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GENE REGULATION INVOLVED IN C5 PATHWAY

FOR SYNTHESIS OF DELTA-AMINOLEVULINIC ACID

by

FANG LIN

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

2002

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ABSTRACT

GENE REGULATION INVOLVED IN C5 PATHWAY

FOR SYNTHESIS OF DELTA-AMINOLEVULINIC ACID

by

FANG LIN

Advisors: Professor Sharon Cosloy and Professor Charlotte Russell

Production of δ-aminolevulinic acid (ALA) is the rate-limiting step in the C5 pathway for tetrapyrrole biosynthesis. In the C5 pathway, glutamate is charged with $tRNA^{Glu}$ by glutamyl-tRNA synthetase (GTS) encoded by *gltX*; glutamyl-tRNA is reduced to glutamyl-1-semialdehyde by glutamyl-tRNA reductase (GTR) encoded by *hemA*; GSA is converted to ALA by an aminotransferase. Regulation of *hemA* most probably controls ALA biosynthesis. There are P1, P2 and putative P3 promoters in the *hemA* upstream region, and two overlapping stem-loops (SL1 and SL2) behind the putative P3. Regulation of *hemA* at the transcriptional level was studied by comparing the chloramphenicol acetyltransferase (CAT) expression of various parts of the *hemA* upstream region inserted in front of the promoterless CAT reporter gene of pKK232-8 — pWH515-CAT (pKK232-8 with the 554 bp of the *hemA* upstream region) and pFL510-CAT (pKK232-8 inserted with the *hemA* upstream region with deletion of

SL1 and SL2). It was found that deletion of the SL1 and SL2 did not change the *hemA* promoter activity. The putative P3 promoter activity was detected by measuring the CAT expression of pFL25-CAT in medium copy plasmids (pKK232-8 inseted with the *hemA* upstream region with deletion of P1 and P2 promoters), which accounts for 1% of the promoter activity of the whole 5' upstream region of *hemA*. The CAT expression did not change when a plasmid harboring *hemA* or *gltX*, coexisted in a strain with pWH515-CAT, pFL510-CAT or pGLTX-CAT respectively. Over-expression of GTS or GTR did not change the promoter activity of *hemA* or *gltX*. HemX in *Bacillus subtilis* is a 32-kDa membrane protein. Expression of *B. subtilis hemX* down-regulates its GTR. *E. coli* does not have *hemX*. In order to understand HemX function, *hemX* was subcloned into modified pUC19 and transformed into SASX41B, an *E. coli hemA*$^-$ heme-impermeable strain. The transformants did not grow in the medium supplemented with hemin. It suggests that HemX is not a heme channel and does not make *E. coli* permeable to heme.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

ALA: δ-aminolevulinic acid

Amp: ampicillin; tet, tetracycline; Cm, chloramphenicol

CAT: chloramphenicol acetyltransferase

GSA-AT: glutamate-1-semialdehyde-2,1-aminotransferase

GTR: glutamyl-tRNA reductase

GTS: glutamyl-tRNA synthetase

MCS: mutiple cloning sites

PCR: Polymerase chain reaction

RBS: ribosome binding site

SL1 and SL2: over-lapping stem-loops

INTRODUCTION

The biological tetrapyrroles include oxidized tetrapyrroles such as heme and chlorophylls, and reduced tetrapyrroles such as siroheme and vitamin B12. Hemes are prosthetic groups for respiratory cytochromes and some catalases, which play an important role in cellular respiration and remove toxic substances such as H_2O_2. Siroheme is the cofactor of sulfite reductase and nitrite reductase, which function in conversion of highly oxidized forms of nitrogen and sulfur in the environment into reduced forms (for review, see Beale, 1996).

1.1. Biosynthetic pathways of the tetrapyrroles

The biosynthetic pathways for tetrapyrrole synthesis have been elucidated (for reviews, see Beale 1996; Porra 1997). The biosynthesis of tetrapyrroles from a first common intermediate precursor, δ-aminolevulinic acid (ALA), is highly conserved in all organisms (Beale, 1996). There are two different pathways to synthesize ALA, named the C4 and C5 pathways (Beale 1996, Porra 1997). Animals, fungi and proteobacteria of the α-subdivision use the C4 pathway to synthesize tetrapyrroles. In the C4 pathway, ALA is synthesized from succinyl-CoA and glycine in a reaction that is catalyzed by ALA synthase. The C5 pathway (Figure 1) is used to synthesize ALA by plants, most bacteria, including *Escherichia coli, Bacillus subtilis*, and archaea (Li *et al.*, 1988; 1989a, b; O' Neill, 1990; Jahn, *et al.*, 1992a).

