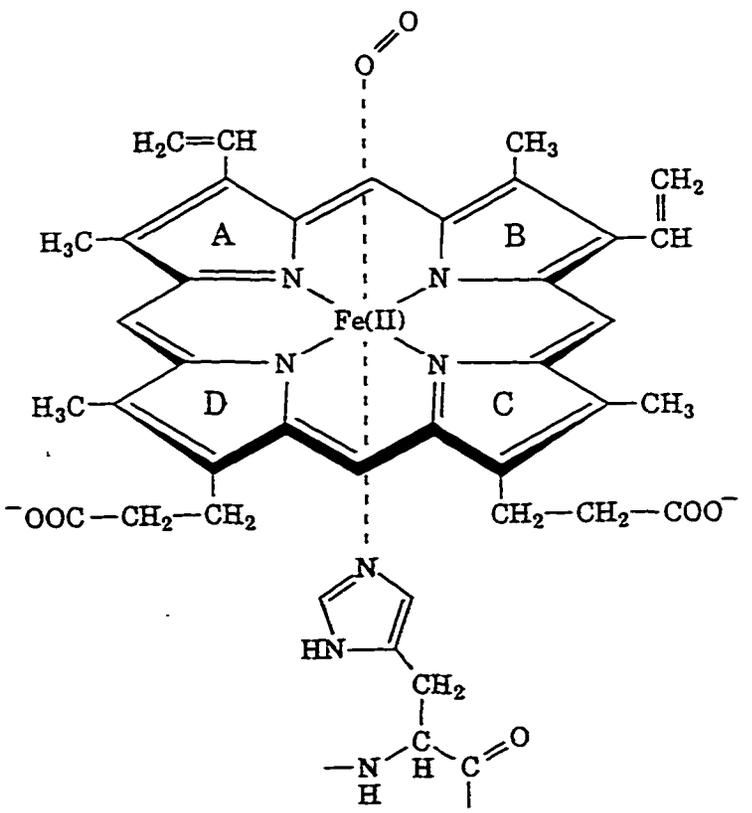



Figure 2. The structure of heme shown liganded to His and $O_2$ as it is in oxygenated myoglobin and oxygenated hemoglobin(adapted from Voet & Voet, 1989).

## 1.2. The heme biosynthetic pathway

The biosynthesis of the hemes, chlorophylls, corrins and other tetrapyrroles (Figure 3) begins with the synthesis of 5-aminolevulinic acid (ALA), the first universal precursor of the pathway. The pathway is highly conserved except for the synthesis of ALA. ALA can be produced by two completely different pathways, $C_4$ (Shemin & Russell, 1953) and $C_5$ (Beale et al., 1975).

In the $C_4$ pathway, ALA synthetase (ALAS) converts succinyl-CoA and glycine to ALA, In the $C_5$ pathway, glutamyl-tRNA is synthesized by glutamyl-tRNA synthetase (GTS) and reduced by glutamyl-tRNA reductase to glutamic semialdehyde (GSA). GSA is converted to ALA by GSA aminotransferase. These will be described in detail in the next section.

The next step is the condensation of two molecules of ALA to generate porphobilinogen (PBG) in a reaction catalyzed by a zinc-containing enzyme ALA dehydratase (ALAD-EC. 4.2.1.24). Four PBG molecules are joined, head to tail, to form a linear tetrapyrrole (bilane) by PBG deaminase (PBGD-EC. 4.3.1.8). This bilane is very unstable. It cyclizes spontaneously to uroporphyrinogen I (urogen I), or to uroporphyrinogen III (urogen III) in the presence of urogen III synthase, enzymatically (EC. 4.2..1.75). The four acetic acid groups of uroporphyrinogen III are decarboxylated to methyl groups by uroporphyrinogen decarboxylase (EC. 4.1.1.37). The result is the formation of coproporphyrinogen III. Coproporphyrinogen III is modified by coproporphyrinogen oxidase (EC. 1.3.3.3); the propionic acid groups of ring A and B of

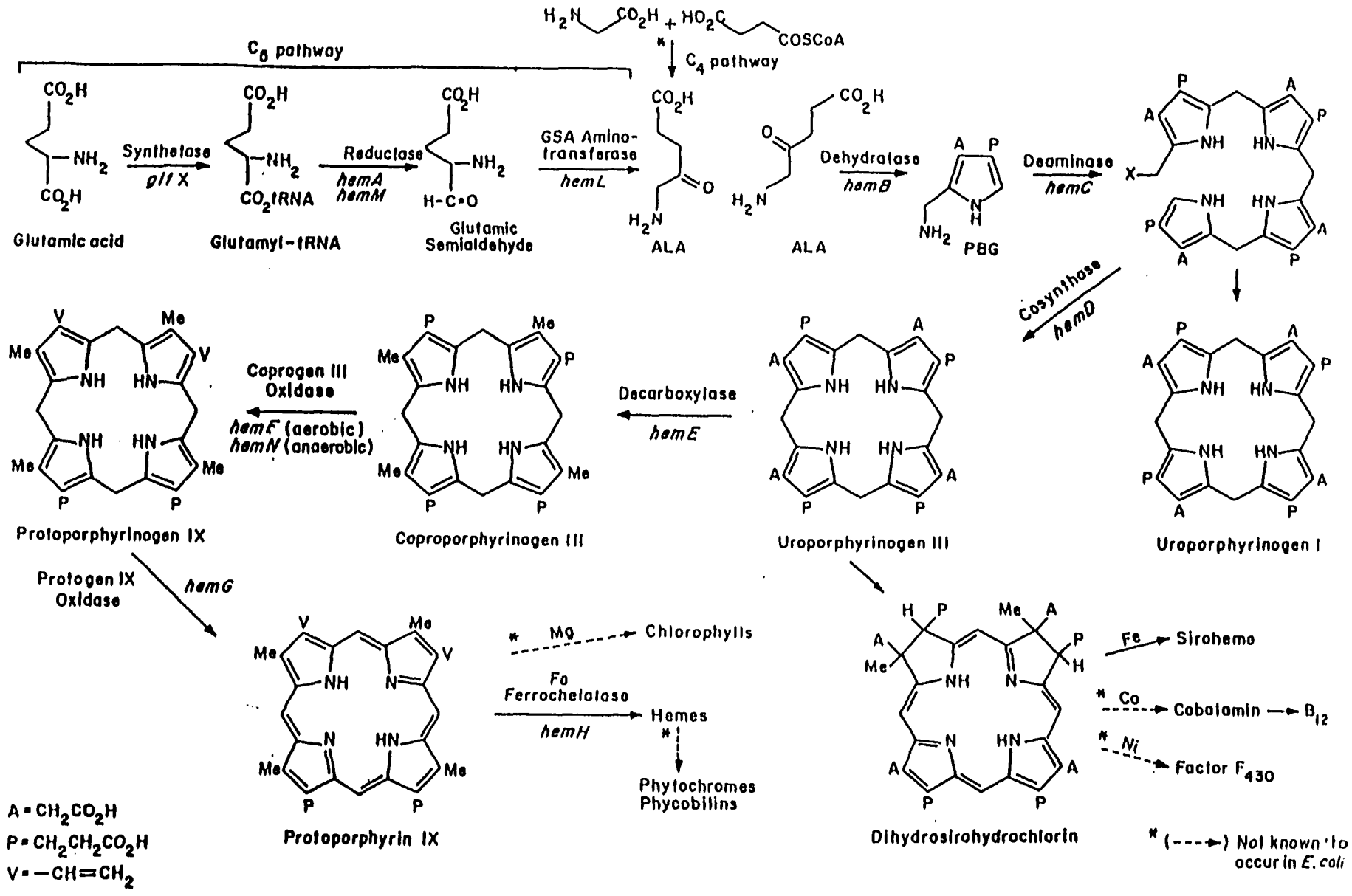



Figure 3. The heme biosynthesis pathway in *E. coli* (by Dr. C. S. Russell).

coprogen III are converted into vinyl groups to produce protoporphyrinogen IX. Protoporphyrinogen oxidase (EC. 1.3.3.4) oxidizes the latter to form protoporphyrin IX.Finally, iron is inserted to form heme. This reaction is catalyzed by ferrochelatase (EC. 4.99.1.1).

There are some interesting branch points in this pathway: at glutamyl-tRNA to form heme or protein, at uroporphyrinogen III to form siroheme, cobalamin or coenzyme $F_{430}$, at protoporphyrin IX to form chlorophylls, bacteriochlorophylls, phycobilins and hemes. *E. coli* synthesizes heme and siroheme.

### 1.3. Genetics of heme formation

In the $C_5$ pathway, there are more than ten genes involved in heme biosynthesis (Figure 4). In *E. coli*, *hem* genes are widely scattered on the genome, except for *hemC*, *hemD* and *hemG* which are linked and appear to form an operon. Most of the *hem* genes have been identified, mapped and cloned. *hemA is* located at *E. coli* K-12 linkage map minute 27 (Guest, 1969; Sasarmann et al., 1968b; Sasarmann 1968a), *hemB* at minute 8 (Sasarmann et al., 1968a), *hemC* at minute 85 (McConville & Charles, 1979b), *hemD* at minute 85 (Chartrand et al., 1979), *hemE* at minute 90 (Sasarmann et al., 1975), *hemF* at minute 53 (Troup et al., 1994), *hemG* at minute 86 (Sasarmann et al., 1978), *hemH at minute* 11 (Cox & Charles, 1973; Powell et al., 1973), *hemK* at minute 27 (Nakayashiki et al., 1995), *hemL* at minute 4 (Powell et al., 1973), *hemM* at minute 27 (Ikemi et al., 1992), and *hemN* at minute 87 (Troup et al., 1995) respectively. *hemA* (Li et al., 1989c),

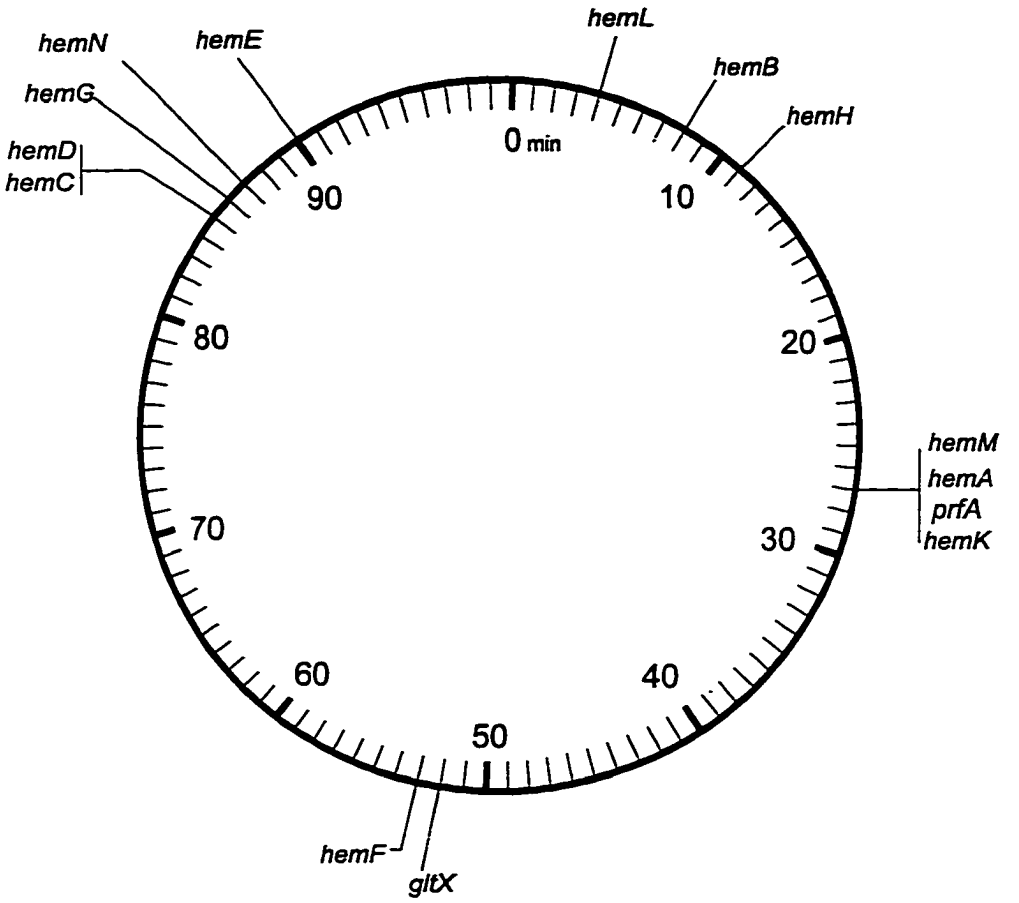



Figure 4. Location of heme biosynthesis genes on the *E. coli* K-12 linkage map.

*hemB* (Li et al., 1989a) and *hemE* (Pido et al., 1994) genes have been cloned and sequenced in this laboratory.

In *Salmonella typhimurium*, *hemN* is required for heme biosynthesis anaerobically. Because *hemN* mutants accumulate coproporphyrinogen III and are auxotrophic for protoheme only when grown anaerobically, it is proposed that *hemN* encodes an anaerobic coproporphyrinogen oxidase (Xu et al., 1992). *hemF* is proposed to be the aerobic coproporphyrinogen oxidase (Xu et al., 1993). There are two different coproporphyrinogen III oxidases in *E. coli* for aerobic and anaerobic conditions (Troup et al., 1994; Troup et al., 1995). These two coproporphyrinogen III oxidases are encoded by *hemF* and *hemN*, respectively. The *hemN* gene product can complement *S. typhimurium* *hemF* and *hemN* double mutants under aerobic and anaerobic conditions. However, *hemN* expression under anaerobic conditions was three fold greater than under aerobic conditions (Troup et al., 1995).

In *E. coli*, *hemK* (Nakayashiki et al., 1995) and *hemM* (Ikemi et al., 1992) are located at minute 27 on the linkage map of the *E. coli* chromosome. *hemK* forms part of the *hemA-prfA-hemK* operon and encodes 225 amino acids. When a light-sensitive double mutant (H103) in *hemA* and *hemK* was transformed with a plasmid that included only *hemA* as an insert, it accumulated protoporphyrinogen and coproporphyrinogen. This indicated that the product of *hemK* might be involved in the oxidation of protoporphyrinogen to protoporphyrin IX. This oxidation step is catalyzed by the product of the *hemG* gene (Sasarman et al., 1978), therefore, the product of *hemK* may be a

subunit of protoporphyrinogen IX oxidase or specific to aerobic or anaerobic conditions as in the case of *hemF* and *hemN*.

The *hemM* gene is adjacent to *hemA* but transcribed divergently. Both genes share 213 bp upstream region. Murooka and coworkers (1992) found that three ALA-requiring mutants were complemented in a heterologous fashion (small and normal colonies) by the *hemM* gene. A plasmid containing *hemA* but not *hemM* gave only small colonies. Therefore, they suggested that the *hemM* gene product is the major glutamyl-tRNA reductase and that *hemA* encodes a protein involved in a minor pathway for ALA synthesis. Results reported in this thesis show that this is not the case.

In *Bacillus subtilis*, a gene called *hemX* is located in the *hemAXCDBL* operon. This gene is not required for heme biosynthesis. However, it negatively affects the steady-state cellular concentration of glutamyl-tRNA reductase (Schroder et al., 1994).

Hansson and Hederstedt (1992) reported that the *hemY* gene is involved in heme biosynthesis in *Bacillus subtilis*. Recently, Dailey et al. (1994) reported that *hemY* could complement a protoporphyrinogen oxidase mutation. The *hemY* sequence has not been observed in *E. coli*.

## 2. 5-Aminolevulinic acid biosynthesis

### 2.1. $C_4$ pathway

In the $C_4$ biosynthesis pathway, ALA is produced by condensation of glycine and succinyl-CoA which is catalyzed by pyridoxal phosphate-dependent ALA synthase (EC 2.3.1.37) (Shemin and Russell, 1953).

Most eukaryotes and α-photobacteria use the $C_4$ pathway to synthesis ALA. The gene coding for ALAS has been cloned and sequenced from different sources and the enzyme has been purified and characterized (Warnick & Burnham, 1971; Ohashi & Kikuchi, 1979; Borthwick et al., 1983; Dzelzkalns et al., 1982 ; Volland & Felix, 1984).

### 2.2. $C_5$ pathway

Beale and Castelfranco (1974) demonstrated that 5-ALA was derived from the carbon skeleton of glutamate in greening cucumber cotyledons. Thus, this pathway was called the $C_5$ pathway. This result accounted for the lack of ALAS activity in plants.

Green plants, algae, archae and most bacteria including *E. coli*, use the $C_5$ pathway to synthesize ALA. The $C_5$ pathway of ALA formation has three steps: synthesis of glutamyl-tRNA by glutamyl-tRNA synthetase (GTS) (EC 6.1.1.17), reduction of glutamyl-tRNA to glutamyl semialdehyde (GSA) by glutamyl-tRNA reductase (GTR), and transamination of glutamyl semialdehyde by aminotransferase (GSA AT)(EC 5.4.3.8) to form ALA.

The first enzyme, GTS (ligase)(the *gltX* gene product) catalyzes the coupling of glutamate to $tRNA^{glu}$ in the presence of ATP and magnesium ions (Schon et al., 1986). The role of glutamyl-tRNA as a precusor of ALA was shown for greening barley by Kannangara et al. (1984) and Peterson et al. (1988), for *Chlorella* by Avissar and Beale (1988), for *Methanobacterium* by Friedmann et al. (1987), for *Chlamydomonas* by Huang et al. (1984); Huang and Wang (1986), and for *Synechocysis* by O'Neill et al (1988). Schneegurt and Beale (1988) showed that the UUC glutamate anticodon in $tRNA^{glu}$ was required for function in ALA synthesis. While *E. coli* $tRNA^{glu}$ could be charged with

glutamate by synthetase from many sources, it stimulated ALA synthesis only in *Chlamydomonas reinhardtii* (Huang and Wang, 1986).

Glutamyl-tRNA is reduced by GTR (the *hemA* gene product) (Lapointe and Soll, 1972), which requires NADPH (Mau et al., 1987). There are two possible structures for the product of this reaction. Houen et al (1984) have proposed glutamate 1-semialdehyde (GSA) as the structure of this intermediate. Jordan et al. (1989) have proposed that this intermediate is 2-hydroxyl-3-amino-tetrahydropyran-1-one (HAT), which is a cyclic form of GSA.

The third enzyme, aminotransferase (GSA-AT), the *hemL* gene product, converts GSA to ALA (Kannangara & Gough, 1978). It was found that GSA could be nonenzymatically converted to ALA at pH 8.0 (Hoober et al., 1988). This may be the reason why *hemL* mutants of *E. coli* and *S. typhimurium* can grow without ALA supplementation (Elliott & Roth, 1989; Ilag & Jahn, 1992).

Some photosynthetic organisms *E. gracilis* (Weinstein & Beale, 1983) and *Sc. obliquus* mutant C-2A (Drechsler-Thielmann et al., 1993) as well as higher plants have both the $C_4$ and the $C_5$ pathways. These organisms can use different ALA synthesis routes depending on the metabolic status. During germination or photosynthetic growth, the $C_4$ pathway will be shifted to the $C_5$ pathway (Yang & Hoober, 1995).

### 2.3. **ALA biosynthesis in *E. coli***

ALA synthesis in bacteria was first studied in *Rhodopseudomonas*, a member of the only bacterial group which uses the $C_4$ pathway. When *E. coli hemA* mutants were

transformed with plasmids carrying the ALA synthase gene from other organisms, they could grow well on the media without addition of ALA (Avissar & Beale, 1989). However, ALA synthase activity has never been detected in wild type *E. coli*. An *E. coli* mutant, which required ALA for growth, was complemented with an *E. coli* genomic library. The gene, which restored ALA synthesis was designated *hemA* and it was cloned and sequenced. Its derived amino acid sequence showed no homology with any known ALA synthase (Li et al., 1989b).

Experiments with [1-$^{14}$C]-labeled glutamate or [2-$^{14}$C]-labeled glycine showed that C-1 of glutamate was preferentially incorporated into $C_5$ of ALA (Li et al., 1989 b). Under the same conditions, an *E. coli* strain TB1(pUI553), a *hemA* mutant harboring a plasmid containing the structure gene for ALA S from *Rhodobacter sphaeroides*, used glycine but not glutamate for ALA synthesis (Avissar & Beale, 1989; O'Neill et al., 1989). These results prove that *E. coli* normally synthesizes ALA by the $C_5$ pathway.

### 2.4. **ALA as a photodynamic agent**

ALA synthesis in most systems is the rate-limiting step in tetrapyrrole biosynthesis. Thus oversupply of ALA causes accumulation of porphyrins and therefore causes fluorescence and photosensitivety (Kennedy et al., 1990). The former is being used for diagnosis and location of tumours (Baumgartner et al., 1996), because tumours take up ALA more than normal tissue. These tumours become photosensitive, so irradiation or photodynamic therapy is used to destroy the tumours (Pier et al., 1995). Plants treated

with ALA become photosensitive and die in the light which suggests potential use of ALA as an herbicide.

a. Herbicide

ALA serves as a building block of tetrapyrrole accumulation. When plants are treated with ALA, they accumulate undesirable amounts of intermediates of the chlorophyll and heme biosynthetic pathways. The high levels of tetrapyrroles can be stimulated to an excited state by absorption of light energy, which can react with ground oxygen to form singlet oxygen or other oxygen free radicals. e.g. superoxide, hydrogen peroxide and hydroxyl radical. The latter can be causing damage of cell by oxidation of their membranes (Rebeiz et al., 1990). ALA has been used as a herbicide for the control of several broadleaf weeds (Rebeiz, 1987b; Rebeiz et al., 1988b).

b. Diagnosis, location of tumours and photodynamic therapy of tumours

ALA synthesis is the first and rate limiting step in the porphyrin synthetic pathway. Under normal conditions the level of heme synthesis and the intracellular concentration of porphyrin intermediates are regulated mainly by heme, which is a negative feedback inhibitor of the ALA synthase (Scholnick et al., 1972). When skin was treated with exogenous ALA, it selectively penetrates through the abnormal skin tumours while normal skin remains completely impervious (Kennedy et al., 1990). ALA, a small, soluble molecule, is an effective and safe alternative in the routine treatment of superficial skin tumor (Pier et al., 1995). ALA-PDT was first proposed in 1990 as a new anticancer therapeutic modality (Kennedy et al., 1990), Recently, topical ALA application followed by photoirradiation has been successfully used for detection and treatment of

thousands of patients with early stage and advanced stage solid tumours (Baumgartner et al., 1996).

## 3. Glutamyl-tRNA reductase, the gene product of *hem A*

### 3.1. The molecular characterization of glutamyl-tRNA reductase

*hemA* is the structural gene for glutamyl-tRNA reductase, because a mutation in this gene in *E. coli* results in a deficiency of glutamyl-tRNA reductase (Avissar & Beale, 1989). *hemA* has been cloned from different sources(Li et al., 1989; ). The gene product has a predicted molecular weight of approximately 45-50 kDa (Drolet et al., 1989; Elliott et al 1989; Verkamp & Beale, 1989; Petricek et al., 1990; Hungerer et al., 1996).

Glutamyl-tRNA reductase isolated (a) from the stroma of greening barley chloroplasts, has a native molecular weight of approximately 250-270 kDa and is composed of identical subunits of 54-60 kDa (Pontoppidan and Kannangara, 1994; Vothknecht et al., 1996), (b) from *Synechocysis* has a molecular weight of 350 kDa with subunits of 47.5 kDa (Rieble and Beale, 1991), and (c) from *Chlamydomonas* has a reported molecular weight and subunit size of 130 kDa (Chen et al., 1990). *E. coli hemA* encodes a protein with a subunit molecular weight of 46 kDa (Li et al., 1989c). Soll and coworkers (1991) isolated two GTR activities from *E. coli*, GTR45 and GTR85, with molecular weights, under both denaturing and non-denaturing conditions, of 45 kDa and 85 kDa respectively. The 45 kDa protein is the gene product of *hemA*. More sensitive analyses failed to observe the 85 kDa species (Verkamp et al., 1992). Although GTR has been partially purified from a number of organisms, the size and the subunits of the enzyme have not been unequivocally

identified. GTR activity from barley was inhibited by hemin. Interestingly, there is evidence that GTR is a heme protein (Vothknecht et al., 1996).