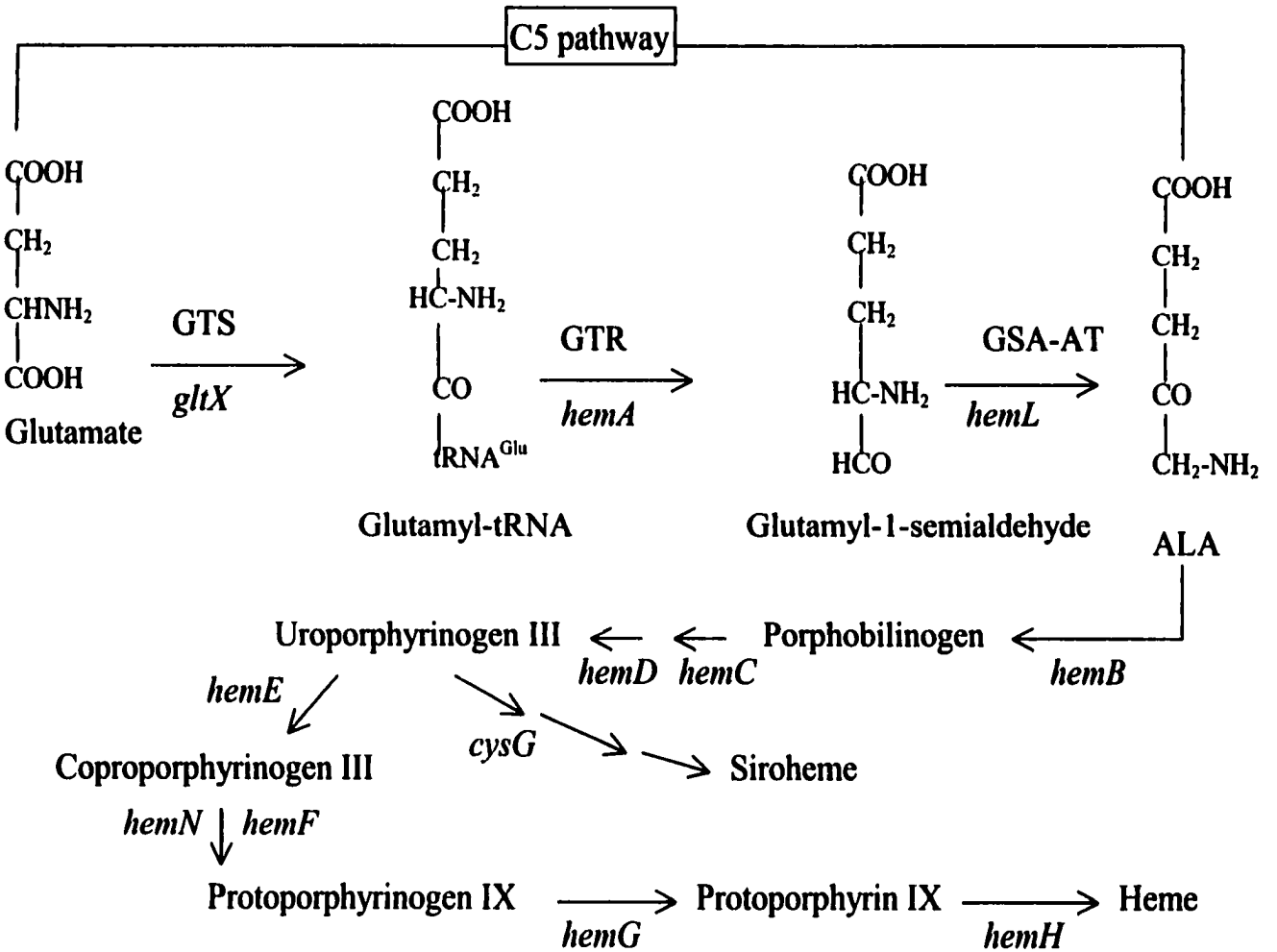


Figure 1: Tetrapyrrole biosynthesis by the C5 pathway in *E. coli*.

In the C5 pathway, there are three steps in ALA production (Figure 1) (Beale, 1996). First, glutamate is charged with $tRNA^{Glu}$ by glutamyl-tRNA synthetase (GTS) encoded by *gltX*. Second, glutamyl-tRNA is reduced to glutamyl-1-semialdehyde (GSA) by glutamyl-tRNA reductase (GTR), encoded by *hemA*. Then, GSA is converted into ALA by aminotransferase (GSA-AT), encoded by *hemL*. In *E. coli*, the genes and their encoded enzymes for tetrapyrrole biosynthesis have been studied. The genes for tetrapyrrole biosynthesis have also been localized on the *E. coli* linkage map (Beale, 1996). The genes for tetrapyrrole biosynthesis are scattered in the *E. coli* linkage map, except *hemC* and *hemD*, which are linked together as shown in Figure 2. After production of ALA, two ALA molecules are asymmetrically condensed into the first pyrrole, porphobilinogen (PBG), by PBG synthase encoded by the *hemB* gene (Beale, 1996). PBG is converted into uroporphyrinogen III, catalyzed by hydroxymethylbilane synthase encoded by *hemC*, and uroporphyrinogen III synthase encoded by *hemD* sequentially (Beale, 1996). From uroporphyrinogen III, reduced tetrapyrroles and oxidized terapyrroles are synthesized by two different sequential enzymatic reactions. In order to synthesize oxidized tetrapyrroles such as heme, uroporphyrinogen III is converted into protoporphyrin IX, catalyzed by three enzymes sequentially: uroporphyrinogen decarboxylase encoded by *hemE*, coproporphyringen oxidase encoded by *hemF* under aerobic conditions or *hemN* under anaerobic conditions, and protoporphyrinogen oxidase encoded by *hemG* (Beale, 1996). From protoporphyrin IX, different oxidized tetrapyrroles are synthesized. Protoheme production from protoporphyrin IX is catalyzed by ferrochelase encoded by *hemH* (Beale, 1996). Other modified hemes are formed from

protoheme. In order to synthesize reduced tetrapyrroles such as siroheme, uroporphyrinogen III is converted into precorrin 2, catalyzed by uroporphyrinogen methyltransferase, encoded by *cysG* (Beale, 1996). Siroheme is further synthesized from precorrin, catalyzed by 1,3-dimethyluroporphyrinogen III dehydrogenase and siroheme ferrochelase (Beale, 1996).

Salmonella typhimurim and *E. coli* are both Gram-negative bacteria and belong to facultative enteric bacteria. *S. typhimurim* shares a very similar terapyrrole biosynthesis pathway with *E. coli* except that *S. typhimurim* produces Vitamin B12, *E. coli* does not (Beale, 1996). Like *E. coli,* all genes for the tetrapyrrole biosynthesis in *S. typhimurim* are scattered on the genetic map (Beale, 1996). The *S. typhimurim hemA* gene is very homologous with the *E. coli hemA* gene. There is 89% amino acid sequence identity between *S. typhimurim* GTR and *E. coli* GTR (NCBI BLAST: www.ncbi.nlm.nih.gov/BLAST/). It suggests their regulation for tetrapyrrole biosynthesis is very similar.

Unlike *E. coli*, in *B. subtilis*, a Gram-positive aerobic bacterium, the genes for tetrapyrrole synthesis are clustered into two groups: the *hemAXCDBL* operon and the *hemEHY* operon at different locations on the genetic map (Petricek *et al.*, 1990; Hansson *et al.*, 1991; and Hansson & Hederstedt, 1992). The *hemAXCDBL* operon encodes all enzymes required for the synthesis of uroporphyrinogen III from glutamyl-tRNA. The *B. subtilis hemA* and *hemL* encode GTR and GSA-AT respectively, which catalyze the production of ALA from glutamyl-tRNA (Hansson *et al.*, 1991; Schröder *et al.*, 1992). The *B. subtilis hemB*, *hemC* and *hemD* encode porphobilinogen synthase, hydroxymethylbilane synthase and uroporphyrinogen III

synthase respectively to synthesize uroporphyrinogen III from ALA. The function of *hemX* in the *hemAXCDBL* operon is not clear. The *hemEHY* operon encodes the enzymes for late steps of heme biosynthesis (Hansson & Hederstedt, 1992). The two clusters of genes for the tetrapyrrole biosynthesis may suggest a more coordinate regulation in *B. subtilis* than in *E. coli.*