### 3.2. **Substrate specificity**

GTR purified from *Chlamydomonas* and from *Synechocystis* has been shown to catalyze the conversion of glutamyl-tRNA$^{glu}$ to GSA in the presence of NADPH (Chen et al., 1990b; Rieble and Beale, 1991). The GTRs from barley and *E. coli* show sequence-specific recognition of glutamyl-tRNA$^{glu}$ (Kannangara et al., 1984; Huang & Wang, 1986; Weinstein et al., 1986; Rieble et al., 1989). The barley enzyme has been shown to discriminate between several glutamyl-tRNA$^{glu}$ or glutamyl-tRNA$^{gln}$ preferring the glutamyl-tRNA$^{glu}$ of its chloroplast (Peterson et al., 1988). Glutamyl-tRNA$^{glu}$ species from bacteria, yeast and other higher plants are poorly recognized by the barley GTR (Kannangara et al., 1984). The glutamyl-tRNA$^{glu}$ from *E. coli* is a good substrate for *E. coli* GTR but not for GTR from *Chlorobium vibrioforme*, *Chlorella vulgaris*, *B. subtilis*, *Euglena* and *Synechocystic* (Avissar & Beale, 1990; Jahn et al., 1991; Beale, 1996).

Jahn (1992) reported that for *Chlamydomonas reinhardtii*, GTR and GTS form a 200 kDa complex in the presence of tRNA$^{glu}$, glutamate and ATP, which suggested that there are specific recognition sites on the tRNA$^{glu}$ for both enzymes. More recently, it was reported that five nucleotides ($G_{10}$, $A_{26}$, $U_{34}$, $U_{35}$ and $A_{37}$) of tRNA$^{glu}$ from various species may be important for recognition and binding of the barley GTS and a further seven nucleotides ($A_7$-$U_{66}$, $U_{29}$-$A_{41}$, $A_{53}$-$U_{61}$ and $A_{37}$) may be required for recognition and

binding the barley GTR (Willows et al., 1995). When $C_{56}$ was replaced with $U_{56}$, ALA formation was inhibited, which may due to the changing GTR binding site to prevent binding of glutamyl-$tRNA^{glu}$ (Stange-Thomann et al., 1994).

### 3.3. **Overexpression of *hemA* in *E. coli***

GTR is the key enzyme of ALA biosynthesis. Despite the fact that the structural gene, *hemA*, has been cloned and sequenced (Li et al., 1989b; Drolet et al., 1989; Verkamp and Chelm, 1989), the gene product has not been well purified and characterized. GTR has been purified from *E. coli* (Jahn et al., 1991), *Chlamydomonas reinhardtii* (Chen et al., 1990) and barley (Pontippidan & Kannangara, 1994). However, the amount of purified enzyme finally obtained was less than 1 mg and the yield was very low. Attempts to overexpress the *hemA* gene in *E. coli*, or *Bacillus subtilis* or yeast have been unsuccessful, even using different promoters and RNA polymerases. Using pUC- or pTZ-derived plasmids containing the *hemA* gene with its own promoter for expression, the activity of GTR was increased only a few-fold (Beale and Weinstein, 1990; O'Neill and Soll, 1990; Verkamp et al., 1992).

*E. coli* expression systems have become powerful tools for the production of proteins. Studier et al. (1990) developed a series of pET vectors for cloning and expressing target DNAs under the control of a T7 promoter. T7 RNA polymerase is a very active enzyme which elongates mRNA chains about five times faster than does *E. coli* RNA polymerase. It is also very selective for specific promoters that are rarely encountered in DNA unrelated to T7 DNA. T7 RNA polymerase is able to make complete transcripts of almost

any DNA that is placed after a T7 promoter. Because the T7 phage promoter is not recognized by the host *E. coli* RNA polymerase, production of the cloned gene occurs only after expression of T7 RNA polymerase, which provides a controlling tool for expression.

Davanloo et al. (1984) cloned the T7 RNA polymerase *gene1* which provides a source for directing selective high-level expression of target genes under control of a T7 promoter. Studier et al. placed *gene1* in the *BamH1* site of the lambda cloning vector D69 to form bacteriophage DE3 in which the expression of *gene1* is under control of the *lacUV5* promoter. The *E. coli B* strain BL21 (F$^-$, *ompT*, r$\beta$ m$\beta^-$) has the potential advantage that it lacks both *lon* and *ompT* outer membrane proteases (Studier & Moffatt, 1986). These proteases can degrade proteins during purification (Grodberg & Dunn, 1988). It also lacks a DNA restriction system. The strain BL21(DE3) was formed by infection of BL21 with DE3 bacteriophage ($\lambda DE_3$). Thus the only promoter known to direct transcription of the T7 RNA polymerase gene in this strain is the *lacUV5* promoter, which is inducible by isopropyl-$\beta$-D-thiogalacto-pyranoside (IPTG). This system is capable of expressing a wide variety of DNAs from prokaryotic and eukaryotic sources.

### 4. Regulation of heme biosynthesis in *E. coli*

It has been demonstrated in different organisms that tetrapyrrole biosynthesis is mainly regulated by the control of ALA formation. In the $C_4$ pathway, ALA synthase was shown to be the rate-limiting enzyme, which is mainly regulated by heme (Schonick et al., 1972; Pirola et al., 1984). However, less is known about regulation of tetrapyrrole biosynthesis

in the $C_5$ pathway. It has been reported that ALA formation is also the rate-limiting step of the heme biosynthesis in the $C_5$ pathway since (i) *E. coli* cells accumulate heme when they are grown in the presence of ALA (Philipp-Dormston and Doss, 1975), (ii) a *hemH* mutant, which can not make ferrochelatase, the enzyme catalyzing the last step in heme synthesis, accumulates porphyrins, (iii) HU227, a *hemA* mutant, accumulates porphyrins as well as ALA when it is transformed with a multi-copy plasmid which contains *hemA* gene (Li et al., 1989; Chen et al., 1994). Thus, control of heme synthesis in *E. coli* appears to be at the level of ALA biosynthesis.

### 4.1. **Regulation of ALA biosynthesis in *E. coli***

In the $C_5$ pathway, ALA biosynthesis requires GRS, GTR, GSA-AT, glutamate, $tRNA^{glu}$, ATP, NADPH and $Mg^{2+}$ (Weinstein & Beale, 1985a, b; Weinstein et al., 1987; Schneegurt & Beale, 1988). $tRNA^{glu}$ and three enzymatic activities were obtained from greening barley (Kannangara et al., 1984; Wang et al., 1981), *Chlorella* (Weinstein et al., 1987; Avissar and Beale, 1988), *Methanobacterium* (Friedmann et al., 1987), *Chlamydomonas* (Wang et al., 1984; Huang et al., 1984; Huang and Wang, 1986) and *Euglena* (Mayer et al., 1987). When glutamyl-tRNA was supplied, glutamate, ATP and $tRNA^{glu}$ were no longer required. When GSA was supplied, NADPH was also not required. In the absence of GSA-AT, GSA accumulation was observed (Mau et al., 1987).

It is well known that three enzymes, glutamyl-tRNA synthetase (GTS), glutamyl-tRNA reductase (GTR) and glutamyl semialdehyde aminotransferase (GSA-AT) are involved in the three steps of ALA formation in *E. coli*. Since GTS is also involved in protein synthesis and GSA can be converted to ALA non-enzymatically (Hoober et al, 1988;

Gough et al, 1989; Mayer et al, 1994), it is highly probable that control of ALA synthesis and thus of the whole pathway residues in the GTR step, in which GTR catalyzes NADPH-linked reduction of glutamyl-tRNA to form GSA (Lapointe and Soll, 1972). However, the details underlying the regulatory mechanisms of this step remain to be determined.

### 4.2. **Effects of hemin on ALA synthesis**

ALA synthesis in the $C_4$ pathway is mainly regulated by heme. However, there was no clear answer whether heme has the same function in the $C_5$ pathway. It was found that hemin had no obvious effect on either the activities of GTR45 purified from *E. coli* even at 100 uM (Jahn et al., 1991). In contrast, it has been reported that the activities of GTRs from different organisms were inhibited by hemin (Wang et al., 1984, 1987; Weinstein and Beale, 1985a; Rieble and Beale, 1988). Heme acts as a feedback inhibitor of activities of GTR in barley (Gough and Kannangara, 1979). It was found that heme at 4 uM caused a 50% inhibition of the activity of GTR purified from barley (Pontoppidan & Kannangara, 1994). Thus, whether or not the ALA synthesis in *E. coli* is regulated by heme and how is a subject of active study.

### 4.3. **Effects of oxygen and glucose on ALA synthesis**

The amounts of heme-containing respiratory enzymes cytochrome *d* oxidase and nitrate reductase have been reported to be different in aerobically and anaerobically grown *E. coli* (Rice et al., 1978). These enzymes contain 3 and 4 heme molecule as their prosthetic

groups, respectively. This may suggest that more heme is required during anaerobic than during aerobic cell growth conditions (Gunsalus, 1992). In contrast, oxygen enhances the formation of enzymes in some steps between ALA and protoheme. Some heme-containing enzymes are present optimally only under oxygen-rich growth conditions (Ackrell et al., 1992; Cotter et al., 1990). However, the absolute amount of heme in the cell under anaerobic or aerobic growth condition is unknown. More recently, a report concluded that expression of *hemA-lacZ* fusion gene was repressed by oxygen (Darie & Gunsalus, 1994, Hua, et al., unpublished). The results showed that expression of *hemA* is 2.5 fold higher for anaerobic versus aerobic growth.

Glucose represses the expression of several catabolic enzymes and some other proteins (Kranz et al., 1984; Cotter et al., 1990). Glucose is a preferred energy source of *E. coli* and has catabolic repressive effects on a wide variety of sugar-metabolizing enzymes. This repression can be reversed by cAMP through the binding of cAMP-CRP (catabolite activator protein or cAMP receptor protein) complex to the promoter region of some genes (Dickson et al., 1975; Movva et al., 1981; Aiba, 1983). The cAMP-CRP complex is a global transcriptional regulator. The binding sites of CRP are located at different positions (40-70 bp) in the 5' upstream regulatory region in different genes. Most binding sites for cAMP-CRP contain the 22 bp inverted repeat consensus sequence: -A-TGTGA-----TCACA-T- (Jansen et al., 1987). Analysis of the 5' upstream regulatory region of the *hemA* gene shows a putative cAMP-CRP binding site with the sequence: TGAGA------GCAGT at nt -182 to -197 (Figure 5). This may suggest that the expression of GTR is under catabolic control.

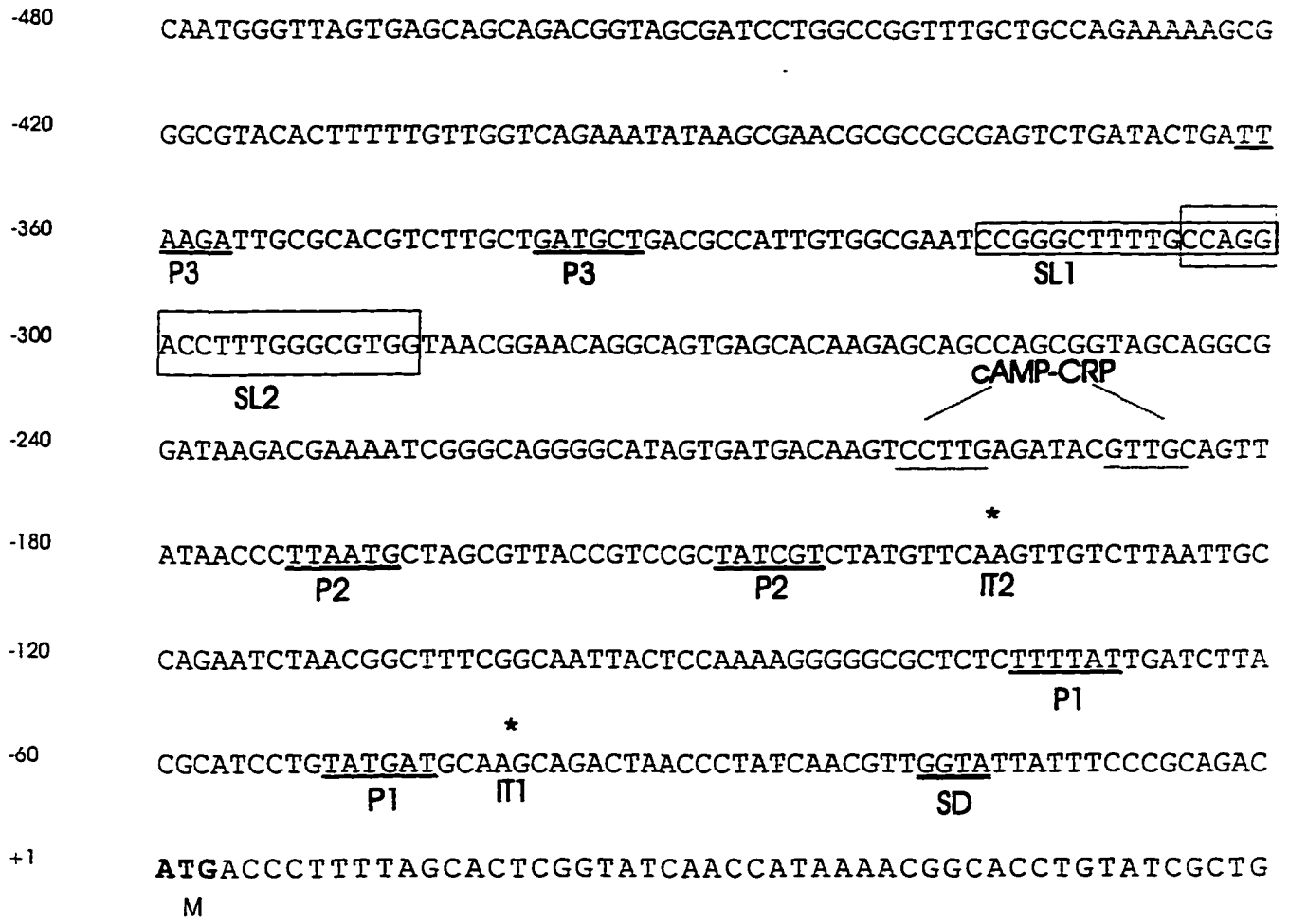



Figure 5. The 5' upstream regulatory region of *hemA* (by Dr. C. S. russell)
Legend: Promoters; underline (P)
Stem-loop; boxed (SL)
cAMP-CRP binding site; dotted line
Shine-Dalgano sequence; double underline (SD)
Initiation of transcription; *(IT)
Initiation of translation; bold

4.4. **Effects of $tRNA^{glu}$ on ALA synthesis**

In *E. coli*, copies of $tRNA^{glu}$ genes are contained in several ribosomal RNA operons between genes encoding 16S and 23S rRNA (Komine et al., 1990). The same $tRNA^{glu}$ is not only used for protein synthesis but also for ALA synthesis in *E. coli*. This was supported by the evidence that ALA formation in extracts of organisms was blocked by preincubation with RNase A and this inhibition was released by addition of RNase inhibitor, Rnasin, plus low molecular weight RNA from the same speices (Schon et al., 1986, Schneegurt & Beale. 1988). There must be a functional excess of $tRNA^{glu}$ in the cell to support the needs of protein and ALA biosynthesis and its traffic must be regulated. The $tRNA^{glu}$ from *E. coli* (O'Nell and Soll, 1990) and barley chloroplast (Kannangara et al., 1988) had equivalent capacities to provide glutamate for protein biosynthesis and support ALA formation *in vitro*. The addition of *E. coli* $tRNA^{glu}$ stimulated ALA synthesis in a coupled *in vitro* cell-free system of JL1268, a strain harboring *hemA* on a multiplecopy plasmid. This enhancement by $tRNA^{glu}$ is more pronounced when endogenous tRNA is first removed from the cell extract by gel filtration (Li et al.1988). This suggests that more $tRNA^{glu}$ is required both for protein and ALA synthesis when the GTR is overproduced than in wild type cells which do not overproduce GTR.

The upstream region of the *hemA* gene sequence for the sense strand contains two stem loop structures: stem loop 1 at nt -316 to -301, CCGGGCTTTTGCCAGGA and stem loop 2 at nt -305 to -287, CCAGGACCTTTGGGCGTGG (Figure 5). These stem loops contain sequences, CCAGGA and CTTTTG or CTTTG. These sequences are similar to the anticodon and a stem loop of *E. coli* $tRNA^{glu}$

(CCAGGACACCGCCCUUUCACGGCGGUA). Brun et al. (1990) reported that the upstream region of *gltX* gene has a similar $tRNA^{glu}$ sequence (CCAGGATTTGCCGGTTGTCCGGCA) and suggested that GTS may function as its own translational down-regulator by binding to the stem loop upstream of *gltX* mRNA that looks like *E. coli* $tRNA^{glu}$ thus preventing translation. This may occur for GTR also and the regulation may be reciprocal. More recently, it was found that $tRNA^{glu}$, GTR and GTS formed a 200 kDa complex in *Chlamydomonas reinhardtii* (Jahn, 1992). Thus, $tRNA^{glu}$ is not only a substrate for GTS but may regulate synthesis and flow of glutamyl-tRNA into protein synthesis and into ALA synthesis.

5. **Purpose of this study**

The major goal of the experiments carried out in this thesis were (i) to find out whether 5-aminolevulinic acid synthesis (ALA) in *E. coli* requires expression of *hemA* or *hemM*, (ii) to express and partially characterize glutamyl-tRNA reductase in *E. coli*. (iii) to determine the effects of various factors that regulate the expression of *hemA* gene and activity of its gene product (glutamyl-tRNA reductase).

# MATERIALS

## 1. CHEMICALS AND ENZYMES

Restriction endonucleases, $T_4$ DNA ligase, calf intestine alkaline phosphatase (CIP) and RNasin were purchased from New England Biolabs (Beverly, MA). The Genius system of nonradioactive detection, RNase inhibitor, DNA markers II and III, phenylmethylsulfonyl fluoride (PMSF), X-Gal, DNaseI, RNase and reverse transcriptase were purchased from Boehringer Mannheim (Indianapolis, IN). Isopropyl-β-D-thiogalactopyranoside (IPTG) was from Jersey Labs (Livingston, NJ). L-[$^{35}$S] methionine, α-[$^{32}$P]dUTP, γ-[$^{32}$P]dATP and Nylon membranes (GeneScreen Plus) were purchased from NEN Research Products (Boston, MA). The WIZARD mini-prep DNA purification system and *E. coli* S30 coupled transcription and translation system, were purchased from Promega (Madison, WI). Lysozyme, protein molecular weight markers, molecular biology grade agarose, acrylamide/bis-arylamide, TEMED, NADPH, *E. coli* $tRNA^{glu}$, L-glutamic acid, casamino acids, levulinic acid, acetylacetone, 5-aminolevulinic acid (ALA), PBG, hemin, ATP, diethylpyrocarbonate (DEPC), proteinase inhibitors, ampicillin, chloraphenicol, Dowex AG 1-8 X and 2', 5'-ADP-Sepharose 4B were obtained from Sigma (St. Louis, MO). Sepharose 6B and HiTrap SP were purchased from Pharmacia Biotech AB (Piscataway, NJ). Media were prepared from Difco products (Detroit, MI). Gradient polyacrylamide slab gels (HF 2.5-27%) were purchased from Isolab (Akron, OH). All other chemicals were from standard suppliers and were reagent grade or better.

## 2. STRAINS AND PLASMIDS

The strains and plasmids used in these studies are shown in Tables 1, 2, 3.

Table 1. The strains and plasmids used in the study of the roles of *hemA* and *hemM* genes in ALA biosynthesis in *E. coli*

| Organism or plasmid | Relevant genotype or phenotype[a] | Source |
|---|---|---|
| ***E. coli* strains** | | |
| JM101 | *supE thi*1 Δ(*lac-proAB*) F'[*traD*36 *proAB*$^+$ *lacI*$^q$ *lacZ* Δ*M*15] | U.S. Biochemical Corp. |
| HU227 | *hemA*[b]; hemin permeable | Umanoff et al. (1988) |
| CSR603 | phr-1, recA, uvrA6 | Sancar and Rupp (1978) |
| JL1268 | HU227(pJL68) | Li et al. [1989b] |
| JL1269 | HU227(pJL69) | Li et al. [1989b] |
| WC1215 | HU227(pWC15) | This laboratory |
| WC1214 | HU227(pWC14) | This laboratory |
| WC1208 | HU227(pWC08) | This laboratory |
| WC1270 | HU227(pWC22) | This laboratory |
| I14 | C600 *hemM* | Ikemi et al. (1992) |
| WC1216 | I14(pJL68) | This laboratory |
| WC1217 | I14(pJL69) | This laboratory |
| WC1218 | I14(pWC15) | This laboratory |
| WC1219 | Hfr Cavalli(pJL68) | This laboratory |
| WC1220 | Hfr Cavalli(pJL69) | This laboratory |
| WC1221 | Hfr Cavalli(pWC15) | This laboratory |
| WC1222 | SASX41B(pJL68) | This laboratory |
| WC1223 | SASX41B(pJL69) | This laboratory |
| WC1224 | SASX41B(pWC15) | This laboratory |
| **Plasmids** | | |
| pUC19 | amp$^R$ | U.S. Biochemical Corp. |
| pJL68 | pTZ19U (*hemA*, *hemM*) | Li et al. [1989b] |
| pJL69 | pTZ19U (*hemA*) | Li et al. [1989b] |
| pWC22 | pTZ19U (partial *hemA* and *hemM* [*hemA'*, *hemM'*]) | This laboratory |
| pWC15 | pUC19 (*hemM*) | This laboratory |
| pWC14 | pUC19 (partial *hemA* [*hemA'*]) | This laboratory |
| pWC08 | pUC19 (partial *hemM* [*hemM'*]) | This laboratory |

a Other genetic markers are as follows: HU227 and its derivatives, Hfr Cavalli, *metB*; for C600 and its derivatives, *supE*44 *hsdR thi*1 *leuB*6 *lacY*1 *tonA*21λ$^-$, F$^-$.

b Strain HU227 was derived from SASX41B, the Sasarman *hemA* mutant, and Hfr Cavalli was obtained from the *E. coli* Genetic Stock Center, Yale University, New Haven, Conn.

Table 2. The strains and plasmids used in the study of overexpression of glutamyl-tRNA reductase in *E. coli*

| Organism or plasmid | Relevant genotype or phenotype[a] | Source |
|---|---|---|
| ***E. coli* strains** | | |
| JM101 | *supE thi* (*lac-proAB*) F'[*traD36 proAB*$^+$ *lacI*$^q$ *lacZ* $\Delta M15$] | U.S. Biochemical Corp. |
| HU227 | *hemA*[b], hemin permeable | Umanoff et al. (1988) |
| BL21($DE_3$) | $F^-$, *ompT*, $\gamma^-_\beta$, $m^-_\beta$,$\lambda DE_3$ | Novagen |
| BL21($DE_3$)/pET14b | | This laboratory |
| WC1201 | BL21($DE_3$)/(pWC01) | This laboratory |
| WC1202 | BL21($DE_3$)/(pWC02) | This laboratory |
| WC1261 | HU227(pWC01) | This laboratory |
| WC1268 | HU227(pWC68) | This laboratory |
| **Plasmids** | | |
| pUC19 | $Amp^R$ | U.S. Biochemical Corp. |
| pTZ19U | $Amp^R$ | U.S. Biochemical Corp. |
| pET14b | $Amp^R$, T7 promoter | Novagen |
| pWC01 | pET14b (*hemA*, *hemM*) | This laboratory |
| pWC02 | pET14b (-T7 promoter)(*hemA*) | This laboratory |
| pWC15 | pUC19 (*hemM*) | This laboratory |
| pWC68 | pUC19 (*hemA hemM*) | This laboratory |
| pWC69 | pUC19 (*hemA*) | This laboratory |
| pJL68 | pTZ19U (*hemA*, *hemM*) | Li et al. [1989b] |
| pJL69 | pTZ19U (*hemA*) | Li et al. [1989b] |
| pTZ7612 | *gltX*, $Amp^R$ | Lapointe et al. [1989] |

a Other genetic markers are as follows: HU227 and its derivatives, Hfr Cavalli, *metB*; for C600 and its derivatives, *supE44 hsdR thi1 leuB6 lacY1 tonA21*$\lambda^-$, $F^-$.

b Strain HU227 was derived from SASX41B, the Sasarman *hemA* mutant, and Hfr Cavalli was obtained from the *E. coli* Genetic Stock Center, Yale University, New Haven, Conn.

Table 3. The strains and plasmids used in the study of regulation of ALA biosynthesis in *E. coli*

| Organism or plasmid | Relevant genotype or phenotype | Source |
|---|---|---|
| ***E. coli* strains** | | |
| C600 | *supE*44 *hsdR thi*-1 thr1 *leuB*6 *lacY*1 *ton*A21 | CGSC |
| RP523 | C600 *hemB*; hemin permeable | Li et al [1988] |
| DH5α | *endA*1 *hsd*$^R$17 *supE*44 *thi*-1 *recA*1 *gyrA*(Nal$^r$) *relA*1 Δ(*lacZYA-argF*) | |
| HU227 | *hemA*, hemin permeable | Umanoff et al. (1988) |
| JL1268 | HU227(pJL68) | Li et al. [1989b] |
| WC1268 | HU227(pWC69) | This laboratory |
| LPL-1 | WC1268(pMO11) | This laboratory |
| **Plasmids** | | |
| pUC19 | Amp$^R$ | U.S. Biochemical Corp. |
| pMO11 | *gltT*, *cm*$^R$ | Gregory et al. [1995] |
| pJL68 | pTZ19U (*hemA*, *hemM*) | Li et al. [1989b] |
| pWC68 | pUC19 (*hemA*, *hemM*) | This laboratory |

3. **MEDIA**

Media (LB and M9) were prepared according to the recipes of Schleif and Wensink (1981) with or without varying combinations of the following supplements: glucose (0.2%), glycerol (1%), fumarate (50 mM), hemin or hemin arginate (4ug/ml), amino acids (50 ug/ml), and thiamine (5 ug/ml) as stated below and in figure legends.