The production of ALA is carefully controlled in heme biosynthesis. When there is over-expression of a heme protein such as a rat cytochrome in *E. coli*, there is an increase of heme production without accumulation of the intermediate products (Woodard *et al.*, 1995). In contrast, when there are large amounts of ALA in the medium, or plasmids with ALA mouse synthase (Woodard *et al.*, 1995), or *E. coli hemA* are over-expressed in *E. coli*, there is porphyrin accumulation (Chen *et al.*, 1994; Li, J-M. *et al.*, 1989a). Therefore, ALA synthesis appears to be the rate-limiting step in tetrapyrrole biosynthesis.

In the C5 pathway of ALA production (Figure 1), the first step and the third step are probably not key regulatory steps (O'Neill and Söll, 1990; Beale, 1996). The enzyme GTS in the first catalytic step is also involved in protein synthesis. In the third step, the reaction can occur nonenzymatically. As a result, the enzyme GTR, encoded by *hemA*, in the second step of ALA production, is believed to be the important regulatory point in *E. coli* heme biosynthesis.

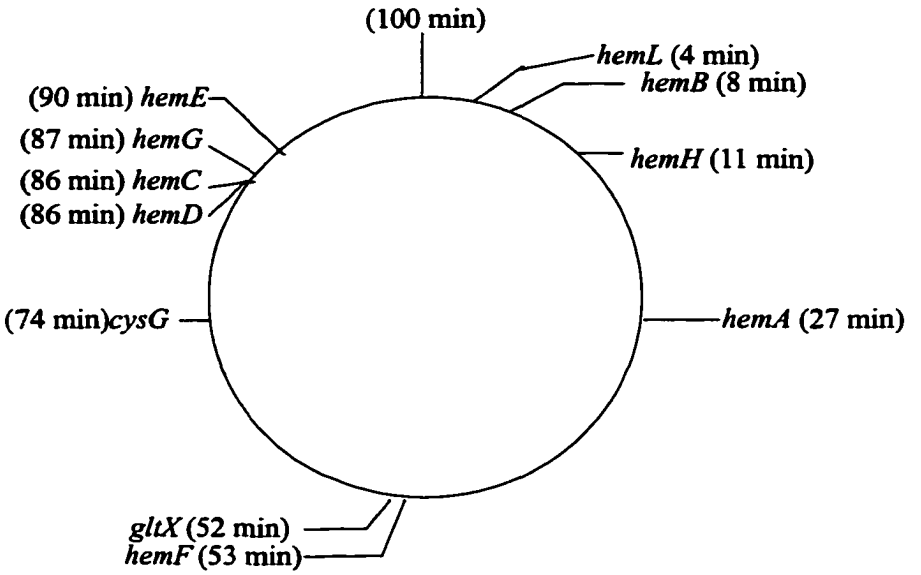


Figure 2: Genetic map of the genes for the tetrapyrrole biosynthesis in *E. coli* The map is calibrated in minutes (Ref. Beale, 1996).

1.2. Regulation of the *hemA* gene expression in *E. coli*

The *E. coli hemA* gene was isolated and sequenced by Li *et al.* (1989b) and Verkamp *et al.* (1989) respectively. *hemA* is located at 26.7 min of the *E. coli* linkage map. Upstream of *hemA* is *hemM*, encoding a 23-kDa protein. *hemA* and *hemM* are transcribed in divergent directions. It was believed that co-expression of *hemM* and *hemA* can cause more ALA production than expression of *hemA* alone (Chen *et al.*, 1994.). However, HemM has been identified as a novel outer membrane lipoprotein, LolB (Matsuyama *et al.*, 1997). LolB plays an important role in the outer-membrane localization of lipoproteins in *E. coli*. Thus it appears that the 5'upstream region of *hemA* which extends into *hemM* is important for *hemA* maximal expression. Downstream of the *hemA* gene is *prfA*, encoding the polypeptide releasing factor-1 (RF-1). Genetic experiments showed that *prf*A and *hemA* share the same promoters as a part of the same operon (Elliot *et al.*, 1992).