Hemin solution (4 mg/ml)was prepared by mixing 0.1 g hemin, 2.5 ml Tween-80, and 22.5 ml 0.05 M Tris pH7.6 and then titrated with 2-4 drops 10 N NaOH until the hemin was completely dissolved. This solution could be stored at 4°C for several months.

Hemin arginate solution (25 mg/ml) was prepared as described by Tenhunen (1987) with some modifications (Hughes & Russell, unpublished). 0.25 g hemin was added to a mixture of 4 g propylene glycerol (1,2-propanediol), 1 ml ethanol, and 5 ml $dH_2O$. After stirring for 4 to 5 hr, the hemin was completely dissolved. This solution could be stored at 4°C for several months and frozen for more than a year.

Modified Ehrlich's stock solution was prepared by dissolving 0.32 g $HgCl_2$ in 81.8 ml glacial acetic acid while stirring on a hot plate in the hood. After cooling, 20 ml 70% perchloric acid was added to the mixture (Lien & Beattie, 1982). This solution could be stored at 4°C for one month. Modified Ehrlich's reagent was prepared fresh by mixing 55 ml of the stock solution with 1 g p-dimethylaminobenzaldehyde. This solution could be stored at 4°C for one week.

X-Gal solution, 20 mg/ml, was prepared by dissolving X-Gal in dimethyl sulfoxide (DMSO).

# EXPERIMENTAL METHODS

## 1. 5-Aminolevulinic acid synthesis in *E. coli* requires expression of *hemA*

### 1.1. Plasmid DNA preparation

#### 1.1.a. Plasmid DNA mini-prep

Plasmid DNA from 5 ml of an overnight culture was obtained using the WIZARD Mini-Prep DNA Purification System from Promega. The instructions of the supplier were followed.

#### 1.1.b. Large scale isolation of plasmid DNA

The procedure of Sambrook et al. (1989) was followed but with the following significant modifications. Briefly, the cell pellet from 250 ml of an overnight culture was resuspended in 6 ml ice-cold freshly-prepared lysozyme in buffer (25mM Tris, 10mM EDTA, 15% sucrose, 2mg/ml lysozyme) and incubated in ice water for 15 min. 12 ml 0.2M NaOH, 1% SDS was added and the mixture was incubated in ice for 10 min. After the addition of 7.4 ml 3.0M sodium acetate (adjusted to pH 4.6 with glacial acetic acid), the mixture was incubated on ice for 20 min and then centrifuged at 10K rpm for 15 min at 4°C to remove the solid residue. The supernatant was mixed with 2 volumes isopropanol and stored at -20°C overnight. The pellet, obtained by centrifugation at 14K rpm at 4°C for 20 min, was dissolved in 0.8 ml TE buffer (pH 7.5) and mixed with 50 ul RNase (DNase free, 10 mg/ml). The mixture was incubated at 37°C for 45 min. Proteins were extracted twice by adding an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), mixing and centrifuging. Then the upper layer was extracted again with an equal volume of chloroform: isoamyl alcohol (24:1) (no phenol), mixed and centrifuged.

The upper layer was transferred and mixed with 2 volumes ice-cold ethanol and the DNA was allowed to precipitate at -20°C overnight. The pellet, obtained by centrifugation at 14K rpm at 4°C for 20 min, was washed with 70% ethanol and centrifuged again. The pellet was dried in a SpeedVac (Savant, NY) and taken up in the appropriate amount of distilled water ($dH_2O$).

Plasmid DNA concentration and purity were estimated by $A_{260}$ and the ratio of the $A_{260}/A_{280}$. One $O.D_{260}$ = 50 ug/ml DNA.

1.2. **Sub-cloning of *hem*A and *hem*M**

1.2.a. **Restriction enzyme digestion**

pJL68 was used for making different plasmids containing only *hem*A, or *hem*M, or *hem*A', or *hem*M', or *hem*A'+ *hem*M' genes (Figure 6). To construct plasmids containing only *hem*A, or *hem*A'+ *hem*M' genes, pJL68 was partially digested with *Sal*I for 10 min at 37°C. The 4.99 and 5.11 kb fragments were used for making pJL69 and pWC22. To construct plasmids containing only *hem*M, or *hem*M', pJL68 was first completely digested with *Pst*I for 2 hr and then partially digested with *Sal*I for 10 min. The digested mixture was directly used for shotgun cloning to make pWC15 and pWC08. To construct plasmid containing only *hem*A', pJL68 was first completely digested with *Bam*HI for 2 hr and then partially digested with *Sal*I for 10 min. The 1.45 kb fragment was used for making pWC14. Digestion conditions varied with the enzyme and were carried out according to the supplier's directions. A typical digestion mixture contained 2 ul of 10 X

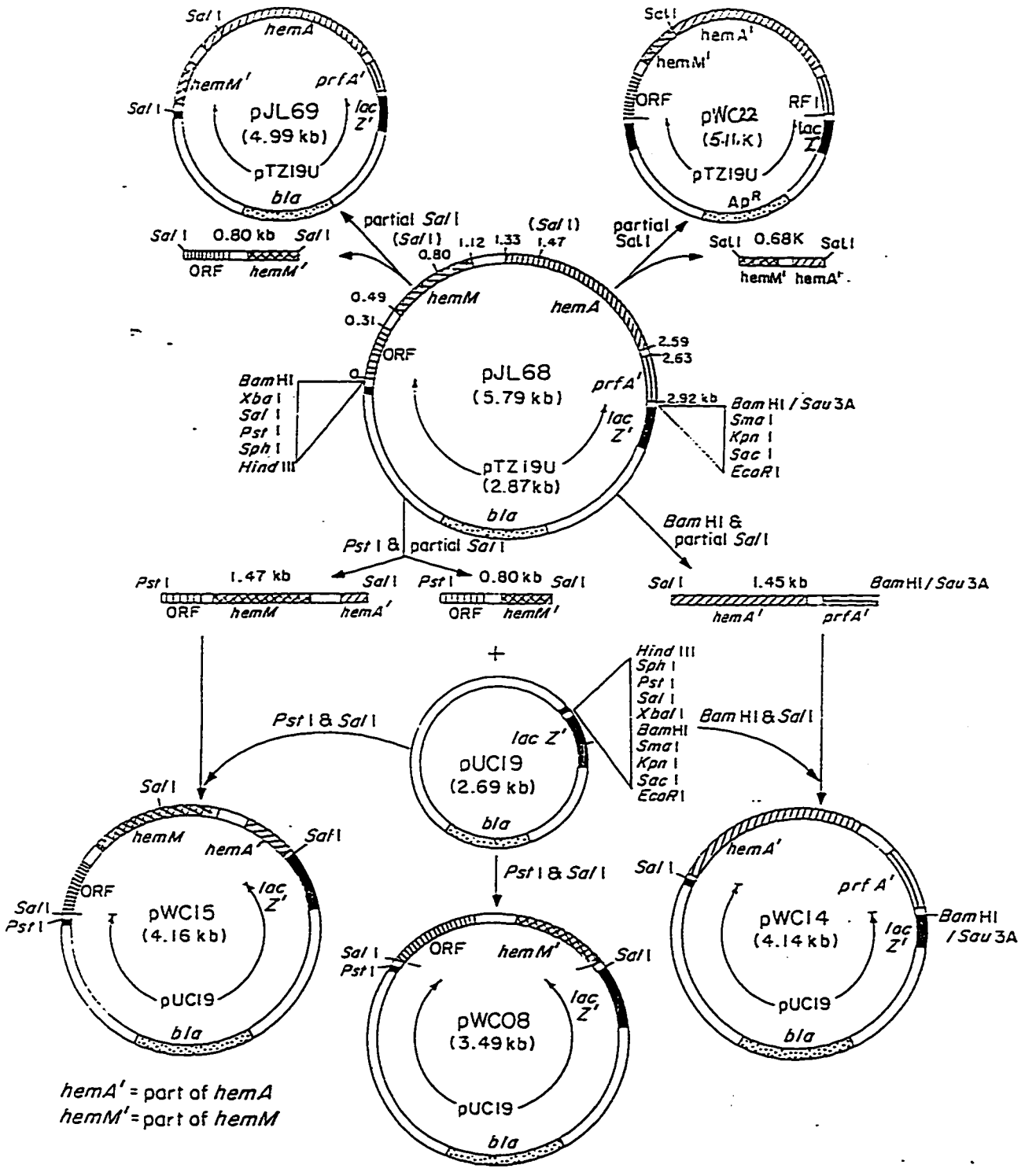



**Figure 6. Strategy for constructing plasmids**

buffer, 1 ug of DNA, 2-4 units of enzyme, and $H_2O$ to bring the total volume to 20 ul. The reaction was carried out at 37°C for different periods of time depending on requirement for complete or partial digestion, and then stopped by heating at 68°C for 5 min. The reaction mixture was transferred to ice immediately and monitored by electrophoresis on 0.7% agarose. 0.7% agarose gel was made with 1 X TBE buffer and 0.5ug/ml ethidium bromide. Then 4 ul of 6 X loading buffer was mixed with 20 ul of the reaction mixture, and the gel was run at 100 V for 1-2 hr (The power supply: Polyanalyst 431 7000, Haake/Buchler Instruments, Inc., Saddle Brook, NJ.).

1.2.b. **Purification of DNA fragments from agarose gel**

The following apparatus was designed for this purpose: The gel piece containing the fragment was placed into the bottom of a 1 ml clear plastic pipette tip by a clean glass bar as a pusher. 100 ul of 2 X TAE buffer was added to the tip. This tip then was put in a 1.5 ml Eppendorf tube, which contained 100 ul 2 X TAE buffer. A small platinum wire circle (anode) was immersed in the buffer in the tube and a small platinum wire circle (cathode) was immersed in the buffer on top of the gel piece. Electroelution was performed at 150 volts for 20-30 min. To make sure the DNA had completely moved out of the gel, the tip could be directly checked under UV light. If the gel slice still contained DNA, the tip could be put back for further electroelution. The bubble produced around the tip during electroelution could be removed by flicking the tube (Figure 7).

When electrophoresis was completed, the DNA in the tube was centrifuged and transferred to a new tube to remove any gel material. The DNA was precipitated at -20°C overnight by mixing with 2 volume of ice-cold ethanol. The pellet was washed with 70%

ethanol, vacuum-dried in the SpeedVac (Savant, NY), then resuspended in the appropriate amount of sterile water.

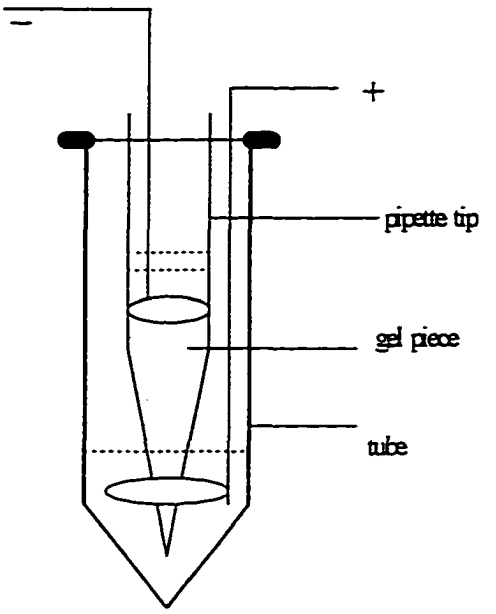



Figure 7. The apparatus for electroelution.

1.2.c. **Ligation**

8 ul of DNA (0.1-1 ug) was mixed with 1 ul 10 X T4 DNA ligase buffer and 1 ul (4000 Units) T4 DNA ligase. The ratios of fragment DNA and vector were 3:1, 1:1, and 1:3 respectively. The mixture was incubated at 16°C overnight.

1.2.d. **Transformation**

Competent cells were prepared by a simple procedure(Hanahan,1983). A fresh

overnight culture was transferred into LB medium and grown at 37°C to log phase ($OD_{600}$ = 0.4) An equal volume ice-cold 2 X TSS (LB with 20% PEG 3350, 100 mM $MgCl_2$, 10% DMSO, pH 6.5) was added and mixed gently on ice. 100 ul competent cells were mixed with plasmid DNA (1-50 ng) in an ice-cold microcentrifuge tube and incubated for 1 hr on ice. 0.9 ml LB medium containing 20 mM glucose was added and the mixture was incubated at 37°C (or 25°C whenever the plasmid containing a *hemA* insert was used) with mild shaking for 2 hr at 37°C or 3 hr at 25°C. 50 ul of the transformation mixture was plated on selective plates (0.005% X-Gal, 0.1 mM IPTG, 50 ug/ml amp for JM101 transformed with recombinant plasmids), ( 20 ug/ml of ALA, 50 ug/ml amp for *hemA*$^-$ strains transformed with recombinant plasmids).

1.3. **Analysis of the plasmids in transformants**

1.3.a. **Southern Blotting**

-Labeling of probes: Two DNA fragments, 0.68 and 0.80 kb *Sal*I restriction fragments containing part of the *hemA* gene from pJL68, were labeled by random prime DNA labeling with digoxigenin (DIG)-11-dUTP using the Genius Kit (Boehringer Mannheim, Indianapolis, IN).

-Southern Transfer: DNA digested with restriction enzymes was separated by agarose gel electrophoresis. The gel was submerged in denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 30 min and then transferred to neutralization solution (1.0 M Tris-HCl, pH 8.0, 1.5 M NaCl) for 30 min. The DNA was vacuum-blotted onto a nylon membrane (NEN Research Products. Boston, MA) by PosiBlot™ Pressure Blotter (American Bionetics, Inc., New Haven, CT) for 2 hr at 75 mm Hg pressure. The DNA was fixed to the

membrane by Stratalinker UV Crosslinker 2400 (Stratagene, La Jolla, CA) at 1200W for 30 sec.

Prehybridization and hybridization: The membrane was prehybridized with hybridization solution (20 ml/ 100 $cm^2$ of membrane) (12.5 ml of 20 X SSC, 2.5g blocking reagent, 0.5 ml N-lauroylsarcosine, 0.01 ml of 10% SDS, 12 ml $dH_2O$ and 25 ml formamide) at 42°C for 1 hr and 68°C for 1 hr. The prehybridization solution was replaced by hybridization solution (containing denatured probe [10 ng/ ml]). After hybridization overnight at 42°C, the membrane was washed 2 X for 10 min at room temperature with 50 ml wash solution I (2 X SSC , 0.1% SDS) and 2 X for 15 min at 68°C with wash solution II (0.1 X SSC, 0.1% SDS). The probe was detected by using an alkaline phosphatase conjugated antibody to DIG. A subsequent reaction with the Lumi-Phos 530 substrate or NBT (nitroblue tetrazolium salt) and X-phosphate (5-bromo-4-chloro-3-indolyl phosphotate) was carried out as described by the manufacturer of the Genius kit (Boehringer Mannheim, Indianapolis, IN).

1.3.b. **Colony hybridizations**

The colonies of transformants on LB plates containing 20 ug/ml ALA and 50 ug/ml amp were chilled at 4°C for 1 hr. Nylon membrane disks (D = 9 cm) were placed on the colony-containing plates for 5 min. The cells on the membrane were lysed by incubating the circular membrane (colony side up) for 15 min at room temperature, on gel-blotting paper saturated with denaturing solution. The DNA on the membrane was neutralized for 15 min on gel-blotting paper saturated with neutralization solution. After this step, DNA

fixing, prehybridization, hybridization, and detection steps proceeded as in Southern Blotting procedure.

1.4. **Analysis of the gene that complements a *hemA* mutant**

1.4.a. **Growth assay**

Transformants of different host strains (JL1268, JL1269, WC1215, WC1219, WC1220, WC1221, WC1222, WC1223, WC1224, WC1216, WC1217, and WC1218. (Table 1.) each containing, variously, a plasmid with *hemA* and *hemM* together or *hemA* or *hemM* separately were streaked on LB plates or inoculated into LB medium containing 50 ug/ml amp with or without 20 ug/ml ALA and incubated at 37°C overnight.

1.4.b. **The feeding of *hemA* mutants**

JL1268 or JL1269 was streaked with WC1215, WC1214 and WC1208 side by side on LB plates containing 50 ug/ml amp. The growth of each strain was checked the next day.

1.5. **Determination of strains which accumulate ALA**

Strains (JL1268, JL1269, WC1215, WC1219, WC1220, WC1221, WC1222, WC1223, WC1224, WC1216, WC1217, and WC1218.(Table 1.) each containing, variously, a plasmid with *hemA* and *hemM* together or *hemA* or *hemM* were inoculated into 10 ml LB medium containing 50 ug/ml amp and 4 ug/ml hemin respectively and incubated at 37°C overnight. 0.4 ml of the overnight culture was transferred into 20 ml of M9 medium supplemented with met, glu, amp at 50 ug/ml of each, and 4ug/ml each of thiamine and hemin. After growth for 20 hr at 37°C, the cells were centrifuged at 0.8K

rpm for 10 min and the cell pellet was washed and resuspended in 10 ml of fresh medium supplemented with levulinic acid (4mM), an inhibitor of ALA dehydratase, to block ALA to PBG. 1 ml aliquots were removed and centrifuged after incubation at 37°C for the following periods 0, 5, 10, 15, and 20 hr. The supernatants of the cell cultures were assayed for ALA accumulation and the pellets were sonicated for protein determination

1.6. **Determination of ALA**

The supernatant of the cell culture or the cell extract was assayed for ALA accumulation using the dichloromethane extraction method according to Lien and Beattie [1982] except that 0.6 ml sample and 0.3 ml 10% acetylacetone in 1 M sodium acetate was heated in a boiling water bath for 15 min. 0.6 ml modified Ehrlich's reagent was added and the spectrum from 450-650 nm was recorded after 15 min. The typical spectra of ALA pyrrole should have a major peak at 552 nm with a shoulder at 525 nm. The ALA concentration was determined using a standard curve for ALA, obtained in the same way, in which the $A_{552}$ was plotted against ALA concentration. The results were corrected by subtracting the background that was caused by growth medium.

1.7. **Preparation of cell extracts**

The cells were washed with Tricine buffer twice, resuspended in 2 ml Tricine buffer, and sonicated in an ice bath in a W-385 sonicator (Heat System Ultrasonics, Inc.) at frequency 50, 5 X 15 sec periods with 15 sec cooling intervals.

1.8. **Determination of protein**

Protein concentrations was determined by the Bio-Rad method (Bio-Rad Laboratories, Richmond, CA). Bovine serum albumin was used as a protein standard.

1.9. **Maxi-cell method**

*E. coli* CSR603 is deficient in all the major repair pathways since it carries *phr*-1, *rec*A, and *uvr*A6 mutations (Sancar & Rupert, 1978). It is therefore extremely sensitive to UV. When irradiated with UV light, the cells stop DNA synthesis and chromosomal DNA is extensively degraded and eventually destroyed. However, if such strains carry a multicopy plasmid, plasmid DNA will survive the irradiation because of its small size and high copy number and if labeled amino acids are added to cells after UV irradiation, the plasmid-encoded proteins will carry the label.

The procedure of Sancar et al. (1979) was followed for the Maxi-cell experiment with slight modifications. Briefly, *E. coli* CSR603 and its plasmid-carrying derivatives were grown overnight in M9 medium with 1% casamino acids and supplements of threonine, leucine, proline, arginine at 50 ug/ml of each, thiamine at 5 ug/ml and ALA at 20 ug/ml. 0.2 ml of overnight culture was transferred to 10 ml of medium as above and incubated at 37°C to $OD_{600}$ 0.4. 5 ml of each culture was transferred into small Petri dishes and irradiated with a UV fluency (with swirling) for 10 sec. at a distance of 60 cm from a Westinghouse Sterillamp G15T8. Cells were then transferred into a 50 ml sterile tube and incubated at 37°C for 1 hr with shaking. D-cycloserine was added to each culture at a final concentration of 100 ug/ml except for the one without plasmid. After incubation overnight, the cells were spun down and washed with 10 ml M9 medium and suspended in 5 ml minimal medium lacking sulfate. After 1 hr of starvation, [$^{35}$S] methionine was added to a final concentration of 5 uCi/ml and incubation was continued for another hr. Cells were harvested by centrifugation and resuspended in 0.2 ml sample buffer (2% SDS, 5%

β-mercaptoethanol, 10% glycerol and 0.0005% bromophenol blue in 0.0625 Tris buffer pH 6.8) and heated for 5 min in a boiling water bath. 30 ul of each sample was loaded onto an SDS-PAGE (10%) gel.

1.10. **Polyacrylamide gel electrophoresis**

The procedure of Laemmli (1970) was used for preparation of discontinuous SDS-PAGE. The final concentration of each component in the separating gel was 10% acrylamide, 0.375 M Tris-Cl, pH 8.8, 0.1% SDS, 0.03% ammonium persulfate (W/V), and 0.03% TEMED (V/V) and in the stacking gel was 4% acrylamide, 0.125 M Tris-Cl, pH 6.8, 0.1% SDS, 0.1% ammonium persulfate (W/V), and 0.05% TEMED (V/V). Electrophoresis was carried out at 24 mA per gel for approximately 2 hr (until the bromophenol blue was 1 cm from the bottom of the gel).

The gel was first fixed with 4 gel volumes of fixing solution [50%(v/v) methanol and 10% acetic acid] for 15 min, and then stained with Brilliant Blue G in 10% acetic acid for 20-30 min and destained in 10% acetic acid for 1 hr with several changes. The gel was soaked in a solution of 1% glycerol for 30 min, dried under vacuum at 60°C for 4 hr and autoradiographed on X-ray film.

## 2. Expression of glutamyl-tRNA reductase in *E. coli.*

### 2.1. Construction of the expression plasmid

The plasmid, pJL68, which contains *hemA* and *hemM* genes on an insert, was digested with *EcoR* I and *BamH* I and the 2.92 kb insert was subcloned into pUC19 which had been digested in the same way. JM101 was transformed and transformants were selected as white colonies and for ampicillin resistance on X-Gal plates. The plasmid isolated from one transformant is pWC68 that contains an insert with *hemA* and *hemM*. pWC68 was digested with *Bam* H1 and *EcoR*1 and the 2.92 kb insert was ligated into the expression vector, pET14b, to produce pWC01 (Figure 8). BL21[$DE_3$] was transformed with pWC01 to produce WC1201 [Studier et al., 1990].

### 2.2. *In vitro* transcription-translation of plasmids

The instructions of the supplier of the kit-*E. coli* S30 coupled transcription translation system (Promega) were followed. Briefly, 2-4 ug plasmid DNA, 20 ul S30 premix without amino acids, 5 ul amino acids mixture without methionine, 15 ul S30 extract, 1 ul RNase inhibitor, 1 ul $^{35}$S-methionine (10.2 mCi/ml) (and where noted, 0.5 U $tRNA^{glu}$) were mixed, made up to 50 ul with DEPC-treated water and incubated at 37°C for 2 hr. The reaction was quenched by placing the tubes in an ice bath for 5 min.