1.2.1. Structure of GTR

hemA encodes GTR, a 45-kDa protein monomer. GTR has been isolated from *E. coli* by gel filtration chromatography (Jahn *et al.*, 1991). GTR activity was tested in the presence of NADPH by converting glutamyl-tRNA^{Glu} to glutamate 1-semialdehyde. GTR recognizes the tRNA^{Glu} in glutamyl-tRNA^{Glu} in a sequence specific manner. Li *et al.* (1989b) and Verkamp *et al.* (1992) demonstrated that the *hemA* gene encodes GTR in *E. coli*. By over-expression of *E. coli hemA* gene in yeast, Verkamp *et al* activity (1992) found that the *E. coli hemA* gene encoded 45-kDa protein with GTR activity. That over-expression of the wild type *hemA* gene can

complement a *hemA* mutant strain also suggested that the *hemA* gene encoded GTR (Li *et al.*, 1989b). Partial-sequencing of GTR from barley chloroplast confirms that *hemA* encodes GTR (Pontoppidan *et al.*, 1994). In *Chlamydomonas reinhardtii*, GTS, GTR, $tRNA^{Glu}$, glutamate and ATP form a complex (Jahn, 1992b). The GTR protein in *E. coli* may exist as an oligomer or a complex with GTS and $tRNA^{Glu}$ (Chen *et al.*, 1996). In *E. coli*, it was found that the 45kDa-GTR existed in both 117kDa- and 175kDa-forms (Chen *et al.*, 1996). In *Methanopyrus kandleri*, a tetrameric GTR was purified (Moser J. *et al.*, 1999). GTR was detected as a pentamer in barley (Vothknecht *et al.*, 1996). In barley, heme was precipitated with GTR by immunoprecipitation, and heme also inhibited the enzymatic activity of GTR (Vothknecht *et al.*, 1996). The barley GTR sequence was aligned for homology with 24 sequences of GTR including the *E.coli* GTR. The protein structure of GTR was predicted based on sequence homologies (Brody *et al.*, 1999). It was predicted that a coiled coil structure from E219 to E232 of GTR may be involved in subunit interaction, or protein protein interaction — such as the interaction with GTS. A possible heme-binding site was also predicted in H99, which is highly conserved in plants, bacteria and archaea.

The crystal structure of GTR from the archaeon *Methanopyrus kandleri* revealed the GTR structure in detail (Moser *et al.*, 2001). Dr. Moser *et al.* (2001) found that GTR formed a V-sharp dimer at C-terminus. The crystal structure confirmed the basic domains in GTR predicted by Brody *et el.* (1999), who suggest that a catalystic domain at N-terminus, and a NADPH-binding domain next to that. Dr. Moser *et al.* (2001) found a dimeriazation domain at C-terminus, in which GTR

forms a V-shape dimer; and gave more precise and detailed structures of GTR (Figure 3). Since heme down-regulates GTR, it is interesting to explore whether heme directly or indirectly changes enzymatic activity of GTR. To understand the regulation of GTR, it is also interesting to study the subunit interactions of GTR and the possible interaction between GTR and GTS.

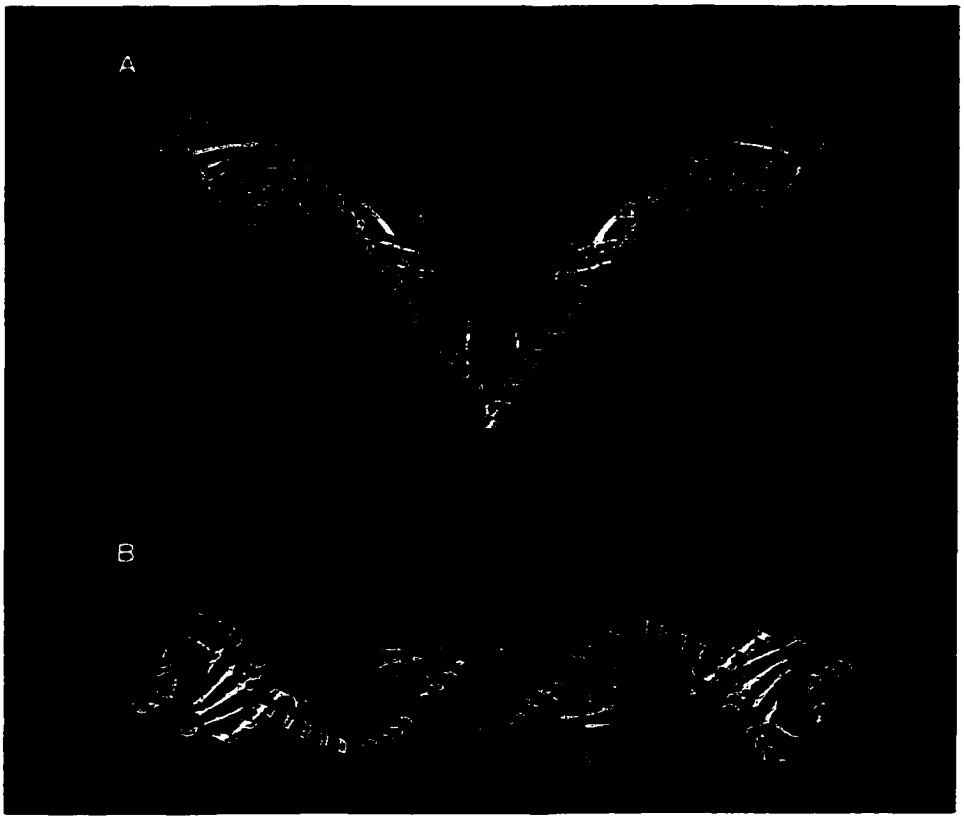


Figure 3: Crystal structure of GTR from archaeon *Methanopyrus kandleri* (Moser *et al.*, 2001).

A: perpendicular to the 2-fold axis; B: perpendicular along the 2-fold axis. The GTR monomer has N-terminal catalytic domain, a NAPDH-binding domain and a C-terminal dimerization domain by which, the GTR forms a V-shaped dimmer.

1.2.2. Transcription of *E. coli hemA*

By using S1 nuclease protection, two mRNA transcripts from *hemA* in *E. coli*, which did not overlap, were detected (Verkamp *et al.*, 1989). Sequence analysis of the 5'upstream region of *hemA* shows the promoter regions (Figure 4 and Figure 8) (Li *et al.* 1989; Verkamp *et al.* 1989). Verkamp *et al.* suggested that there are two transcription initiation sites on the *hemA* gene (Figure 4 and Figure 8). In the P1 promoter, the -10 region sequence is TATGAT which has one base mismatch compared with the consensus sequence of TATAAT (Verkamp *et al.*, 1989; Lisser *et al.*, 1993) (Figure 4). The possible –35 region of the P1 promoter is TTGATC with two mismatches compared with the consensus sequence of TTGACA (Verkamp *et al.*, 1989; Lisser *et al.*, 1993) (Figure 4). The space between the –10 region and possible –35 region of the P1 promoter is 12 base pairs, which is not favorable to the RNA polymerase complex binding (Dombroski *et al.*, 1996). The –10 region of the promoter P2 is TATCGT with two mismatches compared with the consensus sequence of TATAAT (Verkamp *et al.*, 1989; Lisser *et al.*, 1993) (Figure 4). The possible –35 region of the P2 promoter is TTAATG with three mismatches of the consensus sequence TTGACA (Verkamp *et al.*, 1989; Lisser *et al.*, 1993) (Figure 4). The space between the –10 region and possible –35 region is 17 base pairs. The *hemA* P1 promoter is required for *hemA* expression (Hua, 1997; Choi *et al.*, 1996. and Verderber *et al.*, 1997). Figure 4 shows the position and sequence of the 5' upstream region of *hemA*. By using *hemA-lacZ* and *hemA-CAT* fusions respectively, Choi *et al.* (1996) and Hua (1997) found that in the P1 promoter, point mutations and a deletion mutation could abolish *hemA* expression.