### 2.3. Overexpression of *hemA in vivo*

Six LB (50 ug/ml amp) plates were inoculated with frozen cultures of WC1201 and incubated overnight at 37°C. The colonies were washed off the plates with LB broth and transferred to ten 125 ml flasks containing 50 ml LB (each) supplemented with 1% casamino acids and 50 ug/ml amp. The cultures were grown in the dark to $O.D._{600nm}$ 0.6

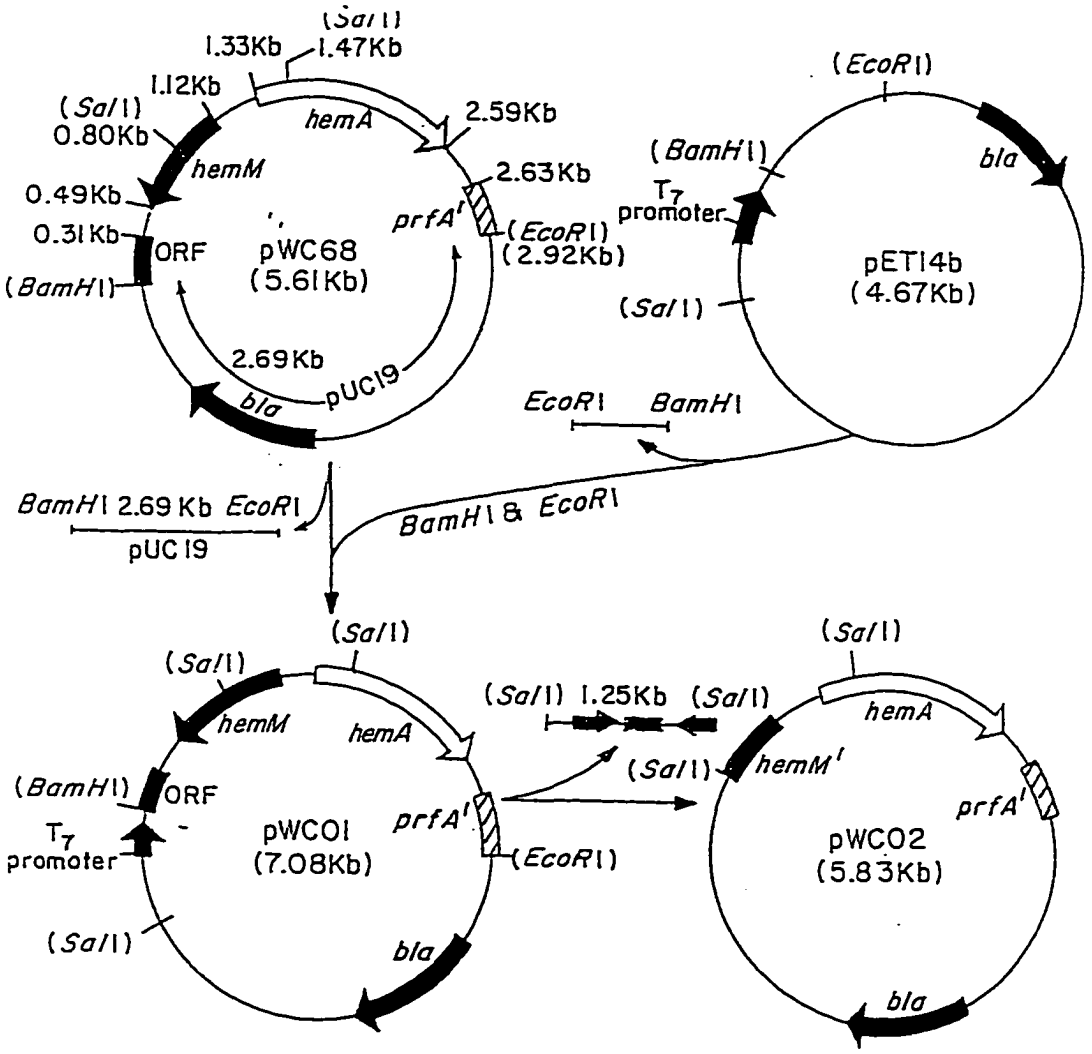



**Figure 8. Construction of plasmids with *hemA***

and centrifuged. The supernatant containing β-lactamase was discarded. The cell pellet was resuspended in 500 ml LB supplemented with 1% casamino acids and 50 ug/ml amp and grown for 1 hr. The cells were induced by the addition of IPTG. The concentrations of IPTG and the induction temperature and time were varied. After centrifugation at 10K rpm for 15 min, the cell pellet was washed twice with Tricine assay buffer (0.1 M Tricine, 25 mM $MgCl_2$, 5 mM dithiothreitol [DTT] and 0.1 M glycerol, pH 7.9) and resuspended in 0.2 ml Tricine assay buffer. When [$^{35}$S] methionine was used to label the induced proteins, casamino acids were omitted from the LB medium. After IPTG induction, 100 uCi [$^{35}$S] methionine was added to 5 ml of WC1201 culture and incubated in the dark for 30 min at 37°C. The culture was then supplied with casamino acids to a final concentration of 1% and grown for 1.5 hr.

2.4. **Preparation of sonicates**

The pellet from the induced culture was washed twice with 5 ml Tricine buffer and transferred to six 2 ml microcentrifuge tubes. Each cell pellet was resuspended in 0.8 ml Tricine buffer containing 0.1 M glycerol and stored overnight at -20°C. After defrosting, 2 ul PMSF (0.017 g/ml ethanol) was added to each tube and the mixtures were sonicated in an ice bath at frequency 50 for six 10 sec periods with 10 sec off intervals. For separation of soluble and insoluble parts of the sonicate, the sonicate was centrifuged at 4°C at 14K rpm in an Eppendorf microfuge for 15 min.

2.5. **Assay for ALA synthesis *in vitro***

The whole sonicate was assayed for ALA synthesis, by the method of Li et al. [1989b] with the following modifications: 0.1-0.4 ml of crude extract and Tricine assay buffer to bring it up to 0.8 ml was added to a 1.5 ml microcentrifuge tube. The Tricine assay buffer pH 7.9 contained 0.1 M glycerol. After addition of 0.2 ml of assay mixture (25 mM ATP, 20mM glutamate, 25 mM levulinic acid-an inhibitor of GTR, and 5 mM NADPH), the reaction mixture was incubation at 37°C for 4 hr. No pyridoxal phosphate (PLP) was added to the assay mixture (we find that PLP at 0.1 mM is inhibitory, unpublished observation) and glutamate was added to the assay mixture to a final concentration of 20 mM. *E. coli* $tRNA^{glu}$ (Sigma) was added to the assay mixture where noted . The reaction was quenched by addition of 0.125 ml 50% TCA. Two control blanks were set up; one contained crude extract but no assay mixture, and the other one contained crude extract and assay mixture but the reaction was stopped at zero time. After centrifugation, ALA was determined in the supernatant as in section 1.2.3.

2.6. **Assay of ALA synthesis activity in gel filtration fractions**

The assay was based on the ability of the gel filtration fraction to enhance ALA synthesis of a sonicate of WC1201 that had been induced with IPTG. The reaction mixture contained 0.2 ml sonicate of WC1201, 0.2 ml of each fraction, 0.2 ml assay mixture, and 0.4 ml Tricine buffer. ALA synthesis was determined as section 1.2.3.

2.7. **SDS-PAGE electrophoresis**

The procedure of Laemmli [1970] was used. Briefly, 5 ul of the *in vitro* transcription and translation mixture was precipitated with 20 ul acetone, kept on ice for 15 min, centrifuged at 14K rpm for 5 min and the pellet dried under vacuum. For induced sonicates, 20 ug protein was used. Gels were stained with Brilliant Blue G. When [$^{35}$S] methionine was used, the protein bands were visualized by autoradiography. In that case the gels were dried on one piece of drying film (Promega) and covered with plastic wrap which was removed for contact with X-ray film.

2.8. **Native gradient PAGE electrophoresis**

The instructions of the supplier of the gels (2.5-27%) were followed. 20 ul induced sonicate or 20 ul *in vitro* transcription and translation mixture was applied to the gel. Importantly, the stacking gel was 4% polyacrylamide gel in 0.125 M Tris-HCl, pH 8.3. Molecular weight standards were thyroglobulin (690 kDa), ferritin (440 kDa) and catalase (232 kDa) all from Pharmacia, and bovine serum albumin (66 kDa monomer and 132 kDa dimer) and ovalbumin (45 kDa) both from Sigma. After destaining, the gel was washed several times with water, soaked in a solution of 30% methanol and 3% glycerol for 30 min, dried by using drying film (Promega) and autoradiographed on X-ray film.

2.9. **Gel filtration chromatography**

For assay of GTR activity as a function of size, Sepharose 6B in a column with bed dimensions: 2.5 cm (i.d.) X 23 cm (ht.) was used, with Tricine buffer pH 7.9 containing glycerol to 0.1 M. 1 ml of sonicate supernatant (50 mg protein) was applied, the flow rate was 1 ml/min and 1.3 ml fractions were collected and assayed as in section 2.6. The

molecular weight standards were thyroglobulin (690 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa) and ovalbumin (45 kDa).

2.10. ***In vitro* transcription-translation of *gltX* and *hemA***

*in vitro* transcription-translation was performed as previously described in section 2.2 with some modifications. The system used was the *E. coli* T7 S30 extract system from Promega. In this kit, the T7 S30 extract was prepared from an *E. coli* strain B deficient in *ompT* endoproteinase and *lon* proteinase activity. The plasmids containing *hemA* (pWC01 or pWC02) or *gltX* (pTZ7612) alone or *hemA* + *gltX* (pWC02 and pTZ7612) together in the same *in vitro* transcription and translation reaction mixture), were expressed in an *in vitro* transcription and translation system in the presence of $^{35}$S-methionine with or without $tRNA^{glu}$. The samples for native gradient PAGE were directly prepared by mixing 40 ul of the reaction mixture and 40 ul of 2X sample buffer except the one sample, in which *hemA* (pWC02) and *gltX* (pTZ7612) were first transcribed and translated in *vitro* separately, and then 20 ul of each reaction mixture was mixed and incubated together at 37°C for 30 min.

## 3. Control of 5-aminolevulinic acid biosynthesis in *E. coli*

### 3.1. Bacterial growth conditions

#### 3.1.a. Aerobic growth

The strain was inoculated into 10 ml of medium (LB or M9) with or without supplements and grown aerobically at 37°C overnight. 0.2 ml of the overnight culture was

transferred to 10 ml medium (LB or M9) with or without varying combinations of supplements and grown aerobically at 37°C to log phase or stationary phase.

3.1.b. **Anaerobic growth**

The cultures were grown in 15 ml Klett tubes fitted with a tight rubber cap. Two needles were placed through the cap; the six inch needle was set to the bottom of the tube for inputting prepurified nitrogen and the one inch needle was used as an outlet for removing the air. The oxygen in the tube was eliminated by bubbling $N_2$ through the sample for 5 min. Then, the needles were removed (Figure 9).

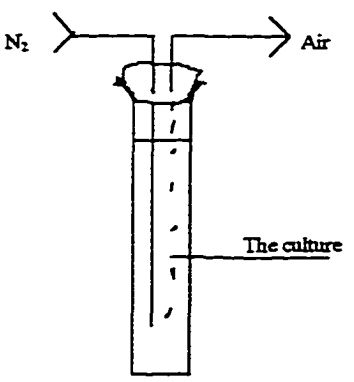



Figure 9. Using Klett tube for anaerobic growth

3.2. **Isolation of ALA**

In order to study the effect of PBG on ALA accumulation, ALA in the supernatant must be separated from PBG, because PBG reacts with Ehrlich's reagent with an absorption maximum at 553 nm which interferes with the determination of ALA. The procedure of Anderson and Desnick [1982] was used to separate ALA and PBG. Briefly,

4 ml of sample (pH 6.7) was put through an anion exchange column (1 cm X 2.5 cm) containing 2 ml Dowex AG 1-8 X resin that had been pre-equilibrated with 0.1 M sodium phosphate buffer, pH6.7. At pH 6.7 ALA does not bind to the resin and is collected by washing with distilled water. The eluate was tested with Ehrlich's reagent to detect whether PBG leaked out. PBG was eluted with 4 M acetic acid.

### 3.3. Construction of a strain with $tRNA^{glu}$ and *hemA* on separate plasmids

To make LPL-1, the plasmid pMO11 containing the $tRNA^{glu}$ gene was isolated from EF41/pMO11 (Gregory & Dahlberg, 1995) and transformed into WC1268 by a one-step method as previously described ( Chung et al. 1989). The temperature for transformation was 25°C and the selecting markers were amp (50 ug/ml) and cam (20 ug/ml).

### 3.4. Assay for glutamyl-tRNA synthetase

Glutamyl-tRNA synthesis was assayed by measuring the rate of formation of [$^{14}$C] glutamyl-tRNA (Hoben & Soll, 1985; Laberge et al., 1989). The incubation mixture was 1 ml containing 50 mM sodium Hepes buffer, pH 7.2, 16 mM $MgCl_2$, 2 mM ATP, 0.8 mM DTT, 50 $A_{260}$/ml of unfractionated tRNA from *E.coli*, 0.1 mM L-[$^{14}$C]glutamate (35 uCi/umol) and 10 mg of proteins in the cell extract. The reaction was incubated at 37°C for 30 min. 50 ul of the reaction mixture was applied to Whatman 3 MM paper disks and washed for 15 min each in 5% trichloroacetic acid at 4°C three times, in 95% ethanol once, and finally in ether once. The dried filter disks were counted for $^{14}$C radioactivity in 4 ml scintillation liquid (Econofluor-NRP) in a Liquid Scintillation Analyzer Tri-CARB 1500 (Packard).

# RESULTS

## 1. 5-Aminolevulinic acid synthesis in *E. coli* requires expression of *hemA*

When *hemA* of *E. coli* was cloned (Li et al., 1989c), an open reading frame (ORF) upstream from it and transcribed divergently was observed. In the maxicell technique, two new bands were observed, a protein corresponding to GTR (MW 41 kDa) and a protein corresponding to the ORF (MW 23kDa). Murooka and coworkers (1992) found three ALA-requiring mutants which were complemented in a heterologous fashion (small and normal colonies) by this ORF. A plasmid containing *hemA* but not the ORF gave only small colonies. The ORF, encoding a 23 kDa protein, was designated *hemM*. Only a plasmid with both *hemA* and the ORF gene gave normal colonies. The investigators suggested that the *hemM* gene product is the major GTR enzyme and that *hemA* encodes a protein involved in a minor pathway for ALA synthesis. The purpose of this study was to find out whether ALA synthesis in *E. coli* requires expression of *hemA* or of *hemM* or of both genes.

### 1.1. Construction of plasmids

pJL68, (Li et al., 1989c) pTZ19U harboring *hemA* and *hemM*, as well as a part of *prfA*, the structural gene for release factor1, and an ORF of unknown function (Nakayashiki et al., 1995) (Figure 10), was used for making different plasmid inserts containing only *hemA* (pJL69), or *hemM* (pWC15), or parts of these genes, such as *hemA'* (pWC14), or *hemM'* (pWC08), or *hemA'+ hemM'* (pWC22) genes (Figures 8 and 10).

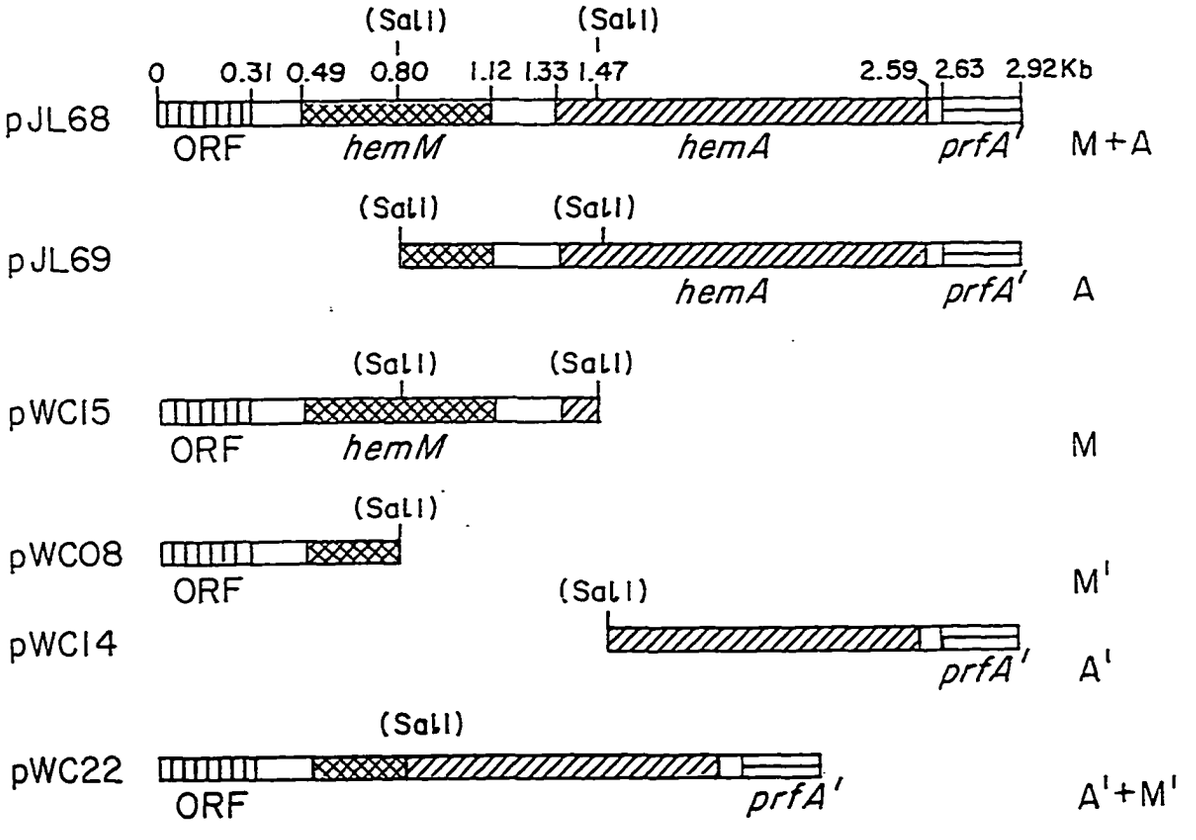



Figure 10. Diagrams showing various inserts derived from pJL68.

When pJL68 was partially digested with *SalI*, seven fragments (0.68, 0.80, 1.48, 4.31, 4.99, 5.11, and 5.79 kb) were generated. Because the 4.99 and 5.11 kb fragments are so close, only six bands were seen clearly on an agarose gel. The 5.11 kb fragment contains an intact *hemA* gene and a part of *hemM* and the 4.99 kb fragment contains a part of *hemA*. In order to make a plasmid with an intact *hemA* gene, the 4.99 + 5.11 kb bands were removed from the gel and ligase added to the mixture. These ligated plasmids were transformed into JM101, which yielded many white colonies. After two days, ten colonies fluoresced. Plasmid DNA (pJL69) was isolated from one of these colonies, and digested with *SalI* , producing 0.68 and 4.31 kb bands after electrophoresis and ethidium bromide visualization (Figure 11 a). Southern hybridization with the digoxigenin (DIG)-labelled 0.68 kb *SalI* restriction fragment, containing part of *hemM* and *hemA* genes from pJL68, as the probe, was positive (Figure 12 a). The blot was stripped and reprobed with the DIG-labelled 0.80 kb *SalI* restriction fragment containing part of the *hemM* gene from pJL68 and was negative (Figure 12 b). Thus, pJL69 has an intact *hemA* gene, but because a 0.68 kb piece has been excised, only a part of *hemM* remains. pWC22 was isolated from one of the white colonies from the transformation which did not fluoresce. Treatment with *SalI* gave 0.80 and 4.31 kb bands (Figure 11 a), indicating that an 0.68 kb fragment containing a piece of *hemM*, a piece of *hemA*, and the non-coding region in between these two genes had been deleted from pJL68.

To make a plasmid without *hemA* but with an intact *hemM* gene, pJL68 was digested with *PstI* and partially digested with *SalI* (Figure 10). Shotgun cloning of the

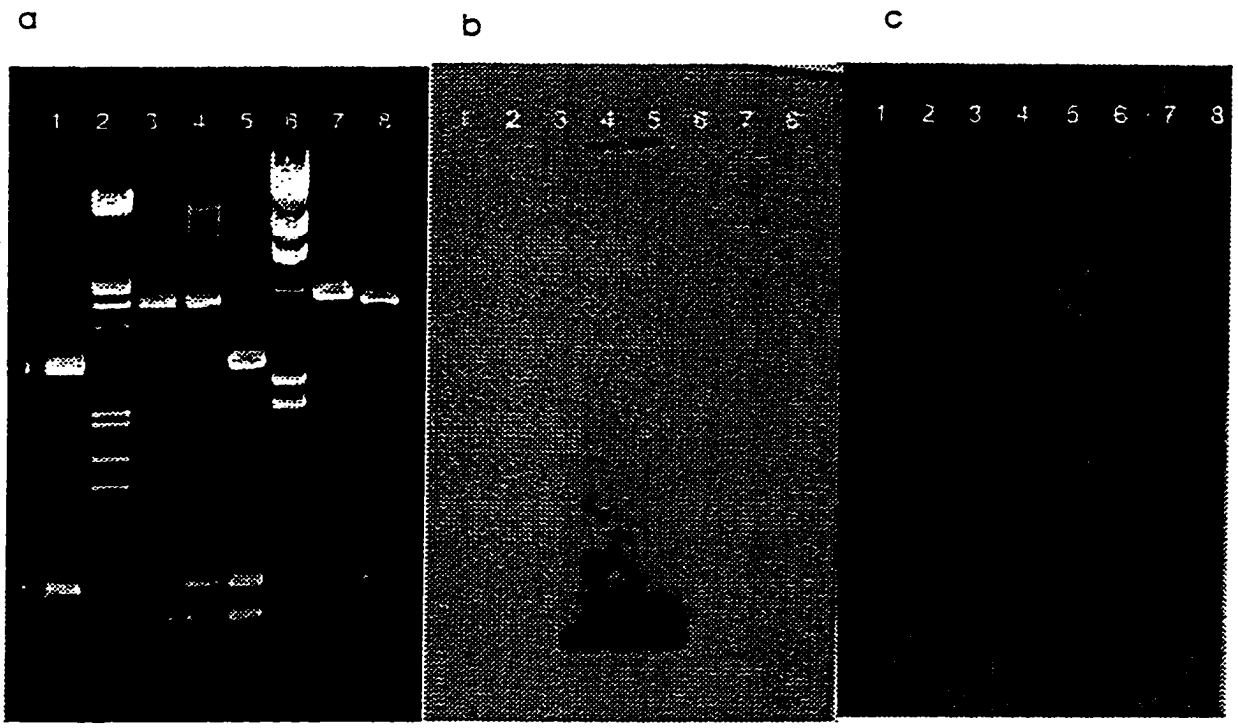



Figure 11. a. Gel electrophoresis of plasmids cut with *Sal* I. Lanes 1 to 8 were pWC08, DNA Marker III[a], pJL69, pJL68, pWC15, DNA Marker II[b], pWC14, and pWC22.
b. Autoradiogram of a Southern Blot probed with digoxigenin-dUTP (DIG) labelled 0.68 kb fragment of a *Sal* I digest of pJL68 and detected with Lumi-phos™530 chemiluminescence (Boehringer Mannheim)
c. Autoradiogram of Southern Blot described in b., but stripped and reprobed with DIG-labelled 0.80 kb fragment of a *Sal* I digestion of pJL68.

a. DNA Marker III contains 21226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564 base pairs fragments.
b. DNA Marker II contains 23130, 9416, 6557, 4361, 2322, 2027, and 564 base pairs fragments.

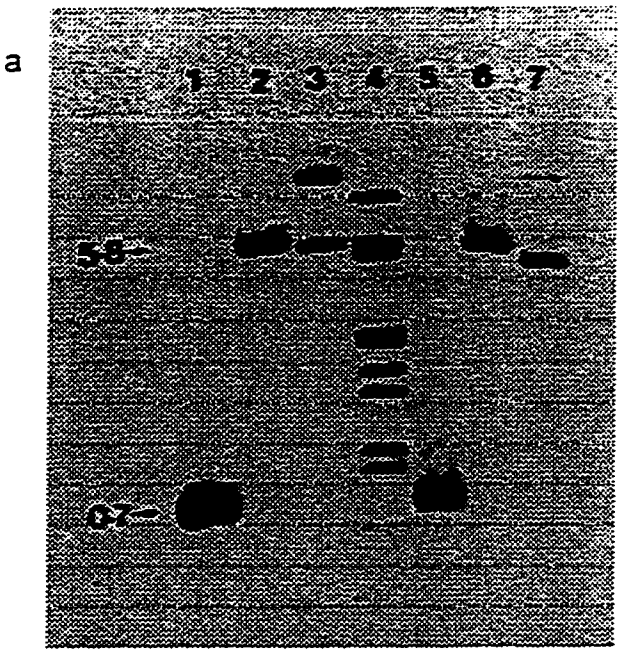



Figure 12 a. Autoradiogram of a Southern Blot probed with the digoxigenin-dUTP (DIG) labelled 0.68 kb fragment of a *Sal* I digestion of pJL68 and detected by the Lumi-phos™530 chemiluminescence (Boehringer Mannheim).
Lanes 1-3, pJL68 digested with *Sal* I, *EcoR* I, and uncut respectively.
Lane 4, DNA marker III labeled with DIG.
Lanes 5-7, pJL69 digested with *Sal* I, *EcoR* I, and uncut respectively.

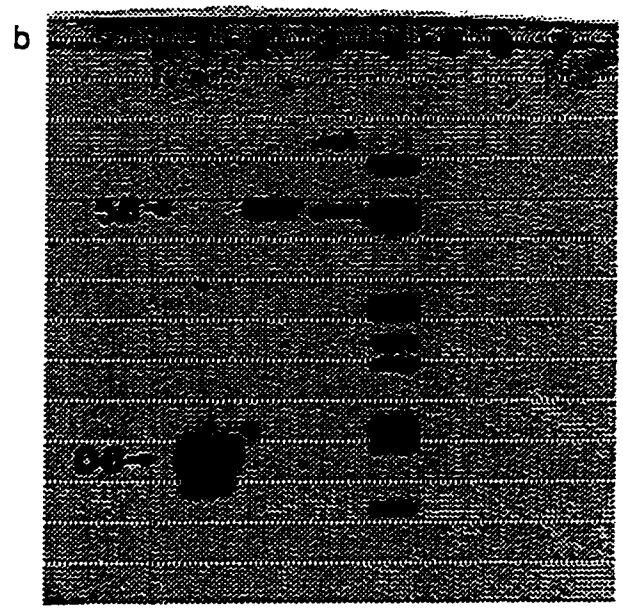



Figure 12 b. Autoradiogram of Southern Blot described in Figure 12 a, but stripped and reprobed with the digoxigenin dUTP (DIG)-labelled 0.80 kb fragment of *Sal* I digestion of pJL68.

digested mixture into pUC19 and transformation of JM101 yielded many white colonies. Colony hybridization with the DIG-labelled 0.68 kb *Sal*I restriction fragment, containing part of *hemM* and *hemA* genes from pJL68 as the probe, gave two positive colonies. DNAs from these colonies were prepared and tested again by slot blot hybridization with the same probe (results not shown). One of the positive plasmids was pWC15. Slot blot hybridization of plasmid DNA with a 0.80 kb DIG-labelled *Sal*I restricted fragment containing part of *hemA* gene from pJL68 was also positive. *Sal*I digestion of the plasmid DNA gave 0.68, 0.80, and 2.69 kb fragments (Figure 11 a-c), confirming that pWC15 contained intact *hemM*.

Two other plasmids, pWC08 containing part of *hemM* and pWC14 containing part of *hemA*, were constructed. pWC08 was obtained from the same restriction digest used to make pWC15 by using the same isolation procedures. However, Southern hybridization was negative when the DIG-labelled 0.68 kb *Sal*I restriction fragment containing part of *hemM* and *hemA* genes from pJL68 was used as the probe and positive when the DIG-labelled 0.80 kb *Sal*I restriction fragment containing part of *hemA* from pJL68 was used as the probe. Digestion of this plasmid DNA with *Sal*I gave two bands, 0.80 and 2.69 kb (Figure 11 a-c), confirming a structure containing a piece of *hemM* (*hemM'*). pWC14 was constructed by digesting pJL68 with *Bam*H I, partially digesting it with *Sal*I, and then cloning it into pUC19. Transformation of JM101 gave many white colonies. Plasmid DNA isolated from one of these colonies was digested with *Sal*I or *Pst*I, producing only one 4.31 kb band (Figure 11 a-c), confirming that this plasmid contains a piece of *hemA* (*hemA'*).

### 1.2. Determination of the gene which complements ALA-requiring mutants

In order to evaluate whether ALA synthesis in *E. coli* requires expression of either *hemA*, or *hemM*, or both, a complementation experiment was performed. HU227 (Table1), a hemin-permeable, *hemA* mutant, whose parent strain is SASX41B (Sasarman et al., 1968), requires hemin or ALA for growth. When HU227 was transformed with pJL69, the plasmid harboring *hemA*, or with pJL68, the plasmid which contains both *hemA* and *hemM*, the resultant strains, JL1269 and JL1268, respectively, no longer required ALA or hemin for growth (Li et al., 1989c). When HU227 was transformed with pWC15, the plasmid harboring *hemM*, the resultant strain, WC1215, required ALA or hemin for growth. WC1214 and WC1208, strains derived from HU227 and transformed with plasmids containing partial *hemA* and *hemM*, respectively, also required ALA for growth. The growth rate dependencies of these three strains for different concentrations of ALA were almost superimposable, and while 25 ug/ml was adequate for optimum growth, 100ug/ml did not appear to be toxic (Figure 13). Unexpectedly, WC1222, a strain derived from HU227 with pWC22 containing part of *hemA* (N-terminal 48 amino acids and 530 bp upstream of initiation of translation were deleted), did not require ALA or hemin for growth.

JL1269 , the strain with *hemA* alone, as well as JL1268 containing both *hemA* and *hemM* genes, accumulated ALA because (i) colonies fluoresced after two days, and it is known that ALA supplementation causes accumulation of porphyrins in wild-type strains (Huang et al., 1986), and (ii) both strains could feed ALA-deficient strains derived from

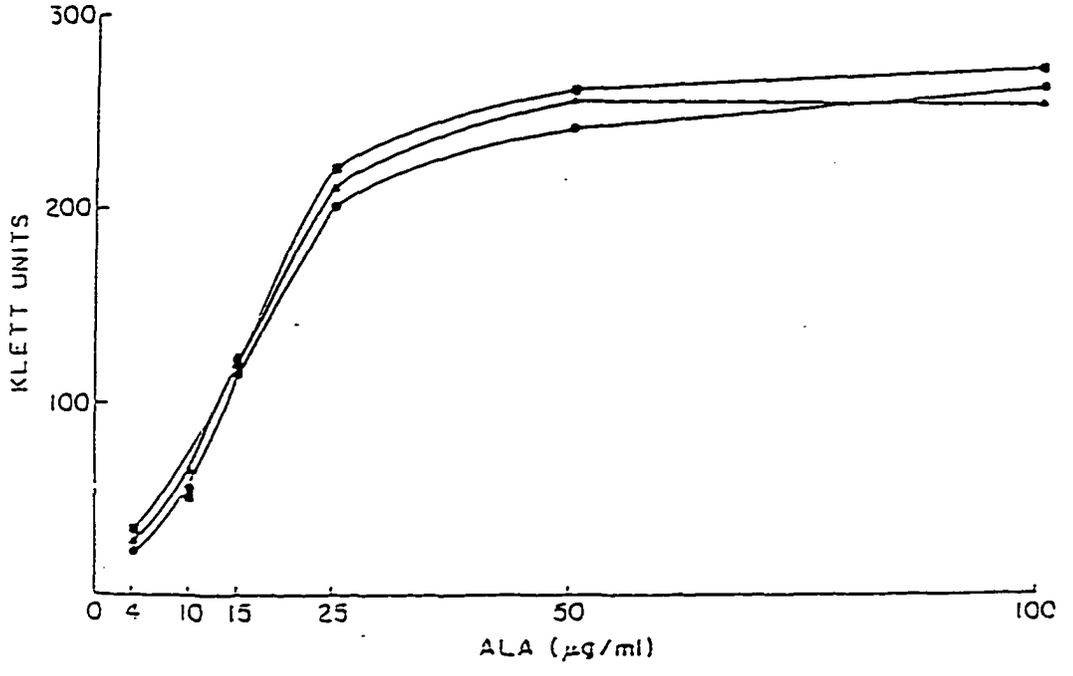



Figure 13. Growth of ALA-requiring strains in LB medium supplemented with ALA at various concentrations[a]. Growth is expressed as Klett units measured after 8 hr. HU227 is the parent strains harboring multi-copy plasmids with *hemM* (•); partial *hemA* (▪); *partial hemM* (▲).

[a] This growth assay was done by Lavern Wright, an undergraduate in the laboratory of Dr. Charlotte S. Russell

HU227 which contain *hemM* (WC1215), or partial *hemA* (WC1214) on a plasmid (Figure 14). In contrast to JL1269, WC1222, the strain with a piece of *hemM* and a piece of *hemA* on a plasmid, could grow without ALA supplementation, but did not fluoresce or feed our ALA-deficient mutants. This result suggests this strain did not accumulate ALA. This may be due to the fact that the structural gene portion of *hemA* was truncated and the upstream region of *hemA* was removed. Therefore, the GTR expressed was less active and/or less GTR was expressed.

1.3. **The product of *hemM* gene and its function**

In order to determine whether *hemM* was expressed, a maxicell experiment was performed using pWC15, which contains *hemM*. A 23-kDa protein, the expected size for the *hemM* gene product, was formed (Figure 15). The only other band, 28-kDa band, was due to expression of β-lactamase.

To determine whether *hemM* is involved in the synthesis of ALA in *E. coli*, plasmids pWC15, pJL69, and pJL68 were introduced into four strains respectively, Hfr Cavalli (wild-type parent), SASX41B (*hemA*; not hemin-permeable parent of HU227), HU227 (*hemA*; hemin-permeable), and I14 (*hemM*; not hemin-permeable) (Table 1). Figure 16 shows the accumulation of ALA by these transformed strains, each containing a plasmid with *hemA* and *hemM* together (pJL68) or *hemA* (pJL69) or *hemM* (pWC15) separately. In every case, the strains with plasmids containing *hemA* and *hemM* together caused the greatest accumulation of ALA. *hemA* resulted in some accumulation while *hemM* resulted in none or a little. pWC15 (*hemM* alone) did not support the growth of

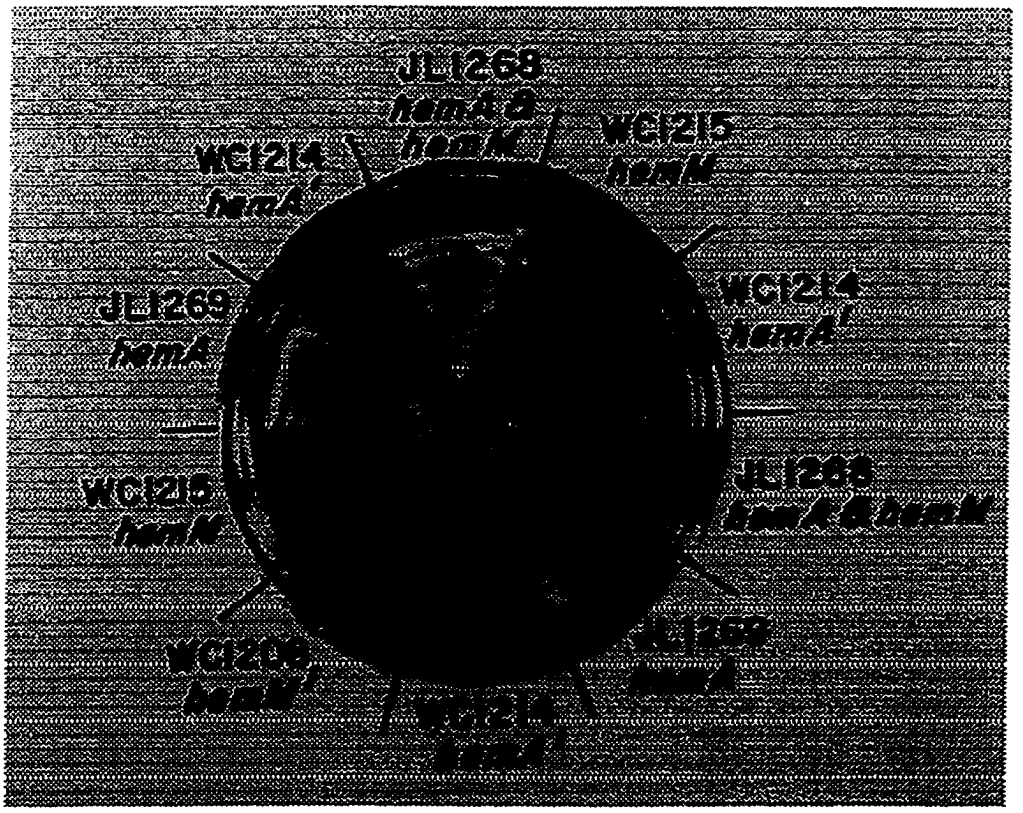



Figure 14. Growth of strains and their ability to feed ALA-requiring strains on LB agar medium

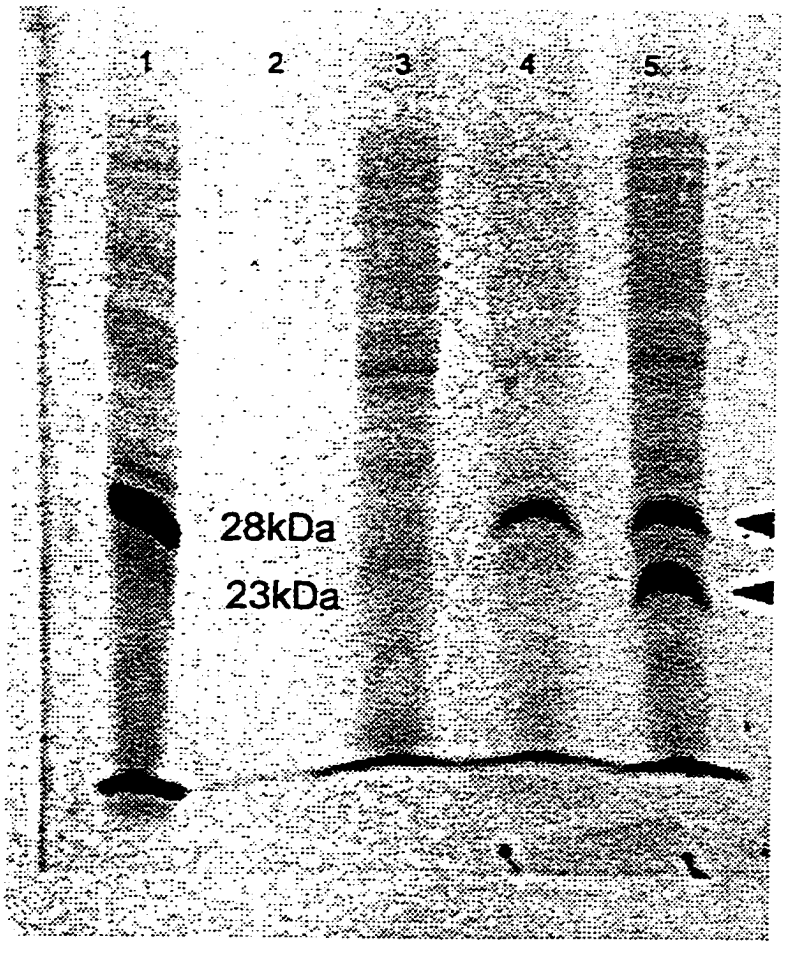



Figure 15. Expression of *hemM*.
An autoradiogram of proteins produced by pWC15 in the maxicell procedure in the presence of [$^{35}$S] methionine is shown. The proteins were analyzed on sodium dodecyl sulfate-10% polyacylamide gels.
Lanes: 1, CSR603/pWC22; 2, protein markers; 3, CSR603;
4, CSR603/pUC19; 5, CSR603/pWC15.
In Lane 5, the band at 28 kDa is β -lactamase and the one at 23 kDa is the *hemM* gene product.

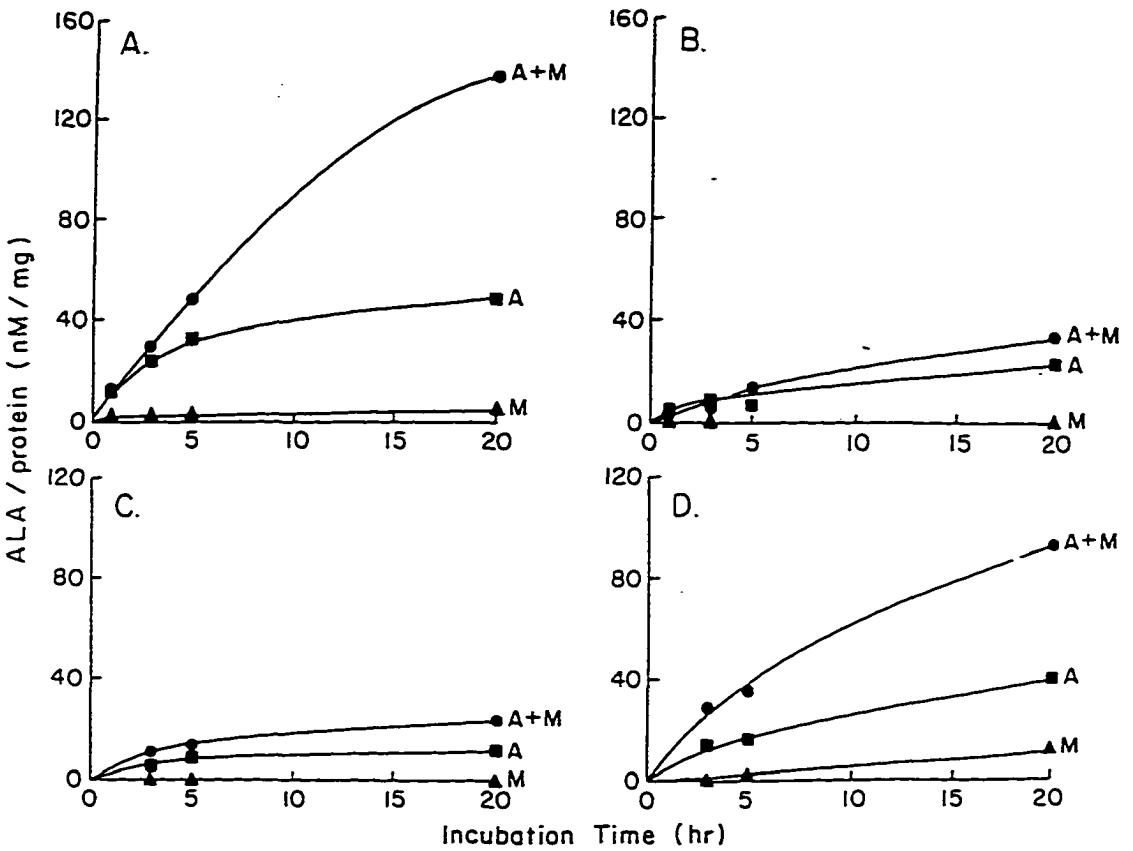



Figure 16. Accumulation of ALA by various strains containing multi-copy plasmids harboring *hemA*, *hemM*, and *hemA* plus *hemM*. Host strains: panel A, HU227; panel B, Hfr *Cavalli*; panel c, SASX41B; panel D, I14.

I14, which is a *hemM* mutant (Ikemi et al., 1992). Thus, for maximum ALA accumulation, both genes are required. Importantly, *hemA* and its upstream region but not *hemM*, is required to complement known mutant strains deficient in ALA synthesis.

## 2. Expression of glutamyl-tRNA reductase in *E. coli*.

The *hemA* gene product, GTR from *E. coli*, is a key enzyme in heme biosynthesis. However, it has not been overexpressed, well purified and characterized. Attempts to overexpress the *hemA* gene in *E. coli*, or *Bacillus subtilis* (Verkamp et al., 1992) have been unsuccessful. GTR has been purified from several organisms but the amount of purified enzyme finally obtained was very little (less than 0.2mg) from *Chlamydomonas reinhardtii* (Chen et al., 1990), *E. coli* (Jahn et al., 1991), and barley (Pontoppidan & Kannangara, 1994). The main reason may be the instability of GTR. The other possibility may be that overexpression is toxic to the cell. The more GTR produced, the more Glu-tRNA will be used for synthesis ALA. Thus there may not be enough Glu-tRNA for protein synthesis. Using an inducible expression system may avoid cytotoxicity. In order to study this very interesting enzyme, the T7 RNA polymerase/promter system, which provides tight regulation, high specificity and high-level of induction, was used to express the *hemA* gene in *E. coli*.

### 2.1. Construction of an expression plasmid

Studier et al (1990) developed a series of pET vectors for cloning and expressing target DNAs under control of a T7 promoter. All plasmids in the pET series contain a T7 promoter inserted into the *Bam*H1 site of the multicopy plasmid pBR322. They carry a

translation initiation signal (Shine-Dalgarno sequence) for the strongly expressed T7s10 protein. Because the T7 phage promoter is not recognized by the host *E. coli* RNA polymerase, expression of the cloned gene occurs only after expression of T7 RNA polymerase, which can be initiated either through induction of strains containing copies of the T7 RNA polymerase gene [e.g. BL21($DE_3$)] or phage infection ($CE_6$) which expresses the T7 RNA polymerase in the host cell (Studier et al. 1990). The pET14b vector also contains the *lacI*$^q$ gene so it has the lowest basal level expression of the pET series.

A 2.92-kb fragment containing the *hemA* and *hemM* genes was obtained when pJL68 was digested with *EcoR* I and *BamH* I. This fragment was ligated into the expression vector pET14b, which had also been digested with *EcoR* I and *BamH* I. Expression of the insert was thus controlled by a T7 promoter (Figure 8). The resulting plasmid is pWC01. When HU227 was transformed with pWC01 at 25°C, many colonies fluoresced after two days. pWC01 DNA was isolated from one of these colonies. Digestion of pWC01 with *Sal* I gave 0.68, 1.25 and 5.15 kb fragments, thus confirming that the *hemA* and *hemM* fragment had been inserted into pET14b (Figure 17). While *hemM* was in the insert upstream from *hemA*, it was transcribed in the opposite direction and therefore was not under the control of the T7 promoter.

2.2. **Construction of a strain for overexpression**

The host strain of choice was BL21($DE_3$) because in addition to having the T7 RNA polymerase integrated into its genome in front of an inducible promoter, it has mutations in *lon* and *ompT* and is therefore significently protease deficient (Studier & Moffatt, 1986; Grodberg & Dunn, 1988). It also lacks a DNA restriction system. When the strain BL21

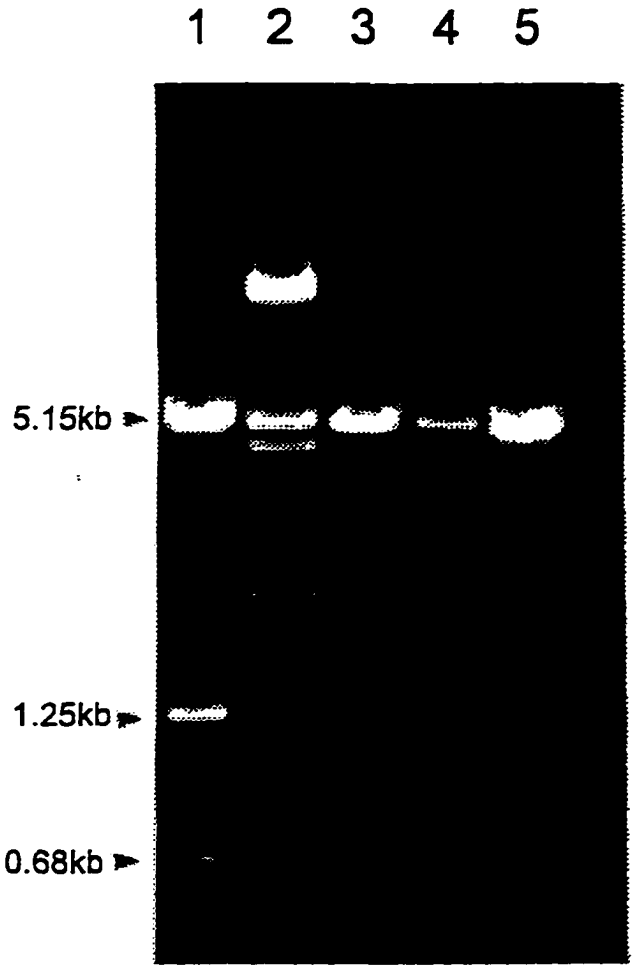



Figure 17. Agarose gel electrophoresis of *Sal* I digestion of plasmids:
Lane 1, pWC01; Lane 2, DNA marker III; Lane 3, pWC02.
Lane 4, pWC02; Lane 5, pET14b.
Arrows indicate 5.15kb, 1.25kb and 0.68kb fragments.

was infected by λDE$_3$, which contains T7 RNA polymerase gene (*gene1*), the resultant lysogen is BL21(DE$_3$), in which the only promoter known to direct transcription of the T7 RNA polymerase gene is the *lac*UV5 promoter, which is inducible by IPTG.

When pWC01 was transformed into BL21[DE$_3$], the resultant strains fluoresced after a day or two. Some colonies fluoresced more strongly than the others. Plasmids isolated from two colonies, which fluoresced to different degrees, were not the same. The plasmid isolated from the more fluorescent colony is pWC01, and the plasmid isolated from a less-fluorescent colony is pWC02. pWC02 contained a 2.12 kb insert with *hemA* but no T7 promoter or *hemM* on the plasmid. When pWC02 was digested with *Sal*I, 0.68 and 5.15 kb bands were observed. Thus, a 1.25 kb fragment which was on pWC01 was lost in pWC02 (Figure 17).

After a day or two, colonies of WC1201 (BL21[DE$_3$]/pWC01) fluoresced much more strongly than those of WC1202 (BL21[DE$_3$]/pWC02 ) (data not shown). Figure 18 shows that colonies of WC1201, WC1261 (HU227/pWC01), and WC1268 (HU227/pWC68) fluoresced strongly, with WC1201 fluorescing earlier and more intensely indicating that *hemA* gene in WC1201 is expressed, probably at a higher level than that in WC1261 or WC1268.

### 2.3. *in vitro* transcription-translation

When pWC01 was transcribed and translated *in vitro*, supplementation with *E. coli* tRNA$^{glu}$ stimulated expression of the insert containing *hemA* and *hemM* (Figure 19). In the presence of $^{35}$S-methionine, protein bands at 46, 24 and 23 kDa were revealed by

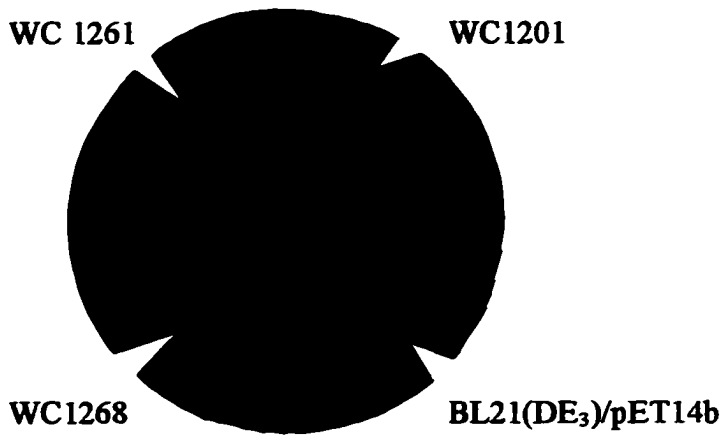



Figure 18. The accumulation of porphyrin by various strains was visualized by photography under long wavelength (366 nm) ultraviolet light. The strains were grown for 2 days in LB medium (50 ug/ml amp).

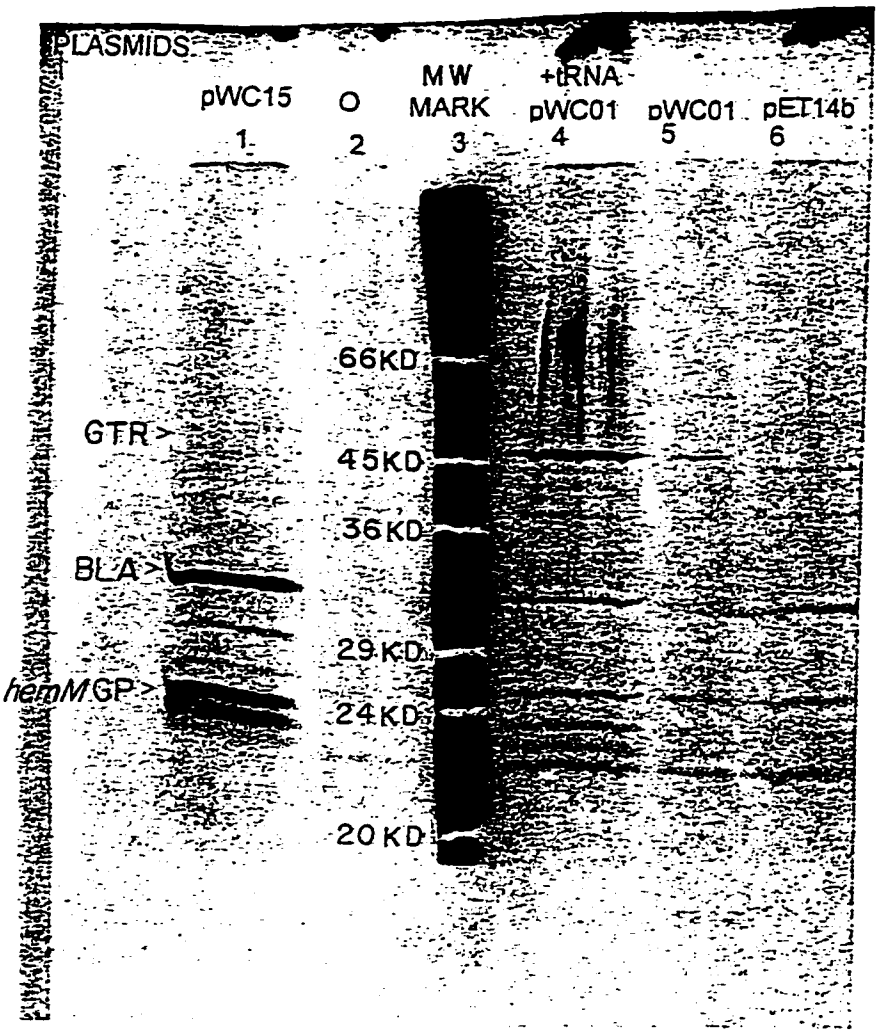



Figure 19. pUC19 and pET14b-based vectors containing *hemA* + *hemM* and *hemM* alone, were expressed in an *E. coli in vitro* transcription and translation system in the presence of $^{35}$S-methionine, resolved on SDS-PAGE and visualized by autoradiography.

autoradiography on SDS-PAGE representing GTR and *hemM* gene products. A plasmid with *hemM* alone, pWC15, showed only the bands at 23 and 24 kDa (Figure 19). The 46 kDa band was observed for pWC69 (pUC19+ *hemA*) and pJL69 (pTZ19U+ *hemA*) but not for pET14b (Figure 20), indicating that these 46 kDa bands were *hemA* gene products.

2.4. **Overexpression of the *hemA* gene product, GTR**

Both transformants, WC1201 (BL21[$DE_3$]/pWC01) and WC1202 (BL21[$DE_3$]/pWC02), accumulated ALA after IPTG induction. This was measured over a three day period (Figure 21). The specific activity of accumulation of ALA for WC1201 was highest after one day. The decline can be attributed to death of cells after IPTG induction, or loss of GTR activity, or conversion of ALA to porphyrin.

The appearance of ALA synthesis activity in sonicates of WC1201 was associated with induction of WC1201 with IPTG. The induction conditions of IPTG concentration, temperature and time were optimized for maximum specific activity (Table 4).

It was found that induction with 5 mM IPTG at 37°C and 2 hr gave the best results. Lowering the IPTG concentration, or the temperature of induction to 25°C, or increasing induction time, or adding sorbitol and betaine (Blackwell & Horgan, 1991) did not improve the specific activity of the sonicate. 1 mM IPTG was almost as good as 5 mM, and was used for larger culture volumes.

The cells of the induced culture were sonicated in the presence of PMSF to prevent proteolysis and the GTR activity of the sonicate was measured as the appearance of ALA after incubation with assay mixture containing glutamate, ATP and NADPH, and

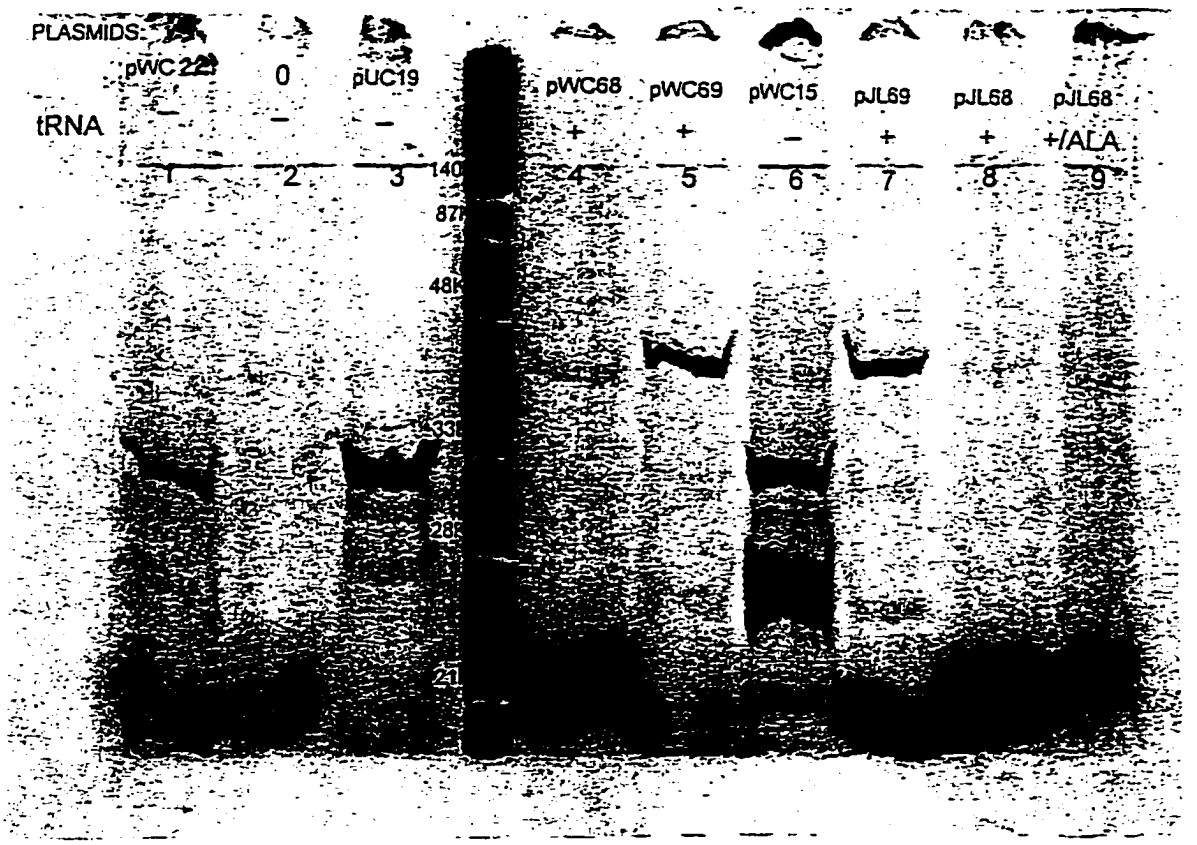



Figure 20. pUC19-based vectors containing *hemA* + *hemM*, *hemA* alone and *hemM* alone, were expressed in an *E. coli in vitro* transcription and translation system in the presence of $^{35}$S-methionine, resolved on SDS-PAGE and visualized by autoradiography.

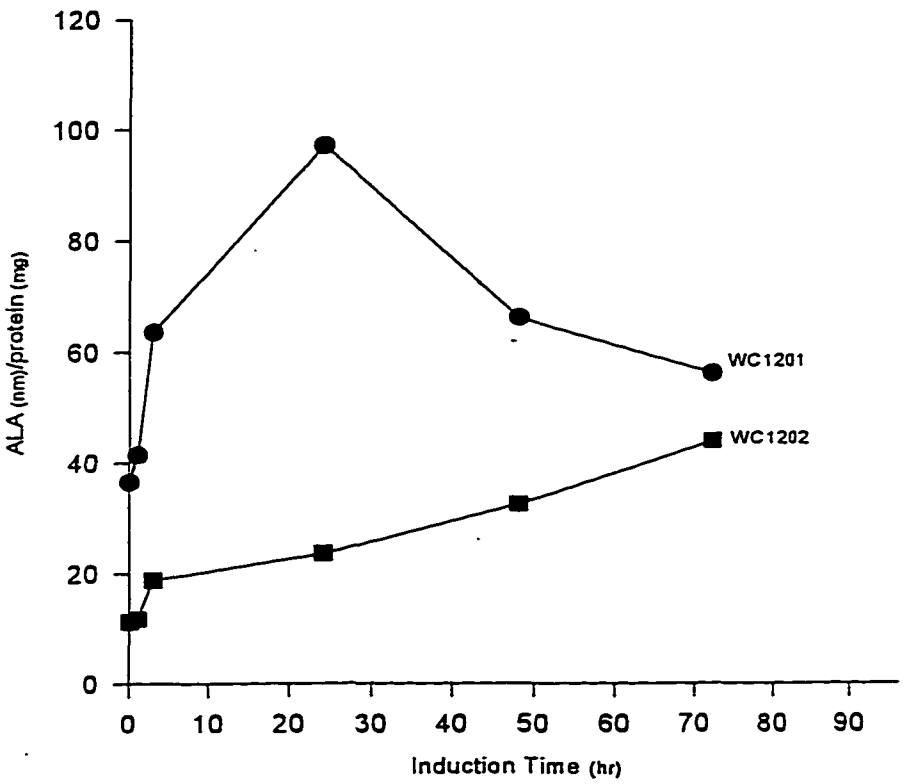



Figure 21. Secretion of ALA into the medium by transformed strains

Table 4. Induction of GTR activity with different conditions [a]

| Medium | $T_{(Growth)}$ | $IPTG_{(mM)}$ | | GTR activity [ALA(nM)/protein(mg)] | | |
|---|---|---|---|---|---|---|
| | | | Induction time (hr.) | 2 | 24 | 48 |
| LB-CA[b] | 37°C | 0 | | 1.35 | 3.00 | 0.52 |
| " | " | 5 | | 8.60 | 0.37 | - |
| " | " | 1 | | 7.40 | 0.00 | - |
| " | 25 | 1 | | 4.30 | 1.30 | 0.20 |
| " | " | 0.1 | | 0.70 | 0.80 | - |
| LB | " | 1 | | 5.65 | 0.00 | - |
| LB-BS[c] | " | 1 | | 4.07 | 0.22 | - |

a. Assay conditions are described in materials and methods.
b. LB + casamino acids.
c. LB + 2.5 mM betaine and 660 mM sorbitol.

$tRNA^{glu}$ when noted. WC1202, the strain without the $T_7$ promoter on the insert, showed lower activity and a slower time course for appearance of activity (Table 5). Additions of sonicate from an uninduced strain to the assay mixture did not increase activity. Thus it appears that the other enzymes required for the coupled assay were not rate-limiting.

When WC1201 was induced with IPTG *in vivo*, the appearance of the *hemA* gene product was monitored by the appearance on SDS-PAGE, which was stained with Brilliant Blue G. A band corresponding to a 46 kDa protein (Figure 22) was present. The color grew stronger with time after induction up to two or three days (Figure 23). The *hemM* gene product was not observed. The β-lactamase band got weaker with time. This result was confirmed by autoradiography, when the induction was initiated in the presence of $^{35}$S-methionine (data not shown).

2.5. **Native molecular weight of the *hemA* gene product, GTR**

When pWC01 and pWC68, each of which contains both *hemA* and *hemM* but on different vectors, were transcribed and translated *in vitro*, a protein at approximately 175 kDa appeared on native gradient PAGE by using the whole *in vitro* reaction mixture. Importantly, when pWC69, which contains *hemA*, but not *hemM*, was transcribed and translated in *vitro*, the same protein band at 175 kDa appeared (Figure 24), which suggests that the *hemM* gene product is not required for this band.

When WC1201 was induced with IPTG *in vivo* in the presence of $^{35}$S-methionine, native gradient PAGE gel electrophoresis of the sonicate supernatant, and subsequent autoradiography, showed a protein band at a molecular weight of approximately 117 kDa (Figure 25), while SDS-PAGE showed a band at 46 kDa.

Table 5. GTR activity in sonicates of induced transformed strains

| Induction time (hr) | 2 | 24 | 48 | 72 |
|---|---|---|---|---|
| Strains[a] | GTR activity [ALA(nM)/protein(mg)] | | | |
| WC1201 | 7.40 | 0.00 | - | - |
| WC1202 | 0.01 | 0.02 | 0.77 | 0.06 |

a The transformed strains were grown at 37°C in LB medium with 1% casamino acids and induced with 1 mM IPTG.


Figure 22. **WC1201 was induced with IPTG and after 2 hr the mixture was sonicated, subjected to SDS-PAGE and stained with Brilliant Blue G.**

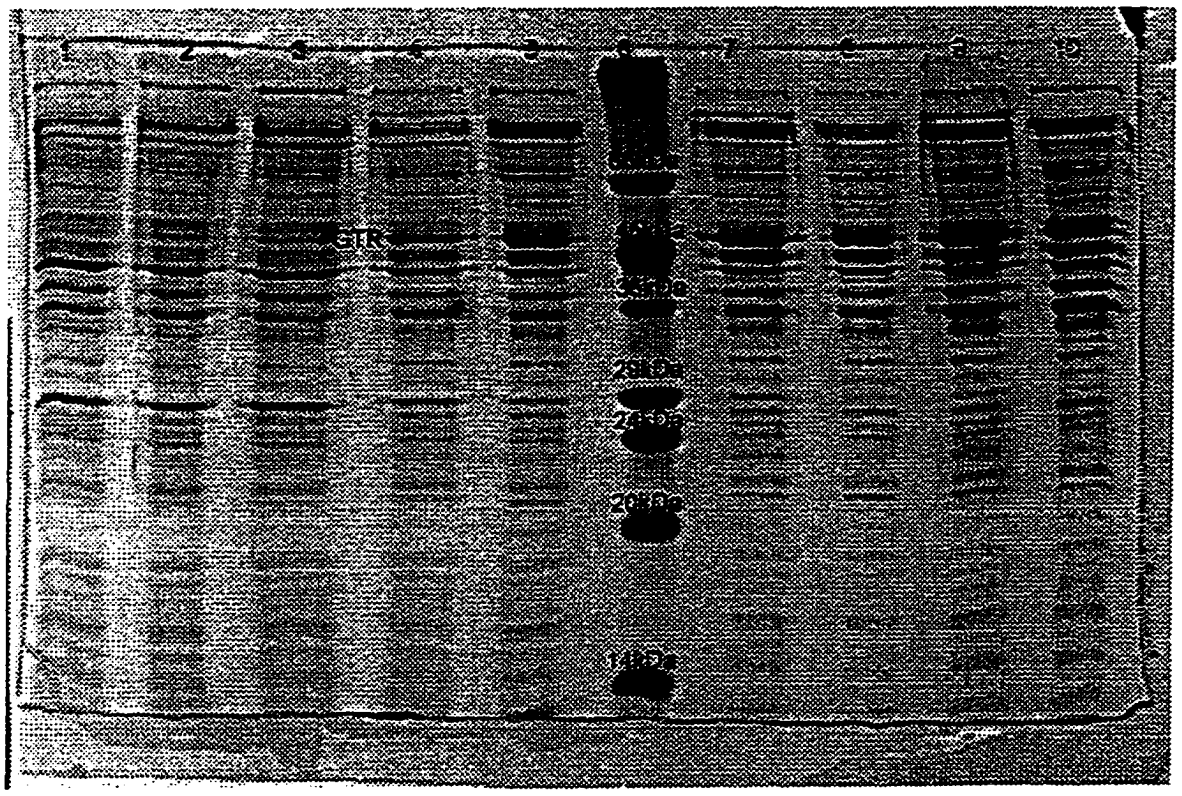
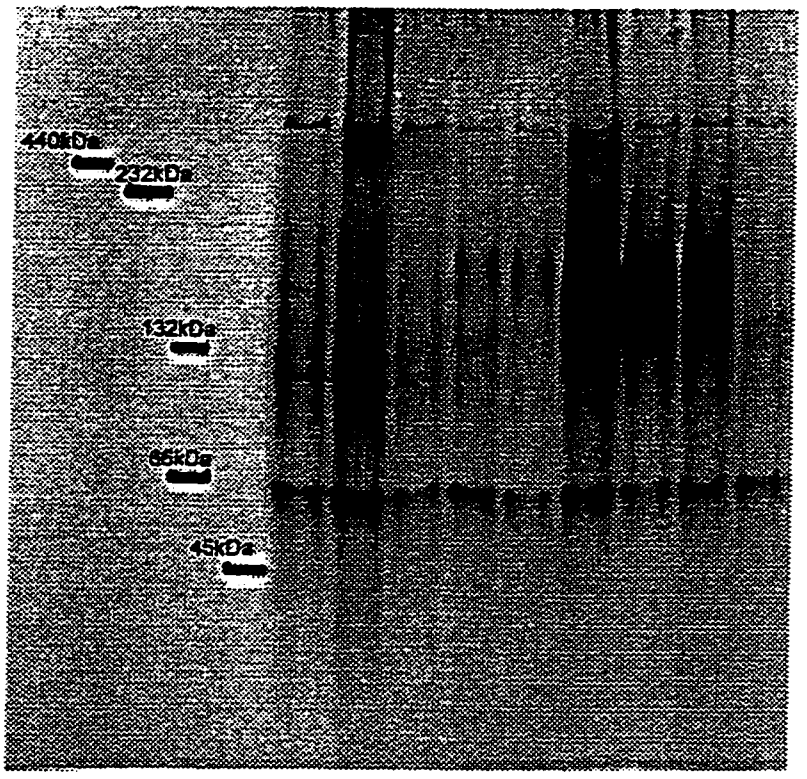
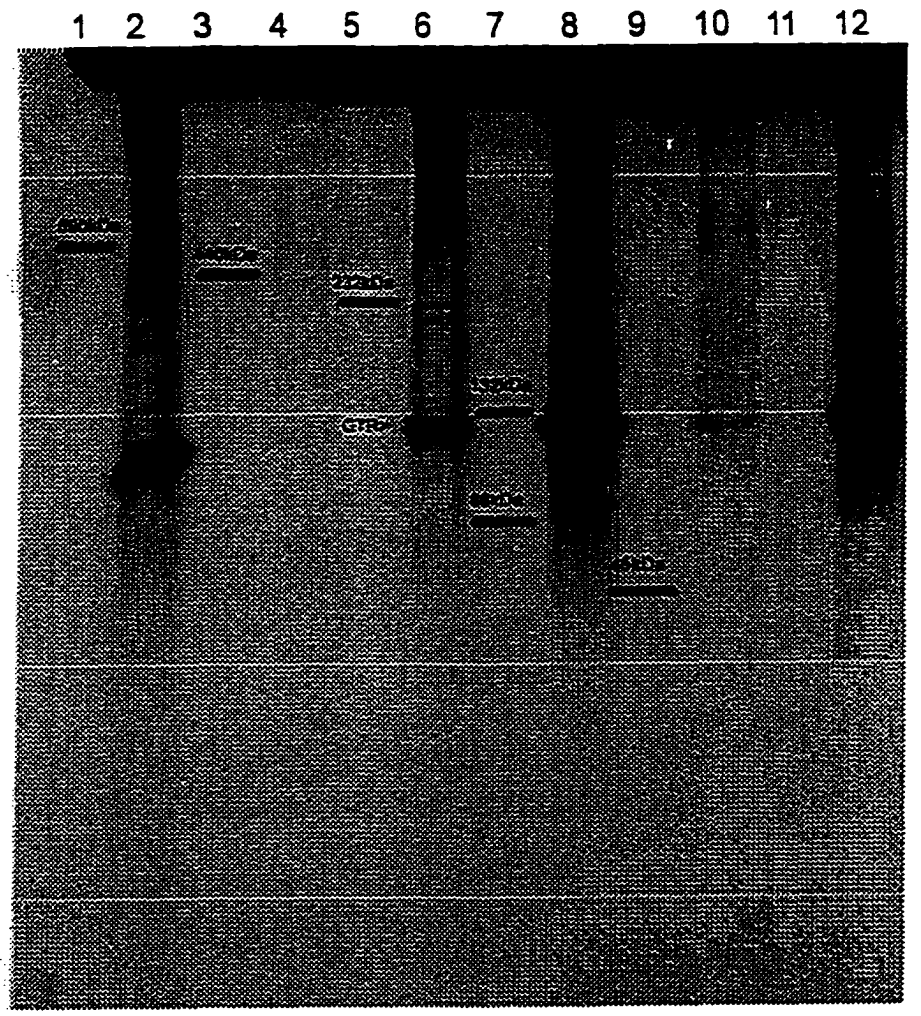



Figure 23. Expression of GTR by WC1201, induced and uninduced cultures were sampled at times given, sonicated, and subjected to SDS-PAGE and stained with Brilliant Blue G. Lanes 2, 4, 7, and 9 represent cultures induced by IPTG. Lane 1 and 2; samples taken out at 0 time; Lanes 3 and 4, 3 hrs; Lanes 5 and 6, 1 day; Lanes 7 and 8, 2 days; Lanes 9 and 10, 3 days.




| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Standards | + | + | + | + | | | | | | | | | |
| pUC19 | | | | | + | + | + | + | + | - | - | - | - |
| *hemA* | | | | | - | - | + | + | + | + | + | + | - |
| *hemM* | | | | | - | + | + | - | + | + | + | + | - |
| pET14b | | | | | - | - | - | - | - | + | + | + | + |
| $tRNA^{glu}$ | | | | | - | - | 0.5 | 0.5 | 0.5 | 0.5 | 1 | 2 | - |

Figure 24. pUC19- and pET14b- based vectors containing *hemA*+ *hemM*, *hemA* alone and *hemM* alone were expressed in an *E. coli in vitro* transcription-translation system in the presence of $^{35}$S-methionine, resolved on native gradient gel PAGE and visualized by autoradiography.
Lane 6, pWC15; Lane 7, pWC15 and pWC68; Lane 8, pWC69;
Lane 9, pWC68; Lane 10, 11, 12, pWC01; Lane 13, pET14b.




| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Standards | + | | + | | + | | + | | + | | | |
| pET14b | | + | | + | | + | | + | | + | | + |
| *hemA* | | + | | - | | + | | + | | - | | + |
| ORF1 | | + | | - | | + | | + | | - | | + |
| Induction time(hr) | | 2 | | 2 | | 2 | | 8 | | 8 | | 8 |

Figure 25. WC1201 was induced with IPTG *in vivo* in the presence of [$^{35}$S]-methionine and after 2 hr and 8 hr, resolved on native gradient PAGE and visualized by autoradiograpgy.

When the 175 kDa band from *in vitro* expression and the 117 kDa band from *in vivo* expression were excised from the respective gels and run on SDS-PAGE, autoradiography showed a band at 46 kDa in both cases (Figure 26). These results suggest that the native form of the *hemA* gene product is as an oligomer or part of a complex.

### 2.6. *in vitro* transcription-translation of *gltX* and *hemA*

The sizes of the native form of *hemA* gene products from *in vivo* or *in vitro* experiments were more than 100 kDa, which suggest that GTR exists as an homooligomer and /or complex. To detect whether GTR is complexed with GTS or not, the plasmids containing *hemA* (pWC01 or pWC02) or *gltX* (pTZ7612) alone, were expressed in an *in vitro* transcription and translation system in the presence of $^{35}$S-methionine with and without $tRNA^{glu}$. Figure 27 shows that protein bands at 46, and 54 kDa were detectable by autoradiography on SDS-PAGE. A plasmid with *hemA* alone, pWC02, showed a 46 kDa band in lane 4. The 54 kDa band was seen in the pTZ7612 lane, which reflected expression of the *gltX* gene. When pWC02 and pTZ7612 were added together in the same *in vitro* transcription and translation reaction mixture, both 46 and 54 kDa bands were seen in lane 7, indicating *hemA* and *gltX* genes were expressed.

Using the same reaction mixture for native gradient PAGE, only one clear 54 kDa band was seen in pTZ7612 lanes (Figure 28). Importantly, this 54 kDa band did not shift when pTZ7612 and pWC02 were mixed together for transcription and translation in *vitro* (lane 9) or first transcribed and translated in *vitro* separately, and then incubated together at 37°C for 30 min (lanes 12 and 13). However, the *hemA* gene product band was not

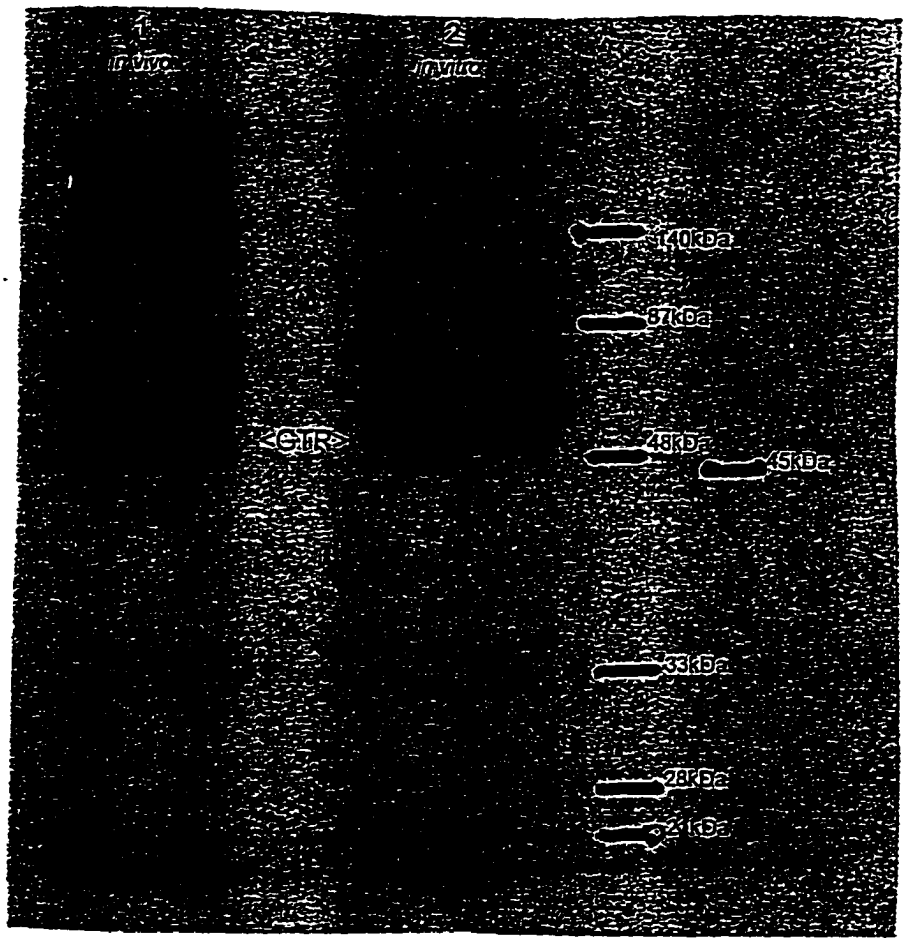



Figure 26. The 117 kDa (from *in vivo* expression) and 175 kDa (from *in vitro* expression) protein bands obtained by *in vivo* and in *vitro* expression experiments which had been observed on native gradient PAGE, were cut out and put on SDS-PAGE and visualized by autoradiography.

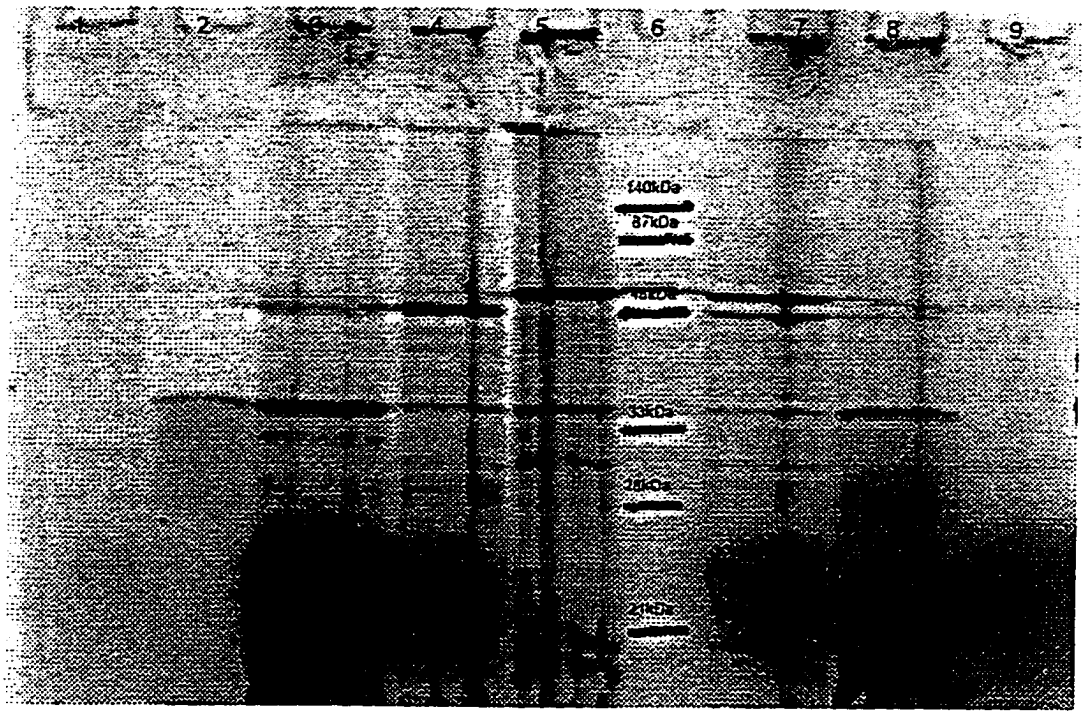



Figure 27. pET14b-based vectors containing *hemA* + *hemM* or *hemA* alone and pBR322-based containing *gltX*, were expressed in an *E. coli in vitro* transcription and translation system in the presence of $^{35}$S-methionine, resolved on SDS-PAGE and visualized by autoradiography.
Lane 1: no DNA; Lane 2: pET14b; Lane 3: pWC01; Lane 4: pWC02; Lane 5: pTZ7612; Lane 6: protein marker; Lane 7: [a]pWC02 + pTZ7612; Lane 8: [a]pWC01 + pTZ7612; Lane 9: pBR322.

a. Two plasmids were mixed together for transcription and translation *in vitro*.


Figure 28. pET14b-based vectors containing *hemA* + *hemM* or *hemA* alone and pBR322-based containing *gltX*, were expressed in an *E. coli in vitro* transcription and translation system in the presence of $^{35}$S-methionine, resolved on native gradient PAGE and visualized by autoradiography. Lane 1: pET14b; Lane 3: pWC01; Lane 5: pWC02; Lane 7: pTZ7612; Lane 9: [a]pWC02 + pTZ7612; Lane 11: [a]pWC01 + pTZ7612; Lane 12: [b]pWC02 + pTZ7612; Lane 13: [b]pWC01 + pTZ7612; Lane 14: [b]pWC02 + pBR322; Lane 15: pBR322.

a. Two plasmids were mixed together for transcription and translation *in vitro*.
b. Two plasmids were transcribed and translated *in vitro* separately and then mixed together and incubated at 37°C for 30 min.

clearly seen and there is a high molecular weight band in every lane except with vector without insert alone. The result suggests that the *hemA* gene product did not form a complex with GTS and might form oligomer, but these are preliminary results.

### 2.7. Partial purification of the *hemA* gene product

When the sonicates from an induced culture of WC1201 were centrifuged and the supernatant and residue assayed for activity separately, most of the activity was found in the supernatant thus it appears that the enzyme is not membrane-bound (Table 6). Thus, the supernatant was used for further purification.

WC1201 was induced with IPTG in the presence of $^{35}$S-methionine. The supernatant from the cell sonicate was analyzed by gel filtration on Sepharose 6B. A peak of radioactivity at about 110 kDa was detected (Figure 29). A peak of enzymatic activity at about 120 kDa was eluted from a Sepharose 6B column (Figure 30). There is a broad plateau of activity at higher sizes. Thus a molecular weight of 110-120 kDa is suggested for GTR.

An attempt to purify GTR by affinity chromatography on the immobilized NADPH analog, 2',5'-ADP-Sepharose (Pontoppidan & Kannangara, 1994) was unsuccessful.

Activity assays were coupled to ALA synthesis by adding sonicate of WC1201 to the assay mixture and measuring enhancement of ALA synthesis. It is significant that extracts of *hemA*$^-$ or wild-type strains which did not overexpress *hemA*, could not be used in the assay of gel filtration and affinity chromatography eluates because no ALA synthesis was observed.

Table 6. Most GTR activity is located in the soluble fraction of the sonicate of WC1201

| Expt | GTR activity [ALA(nM)/protein(mg)] | | |
|---|---|---|---|
| | Sonicate | Supernatant | Residue |
| I | 3.96 | 3.03 | - |
| II | 2.65 | 1.69 | 0.99 |

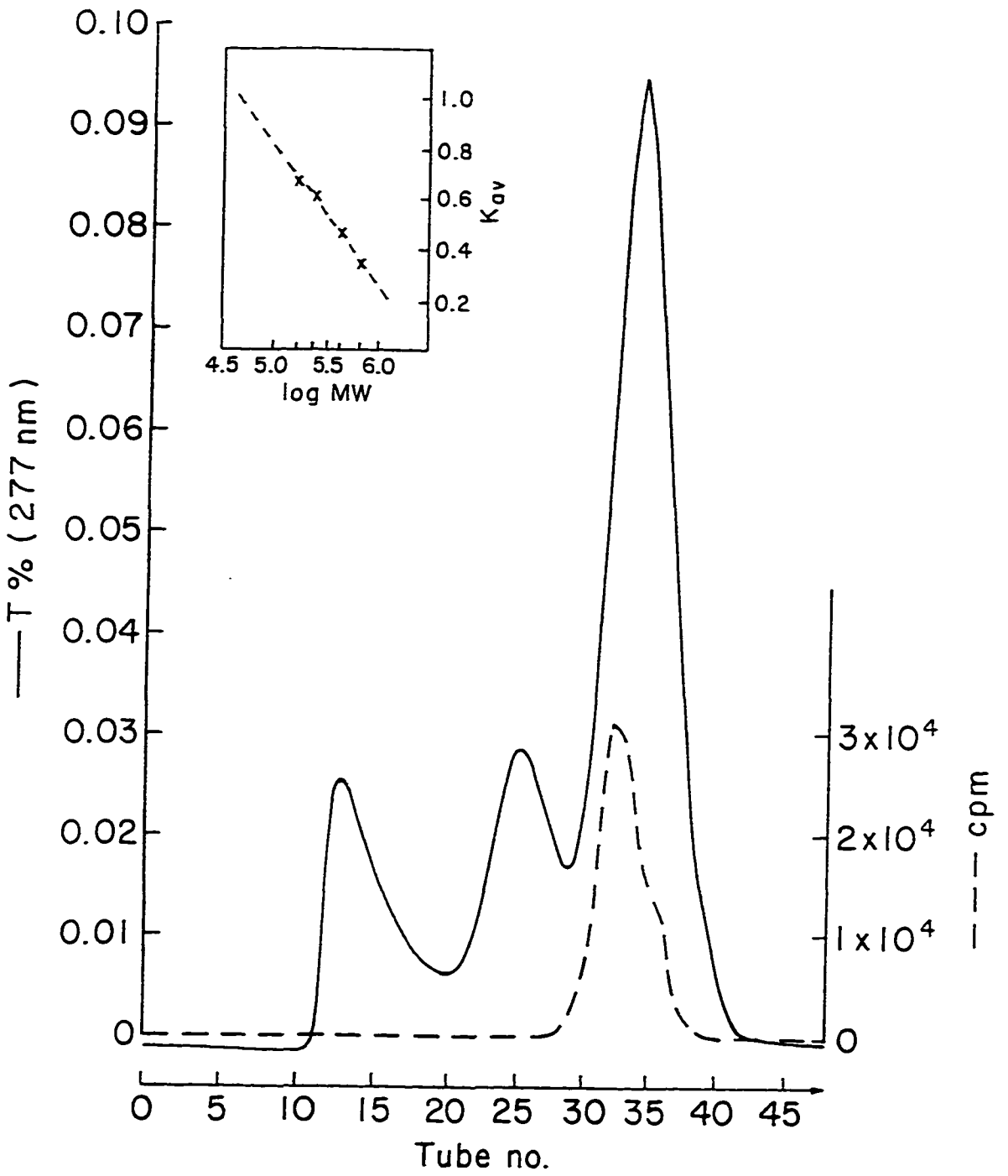



Figure 29. WC1201 was induced with IPTG in the presence of $^{35}$S-methionine and the supernatant from the sonicate of the cells was analyzed by gel filtration on Sepharose 6B. The insert shows the calibration of the column with standard proteins.

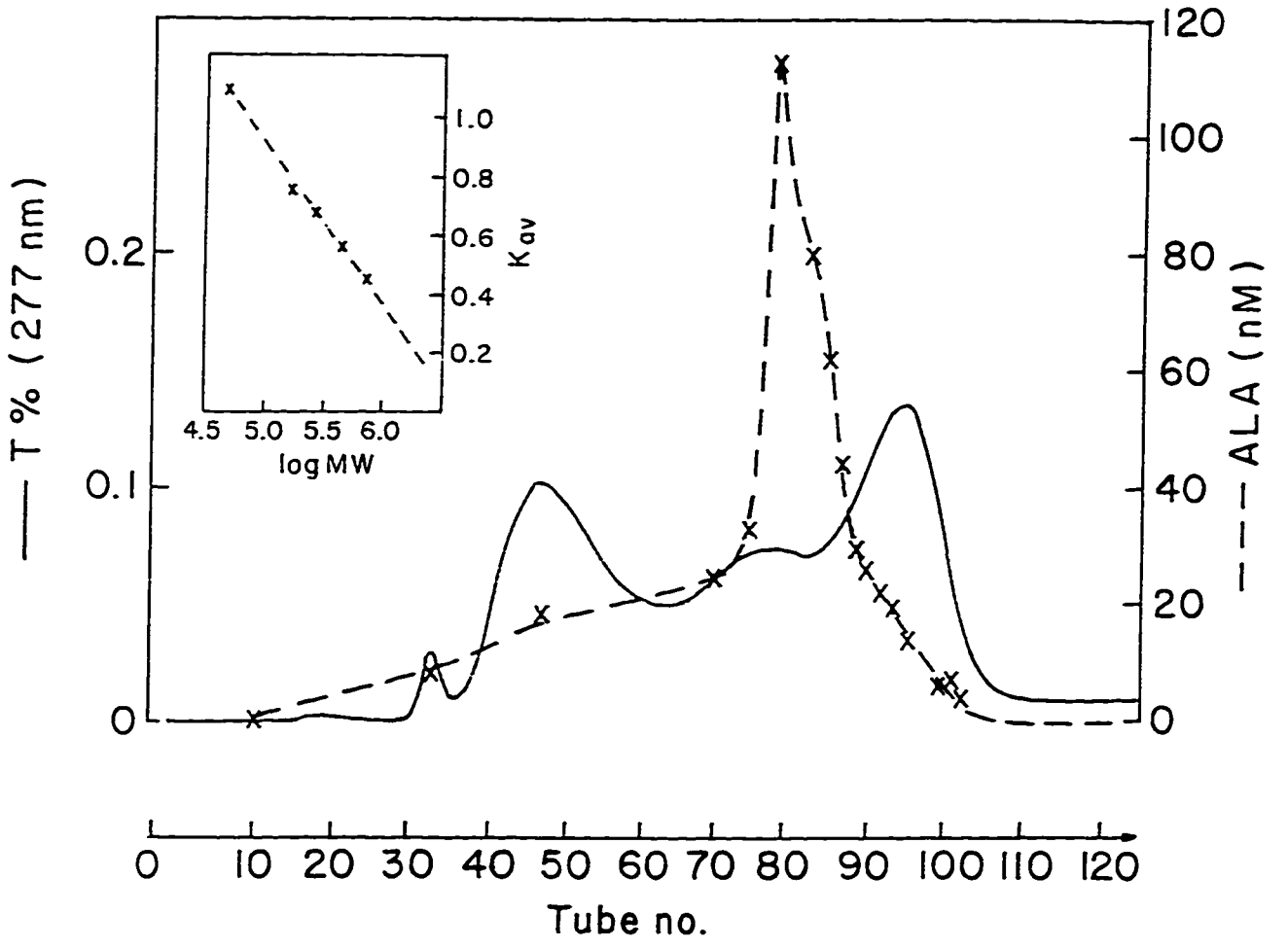



Figure 30. The supernatant from a sonicate of induced WC1201 was analyzed by gel filtration on Sepharose 6B as described in Materials and Methods. The inset shows the calibration of the column with standard proteins. ( X) indicates tubes which were analyzed for their ability to enhance ALA synthesis activity in IPTG-induced sonicates of WC1201.

## 3. Control of 5-aminolevulinic acid biosynthesis in *E. coli*

Because addition of ALA to wild-type cultures of *E. coli* causes the accumulation of porphyrins, it appears that regulation of the pathway is at the level of ALA synthesis. Since glutamyl-tRNA synthetase serves heme and protein synthesis and GSA can be converted to ALA non-enzymatically, regulation is probably at the GTR step. Thus in order to study regulation of heme biosynthesis in *E. coli*, we studied the effects of heme, glucose, cAMP, PBG, $tRNA^{glu}$ levels on ALA synthesis.

RP523, a strain (Table 3), which is hemin-permeable and cannot utilize ALA because there is a mutation in the structural gene, *hemB*, for the next enzyme in the pathway, ALA dehydratase, accumulates ALA. Thus RP523 can be used as an indicator strain for the effects of various factors on ALA synthesis.

### 3.1. Effects of hemin on ALA biosynthesis

#### 3.1.1. Effect of hemin on ALA accumulation by a *hemB* mutant

Parallel cultures of RP523 were grown with increasing hemin concentrations. Thus the effect of hemin could be studied by determining ALA accumulation in the growth medium.

When RP523 was grown areobically or anaerobically in LB medium, the specific activity of accumulation ALA was lowered in the presence of hemin or heme arginate. This effect was concentration-dependent (Figure 31). These results indicate that hemin is an inhibitor of ALA biosynthesis.

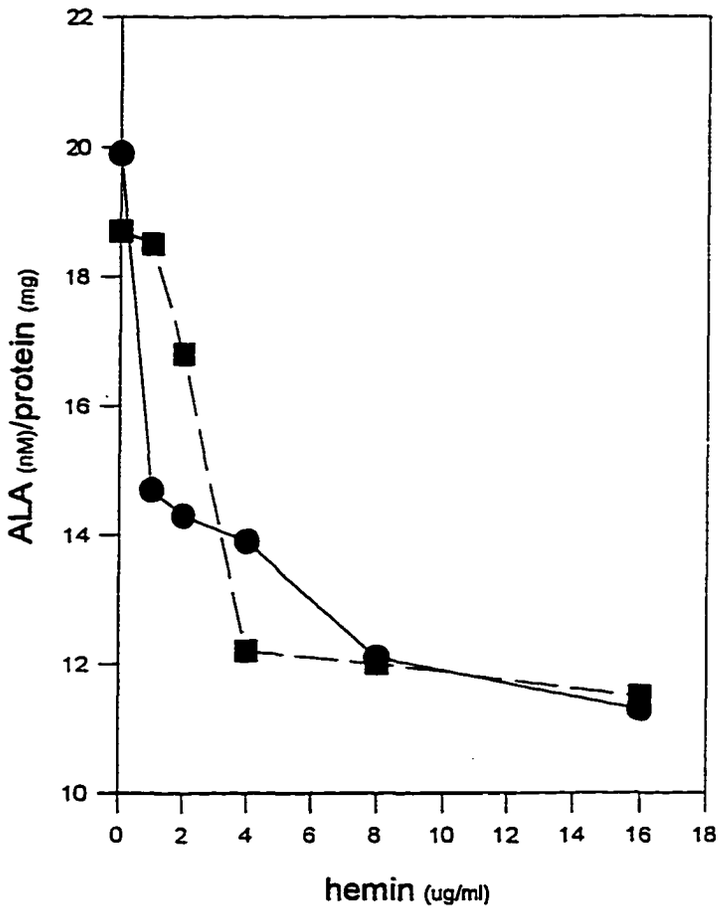



Figure 31. Effect of hemin on ALA accumulations in RP523[a].

[a] Cells were grown aerobically (■) or anaerobically (●) on 10 ml of LB supplemented with 1% glycerol and 50mM fumarate for 20 hrs.

### 3.1.2. Effect of hemin on activity of ALA synthesis in cell-free extracts of a GTR expression strain

When the sonicates from an induced culture of WC1201 were used in a coupled assay, a strong ALA synthesis activity was detected without the use of radioactivity in the substrate. Therefore, the possible regulator role of hemin on enzymatic activity in the synthesis of ALA in a cell free system could be investigated by increasing hemin concentration in the assay mixture. Figure 32a shows that the activity of ALA synthesis in the extract of WC1201 was inhibited by hemin.

In aqueous solution, hemin can form a highly soluble complex with arginine, hemin arginate, which is more stable than hemin solutions formed in strong base. It was found that hemin arginate is a stronger ALA synthesis inhibitor than hemin (Figure 32b). This is probably because hemin as prepared in NaOH is probably aggregated.

## 3.2. Effect of glucose on ALA biosynthesis

### 3.2.1. Effect of glucose on ALA accumulation by a *hemB* mutant

Growth on glucose prevents the synthesis of mRNA for a wide variety of sugar-metabolizing enzymes in a process known as catabolic repression, a process often mediated by cAMP levels. Glucose lowered the specific activity of accumulation of ALA in RP523 during anaerobic growth on LB medium (Table 7). The effect was greater in the absence of glycerol and fumarate. This effect was much weaker under anaerobic growth conditions in minimal medium (Table 8). However, ALA accumulation is very low in minimal medium.

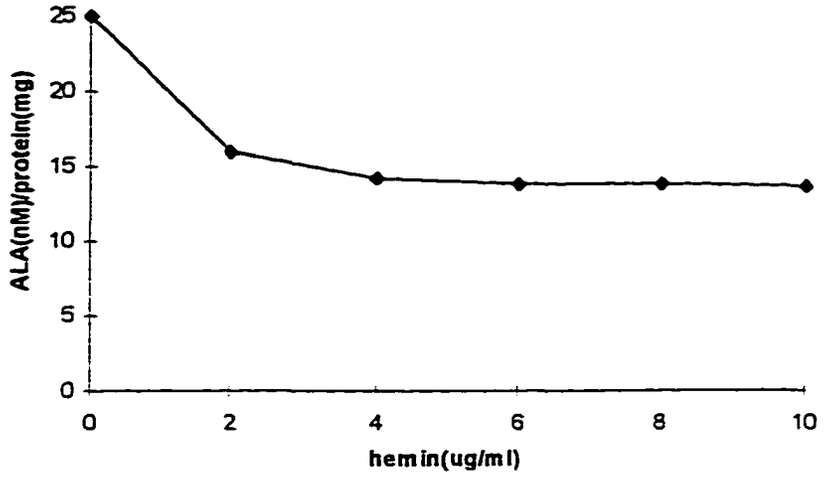



Figure 32 a. Effect of hemin on ALA synthesis in the enzyme assay using the extract of WC1201[a].

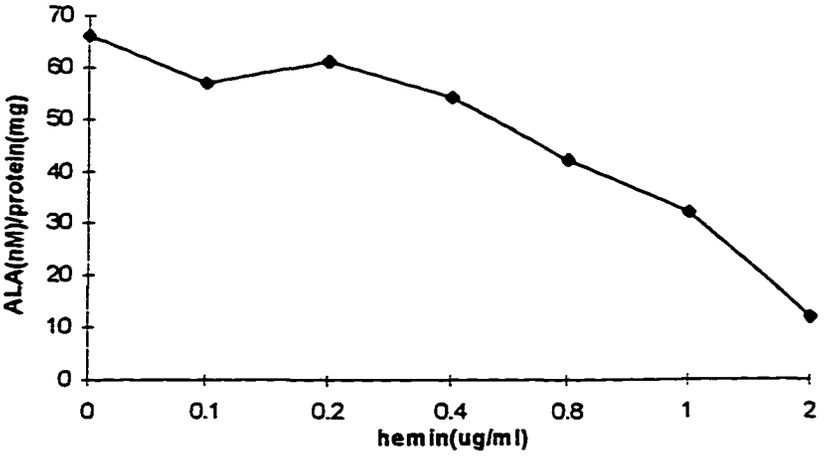



Figure 32 b. Effect of hemin arginate on ALA synthesis in the enzyme assay using the extract of WC1201[a].

a. Hemin or hemin arginate was added to the extract of WC1201 for ALA synthesis assay as in section 1.5.

Table 7. Effect of glucose on ALA accumulation by RP523 in LB medium under anaerobic growth condition[a].

| Additions to medium | Experiments | I | | II | | III | |
|---|---|---|---|---|---|---|---|
| Glucose (0.2%) | | + | - | + | - | + | - |
| Glycerol (1%) | | - | - | - | + | + | + |
| Fumarate (50mM) | | - | - | - | + | + | + |
| ALA(nM)/protein(mg) | | 36 | 68 | 28 | 42 | 53 | 69 |
| | | (53%) | (100%) | (67%) | (100%) | (77%) | (100%) |

[a] Cells were grown in 10 ml LB medium supplemented with 4 ug/ml hemin at 37°C for 20 hrs.

Table 8. Effect of glucose on ALA accumulation by RP523 in minimal medium under anaerobic growth condition[a].

| Additions to medium | Experiments | I | | II | | III | |
|---|---|---|---|---|---|---|---|
| Glucose (0.2%) | | + | - | + | - | + | - |
| Glycerol (1%) | | - | + | - | + | + | + |
| Fumarate (50mM) | | - | - | - | + | + | + |
| ALA(nM)/protein(mg) | | 9.4 | 10.7 | 9.8 | 11.8 | 8.6 | 10.8 |
| | | (88%) | (100%) | (83%) | (100%) | (80%) | (100%) |

[a] Cells were grown in 10 ml LB medium supplemented with 4 ug/ml hemin at 37°C for 20 hrs.

### 3.2.2. Effect of cAMP on ALA accumulation in RP523

cAMP plays a direct role in overcoming the catabolic repression of several different enzymes and participates in the transcription of a wide variety of genes. When RP523 was grown anaerobically in LB medium containing 0.2% glucose, the glucose effect was slightly reversed by dibutyl cAMP (Table 9).

## 3.3. Effect of $tRNA^{glu}$ on ALA biosynthesis

### 3.3.1. Effect of $tRNA^{glu}$ on ALA synthesis *in vitro*

The production of ALA *in vitro* requires addition of glutamic acid, ATP, NADPH and $tRNA^{glu}$ to the cell extract. $tRNA^{glu}$ is a substrate for glutamyl-tRNA synthetase. It was found that ALA formation from glutamate was blocked by preincubation cell extracts with RNase A (Huang et al. 1984, Weinstein & Beale. 1985, Breu & Domemann.1988, Li et al. 1989b,). The addition of *Escherichia coli* $tRNA^{glu}$ to a cell-free extract, in which endogenous $tRNA^{glu}$ had been removed by gel filtration, increased the production of ALA about 3 fold in the *in vitro* assay for ALA synthesis (Li et al., 1989b). Using a cell-free extract of WC1201, ALA synthesis activity was increased by adding *Escherichia coli* $tRNA^{glu}$ (Table 10). However, there is a smaller increase than observed by Li et al. (1989b). This may be due to the fact that endogenous $tRNA^{glu}$ had not been removed, which would decrease the effect.

### 3.3.2. Effect of $tRNA^{glu}$ on *hemA* gene expression

When plasmids with *hemA* alone or *hemA* and *hemM* genes were transcribed and translated *in vitro*, supplementation with *E. coli* $tRNA^{glu}$ stimulated expression of the

Table 9. Effect of dibutyl cAMP on ALA accumulation by RP523[a]

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| Additions to LB medium | | | | | | | | |
| dibutyl cAMP (ug/ml) | 0 | 5 | 10 | 20 | 0 | 5 | 10 | 20 |
| Glucose (0.2%) | - | - | - | - | + | + | + | + |
| Glycerol (1%) | + | + | + | + | - | - | - | - |
| Fumarate (50mM) | + | + | + | + | - | - | - | - |
| ALA(nM)/protein(mg) | 17.1 | 19.1 | 18.2 | 20.4 | 10.7 | 10.4 | 13.9 | 15 |
| Effect (%) | 100 | 112 | 106 | 119 | 62.6 | 60.8 | 81.3 | 87.7 |

[a] Cells were grown in 10 ml LB medium supplemented with 4 ug/ml hemin at 37°C for 20 hrs. The data are average of two determinations.

Table 10. Effect of $tRNA^{glu}$ on ALA synthesis *in vitro* using the extract of WC1201[a]

| Expt. | $tRNA^{glu}$ (0.5U/ml) | |
|---|---|---|
| | - | + |
| | [ALA (nM)/ protein (mg)] | |
| I | 8.60 (100%) | 11.00 (128%) |
| II | 9.06 (100%) | 13.95 (154%) |
| III | 7.70 (100%) | 9.40 (122%) |

[a] WC1201 was grown at 37°C in LB + 1% casamino acids and harvested 2 hr after induction with 5mM IPTG.

insert containing *hemA* and *hemM* (Figures 19 and 20). Without addition of *Escherichia coli* $tRNA^{glu}$ in the reaction mixture, the 46 kDa band, which is the *hemA* gene product, was weak (Figure 19) or missing (data not shown). The basis of this stimulation may be transcriptional or translational regulation.

### 3.3.3. Effect of $tRNA^{glu}$ expression on ALA synthesis *in vivo*

In order to study the effect of $tRNA^{glu}$ on ALA synthesis *in vivo*, a strain containing two plasmids, one harboring *hemA* (pWC68) and one harboring $tRNA^{glu}$ (pMO11), generously supplied by Dr. Gregory, was constructed. pMO11 harboring Cam resistance, was transformed into WC1268 which contained a plasmid containing *hemA* and *hemM* and conferring Amp resistance. When the plasmids isolated from the transformant (LPL-1) were digested with *BamH* I, three bands were clearly seen on an agarose gel. The 7.5 and 3.6 kb bands came from pMO11 and the 5.7 kb band came from pWC68 (Figure 33). Thus this strain contains both plasmids. The strain produced 30% more ALA than WC1268, the host strain with *hemA* + *hemM* plasmid alone (Table 12), indicating that $tRNA^{glu}$ can stimulate ALA synthesis *in vivo* presumably by stimulating expression of GTR and raising levels of glutamyl-tRNA. In this regard, it is significiant that LPL-1 accumulates more ALA when LB medium is supplemented with casamino acids, but not with glutamate alone (Lavern Wright, unpublished result). This suggests a growth rate effect.

### 3.4. Effect of GTS on ALA biosynthesis

The *gltX* gene product is GTS. In order to study whether the *gltX* gene is also overexpressed in cells when the *hemA* gene is overexpressed, the activity of GTS was

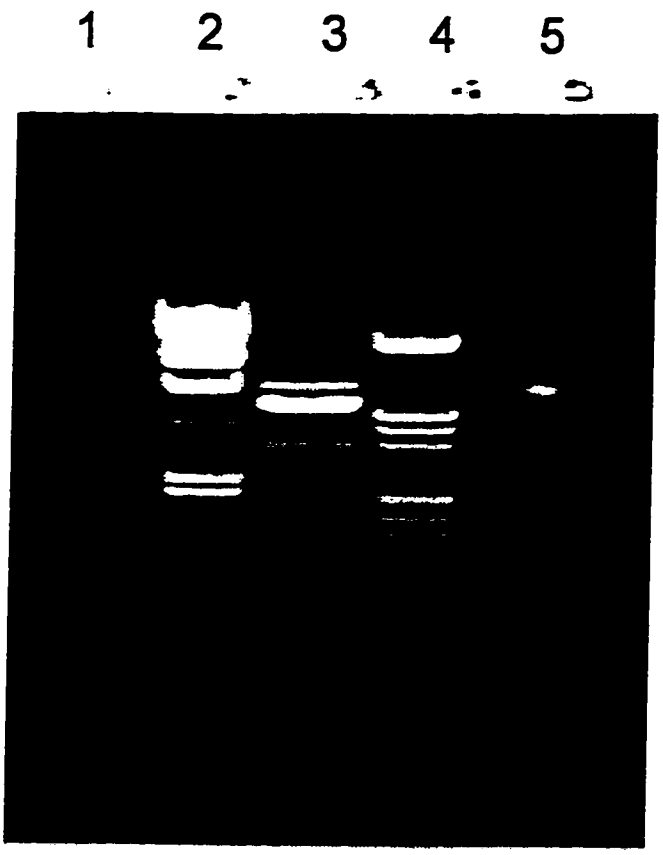



Figure 33. Agarose gel electrophoresis of *BamH* I digestion of plasmids:
Lane 1, pWC68; Lane 2, DNA marker II;
Lane 3, plasmids isolated from LPL-1 (pWC68 and pMO11);
Lane 4, DNA marker III; Lane 5, pMO11.

Table 11. The effect of $tRNA^{glu}$ expression on ALA synthesis *in vivo*[a]

| Expt. | WC1268[b] | LPL-1[c] |
|---|---|---|
| | ( nmol of ALA/mg of protein) | |
| I | 169.2 (100%) | 224.8 (133%) |
| II | 169.7 (100%) | 223.1 (132%) |

a. This assay was done by Lavern Wright, an undergraduate in the laboratory of Dr. Charlotte S. Russell.

b. pWC68 (*hemA* + *hemM*)

c. both pWC68 (*hemA* + *hemM*) and pM011($tRNA^{glu}$)

measured in the wild-type parent strain BL21(DE3)/pET14b, in the same strain with pET14b harboring *hemA* + *hemM* (WC1201) before and after induction, and in a strain containing *gltX* on a plasmid (pTZ7612). The results are shown in (Table 13). It is apparent that expression of *hemA* increased the activity of glutamyl-tRNA synthetase especially after induction for overexpression. DH5α/pTZ7612 has *gltX* on a multi-copy plasmid and would be expected to give the highest value.

Table 12. The effect of *hemA* expression on the activity of glutamyl-tRNA synthetase[a]

| Strain | Glutamyl-tRNA synthetase activity (cpm/mg of protein,hr.) |
|---|---|
| BL21(DE3)/pET14b | 8610 (100%) |
| WC1201 | 14182 (165%) |
| WC1201(induction)[b] | 21802 (253%) |
| DH5α /pTZ7612[c] | 37182 |

a. The data are the average of two determinations.
b. After induction by 1 mM IPTG
c. pTZ7612 has a *gltX* insert

# Discussion and Conclusions

## 1. ALA synthesis in *E. coli* requires expression of *hemA*

Glutamyl-tRNA reductase catalyzes reduction of glutamyl-tRNA to form glutamic acid semialdehyde (Avissar & Beale 1988). This enzyme is encoded by the *hemA* gene in organisms that use the $C_5$ pathway (Li et al., 1989a, Drolet et al., 1989, Verkamp & Chelm 1989). It was found in this laboratory that when a multicopy plasmid, pJL68, containing *hemA* gene, was transformed into a hemin-permeable *hemA E. coli* mutant, HU227, the resulting transformant, JL1268, no longer required ALA or hemin for growth and even excreted ALA into the medium (Li et al., 1989b). Avissar and Beale (1989) found that SASX41B, the parent strain of HU227, which was isolated by Sasarman and coworkers (1968), was GTR deficient. Yeast uses the $C_4$ pathway to make ALA. Therefore, it does not contain GTR activity. However, when *E. coli hemA* was expressed in yeast, the extract of the transformant could convert glutamyl-tRNA to GSA. It was demonstrated by gel filtration experiments that *E. coli hemA* gene products were the same when expressed in *E. coli* or in yeast (Verkamp et al. 1992).

Although Murooka and coworkers (1992) claimed that *hemM*, upstream of *hemA*, and expressed divergently, was the major gene for ALA synthesis, we found that *hemA* expression is sufficient for ALA synthesis. *hemA*, but not *hemM*, could complement ALA-deficient *E. coli* mutants. These results clearly demonstrated that *hemA* is required for ALA synthesis.

The apparent contradiction between our results and those of Murooka and coworkers (1992) may reflect the need for both *hemM* and *hemA* gene products, although the *hemA* gene product appears to be the only protein with GTR activity. The mutation in *hemA* is most likely in the structural gene because if the promoter region did not function, the mutation would be lethal since release factor I depends on the same promoter region (Elliott, 1989), or it may be in an upstream promoter which is inducible and not constitutive. The mutant studied by Murooka and coworkers may be a *hemM* mutant with a normal *hemA* gene. However, our *hemM*-containing plasmid did not complement this strain. It is possible that the mutant studied by Murooka and coworkers has a mutation in the upstream region of *hemA* which is complementary to *hemM*, and their plasmids containing *hemM* may correct the mutation by recombination. Other workers have observed a certain "leakiness" in the *hemM* mutation (personal communication).

The function of the *hemM* gene product is not known. It was shown both in maxicell and *in vitro* transcription and translation experiments that the open reading frame of *hemM* encodes a 23 kDa polypeptide. The accumulation of ALA in these studies indicates that while a normal *hemM* gene and many copies of *hemA* are sufficient for accumulation, the strain with many copies of both genes accumulated more ALA. The reason is not clear. It is possible that the *hemM* gene product is a regulatory protein or a regulatory subunit of GTR reductase. Since *hemA* and *hemM* are 213 bp apart but they are read in the opposite directions, one transcript may influence the expression of the other or the product of one transcript may be a transcriptional regulator of the other. It may be that the

upstream region of *hemA*, which is complementary to *hemM* , is required for maximum expression. Therefore, the regulation could be at the level of transcription or translation.

2. **Expression of glutamyl-tRNA reductase in *E. coli***

It is known that GTR is present in low concentration and is unstable. To overexpress GTR, an expression system using T7 RNA polymerase promoter system offers tight regulation, high specificity and high level of induction.

In order to overexpress *hemA*, the following strategies were used: increasing the amp concentration, replacing amp with carbenicillin, changing the medium regularly to remove β-lactamase, and using a different host, B834($DE_3$), the parent strain of BL21($DE_3$) (Doherty et al. 1995). However, the expression level was still very low. The colonies of WC1201 on LB plates containing amp all fluoresced under UV, which suggested that the selection for plasmid-containing cells is much more stringent on LB plus amp plates in comparison to liquid cultures. We therefore tried first to harvest a large number of plasmid-containing cells on plates and then induced in liquid medium, in such a fashion that plasmid was not lost or the cells killed too soon. Overexpression of *hemA* resulted in high GTR activity, as judged by accumulation of ALA in the culture medium and by enhanced ALA -accumulating activity of sonicates from induced cultures and by visualization on electrophoresis without radioactivity labelling. GTR, whether it functions alone or as part of a complex, is associated mainly with the soluble fraction of the sonicate.

GTR isolated from many different sources has a molecular weight of approximately 45-54 kDa. In our hands overexpression of *hemA* resulted in products which showed native molecular weights of 175 kDa (from the whole mixture from *in vitro* transcription-translation) and 117 kDa (from sonicate supernatants from *in vivo* induction), on native gradient gel electrophoresis. The subunit size, 46 kDa, observed on SDS PAGE, corresponds to the size calculated from the DNA sequence-derived amino acid sequence. Gel filtration of an induced sonicate of WC1201 showed activity in the region of 126 kDa. Thus it appears that the *hemA* gene product, GTR, does not exist as a monomer in its native form in *E. coli*. It may be oligomeric in its native form and/ or exist as a complex with GTS and glu-tRNA. The 175 kDa species observed by us may represent the *E. coli* counterpart of the 200 kDa complex of GTS, GTR and glu-tRNA in *Chlamydomonas* (Jahn 1992) and the 126 kDa species may represent the homo-oligomer counterpart of the 130 kDa GTR in *Chlamydomonas* (Chen et al. 1990) (although this value is reported to be for monomeric enzyme), or a GTR dimer associated with glu-tRNA$^{glu}$, or a complex of one monomer each of GTR, GTS and glu-tRNA. More recently, barley GTR expressed in *E. coli* as a fusion protein with glutathione S-transferase was reported to be a heme protein. After removal of the glutathione S-transferase by thrombin, the protein migrated as an 60 kDa protein on SDS-PAGE and 250 kDa on gel filtration (Vothknecht et al., 1996)

For the gel filtration experiment, the activities in the eluate fractions were measured as enhancement of ALA accumulation by a sonicate of IPTG-induced WC1201. Stimulation by extracts from wild-type strains was much lower for the same fractions. This

suggested that GTS and GSA-AT are overexpressed when GTR is overexpressed and that the gel filtration fractions are oligomers of GTR alone. Our observation of higher GTS activity in induced sonicates lends support to this supposition. It is reported that the specific activity of GTS in a *hemA*⁻ strain was lower than its isogenic wild-type parent (Avissar & Beale. 1989). However, the GTS band did not shift when GTR was added in native gradient PAGE, which may suggest that the GTR does not form a complex with GTS. This is a preliminary result, therefore it needs further investigation.

The structure of GTR has not been as thoroughly studied as ALA synthase of the $C_4$ pathway, since hitherto the amounts of GTR available for study have been very limited. When HU227, a *hemA* mutant was transformed with pWC22, the transformant, WC1270, no longer needed ALA or heme for growth, but did not accumulate ALA. These results suggest that truncated GTR still has some enzymatic activity. It will be of interest to make more conservative truncations at the N- and C- terminal ends of the structure gene to see if that will improve the stability of the protein while preserving its catalytic activity.

**3. Studies on regulation of ALA synthesis**

Philipp-Dormston and Doss (1975) observed that *E. coli* can accumulate all the porphyrin intermediates in the pathway when the growth medium is supplemented with ALA. When a *hemA* mutant is transformed with a multi-copy plasmid harboring *hemA*, it accumulates ALA and porphyrins (Li et al., 1989b; Chen et al., 1994). Woodard and

Dailey (1995) concluded that heme biosynthesis pathway is regulated by ALA levels. They and Rivera and Walker (1995) observed that over-expression of a rat cytochrome in *E. coli* resulted in an increase in cellular heme withoout accumulation of pathway intermediates which suggests control of ALA production by the free heme pool. These observations support the proposition that ALA production is the limiting factor in the pathway. They also suggest that regulation of ALA levels may be mediated by a heme sensor. Since GTS is also involved in protein synthesis and GSA can be converted to ALA non-enzymetically, it is highly probable that control of ALA synthesis and thus of the whole pathway resides in the GTR step. Regulation of GTR activity may be at the level of transcription, translation or enzyme activity, or a combination of these.

**Effect of heme**

Choi et al (1996) observed a small (less than two-fold) effect of ALA- or heme-starvation on the expression of *hemA-lacZ* fusions, in *E. coli* and *S. typhimurium*. Gunsalus and coworkers (McNicholas et al., 1997) found a three-fold increase on heme starvation in a similar system, correcting their previous observations (Darie & Gunsalus (1994) and confirming the observation of Choi et al (1996) and that of our laboratory (Hua, unpublished results). However, Wang et al. (1997) observed a ten- to twenty-five-fold stimulation of GTR activity in heme-deficient mutants of *S. typhimurium* ( *hemB*, *hemE* or *hemH*) or "adapted" *hemL* mutants of *E. coli*, all grown in the absence of heme. The increased activity was due to an abundance of enzyme protein. Under the same conditions, *hemA-lac* operon fusions showed less than two-fold increase in expression. Significant regulation of GTR appears to be at a post-transcriptional step.

It is apparent that the regulation of *hemA* is complex. A hemin-permeable, *hemB* mutant strain, RP523, accumulated ALA because it cannot convert it to PBG, the next intermediate in the pathway. We studied the effects of growth conditions and supplements on this accumulation. In addition, a strain which overexpressed GTR gave cell-free extracts which synthesize ALA. The effect of hemin on this activity, which is separated from transcription and translation , was also studied. Hemin lowered the accumulation of ALA by RP523, as a function of hemin concentration (Figure 31). This suggests that hemin is a regulator of ALA synthesis.

When the structural gene for *hemA*, without its upstream region, was cloned into an overexpression vector and transformed into HU227, which is *hemA* and hemin-permeable, the resultant strain accumulated less ALA when grown on hemin. This effect was concentration-dependent (Colette Knight, unpublished observations). Since the *hemA* upstream region is not present, the effect of heme must be post-transcriptional.

When *hemA* was overexpressed *in vivo* in an overexpression strain, cell-free extracts of the expression mixture had ALA synthesis activity (Chen et al., 1996). This activity dropped as a function of hemin concentration. The effect was stronger with hemin arginate, than with hemin prepared in sodium hydroxide. This latter result may be due to hemin aggregation in the latter prepartion. Thus hemin can inhibit ALA synthesis on the enzymatic level, possibly by feed-back inhibition.

In summary, the work of others, and results from our laboratory, suggest that while hemin has some effects on transcription of *hemA*, there is significant regulation at the level of protein (GTR) synthesis and of GTR enzymatic activity. The observation that GTR may

be a heme protein (Vothknecht et al., 1996) means that heme may be a prosthetic group for GTR, or use the enzyme as a heme sensor protein.

**Effect of glucose**

In *E. coli*, glucose is known to repress the expression of cytochromes and some Krebs cycle enzymes (Kranz et al., 1984; Cotter et al., 1990) through cAMP-mediated regulation (Dickson et al, 1975). When RP523, a hemin-permeable, *hemB* mutant strain, was grown anaerobically on 0.2% glucose and LB medium, ALA accumulation was lower than anaerobic growth on LB medium alone or LB with glycerol and fumerate. The effect was slightly reversed by dibutyl cAMP.

As further evidence of the role of cAMP-CRP, various putative promoter regions in the 5'upstream region of *hemA* were fused to a promoterless *cat* gene. Growth on glucose under aerobic conditions lowered CAT expression for all the constructs which showed promoter activity (Wei Hua, unpublished observations). By using isogenic $CRP^+$ and $CRP^-$ strains it was shown that the effect is mediated by cAMP-CRP.

**Effect of $tRNA^{glu}$**

*E. coli* $tRNA^{glu}$ and not any other tRNA, stimulates ALA synthesis in a coupled *in vitro* cell-free system of a strain which overproduces ALA and porphyrins (Li et al., 1989). This is undoubtedly due to the role tRNA plays as a substrate for glutamyl-tRNA synthetase to produce glutamyl-tRNA, the substrate for GTR. Moreover, *E. coli* $tRNA^{glu}$ stimulates expression of *hemA* in an *in vitro* transcription and translation system. The mechanism for this stimulation is unknown. Regulation of the synthesis and flow of

glutamyl-tRNA into protein synthesis and into ALA synthesis is probably complex and would have to respond to the growth rate and the tetrapyrrole requirements of the cell. The sense strand of the upstream region of *hemA* contains a stem-loop which looks like $tRNA^{glu}$. This suggests that GTR may function as its own translational down-regulator by binding to this region of *hemA* mRNA. This mechanism for regulation is similar to the proposal for glutamyl-tRNA synthetase (GTS) and *gltX* (Brun et al., 1990) and for threonyl-tRNA synthetase and *thrS* (Springer et al., 1986; Brunel et al., 1995). Reciprocal binding of GTS to *hemA* mRNA and of GTR to *gltX* mRNA could serve as a mechanism for coordinate regulation. In addition, $tRNA^{glu}$ stimulation may be due in part to maintaining a high level of glutamyl-tRNA for protein synthesis and for ALA synthesis.

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