-561 ATC GGC GGT ATA ACG CTG ACC TTT ATT GTC GAC TAA CTG CAC GTT ACC CGG

-510 TTG AGC ATT CAG CTC CAG TTC CGT GCT GCC CAA TGG GTT AGT GAG CAG CAG

-459 ACG GTA GCG ATC CTG GCC GGT TTG CTG CCA GAA AAA GCG GGC GTA CAC TTT

P24[a]

-408 TTG TTG GTC AGA AAT ATA AGC GAA CGC GCC GCG AGT CTG ATA CTG ATT AAG

P24[a] P3[b]

-357 ATT GCG CAC GTC TTG CTG ATG CTG ACG CCA TTG TGG CGA (ATC CGG GCT TTT

P32[c] P3[b] T(-337)[d] P32[c] SL1[5]

-306 G{CC AGG A) CC TTT GGG CGT GG}T AACGGAACAGGCAGTGAGCACAAGAGCAGC

SL2[5]

-255 CAG CGG TAG CAG GCG GAT AAG ACG AAA ATC GGG CAG GGG CAT AGT GAT GAC

-204 AAG TCC TTG AGA TAC GTT GCA GTT ATA ACC CTT AAT GCT AGC GTT ACC GTC

P2[2] TTGACA

-35 consensus sequence

-153 CGC TAT CGT CTA TGT TCA AGT TGT CTT AAT TGC CAG AAT CTA ACG GCT TTC

P2[2] TATAAT IT2[3]

-10 consensus sequence

-102 GGC AAT TAC TCC AAA AGG GGG CGC TCT CTT TTA TTG ATC TTA CGC ATC CTG

C(-78)[e] P1[2] TTGACA

-35 consensus sequence

-51 TAT GAT GCA AGC AGA CTA ACC CTA TCA ACG TTG GTA TTA TTT CCC GCA GAC

P1[2] TATAAT IT1[3] RBS[4]

-10 consensus sequence

+1 ATG ACC CTT TTA GCA CTC GGT ATC AAC CAT AAA ACG GCA CCT GTA TCG CTG

+1[1] (Start cordon) (Continue)

Figure 4: The 5' upstream region of *hemA*[1] (Ref. Li *et al.*, 1989b; Verkamp *et al.*, 1989; Hua, 1997) (Continued)

1. The sequence is numbered with the first nucleotide of the *hemA* coding position as +1.
2. P1, P2 (σ^{70}-dependent promoters) and the consensus sequences of P1 and P2 are indicated.
3. IT1 and IT2: transcription initiation sites (Verkamp *et al*, 1989).
4. RBS: ribosome binding site.
5. SL1 and SL2: overlapping stem-loops.

The putative P3 promoter includes a, b and c:

a. P24-the putative σ^{24}-dependent promoter is proposed in this work.

b. P3-the putative σ^{70}-dependent promoter (Hua , 1997, page10).

c. P32- the putative σ^{32}-dependent promoter (Hua , 1997, page10).

d. Fragment FL509 had a mutation on the putative P3 promoter (G→T at –337).

e. Another mutation on FL509 created a stem (T→C at –78).

Verderber *et al.* (1997) compared the strength of the P1 and P2 promoters. They constructed operon fusions: *hemA1A2-lacZ* (*hemA*P1&P2-*LacZ*), *hemA1-lacZ* (*hemA*P1-*lacZ*), and *hemA2-lacZ* (*hemA*P2-*lacZ*). In the *hemA1hemA2* operon fusion, the *hemA* upstream region including the P1 and P2 promoters were inserted in front of the *lacZ* reporter gene. In the *hemA1-lacZ* operon fusion, the 5' fragment upstream from the P1 promoter of *hemA* was deleted. In this fusion, there was only the P1 promoter of *hemA*. In the *hemA2-lacZ* operon fusion, there was a deletion of the P1 promoter. So, in this fusion, there was only the P2 promoter of *hemA*. About 81 codons of the *hemA* structural genes were included in the three constructs. The promoter strength of P1, P2 and both P1 and P2 were analyzed by measuring the amount of *lacZ* reporter gene expression. They found that the P1 promoter of *hemA* is stronger than the P2 promoter. The result is contradictory to Hua's results (Table 1; Figure 5; Hua, 1997), which indicated that both P1 and P2 promoters are required for *hemA* expression. In Hua's construct, the reporter gene used its own Shine-Dalgarno region, but Verderber *et al.* made the constructs with *hemA*'s Shine-Dalgarno region and a piece of the *hemA* structural gene. So, whether the difference is caused by different constructs has to be investigated.

(a) A putative P3 promoter. Evidence exists that transcripts from the putative P3 were produced (Choi *et al.* 1996). By primer extension study of the *hemA* transcripts, Choi *et al* (1996) found a third transcript during ALA starvation. They explained it as an artifact.

According to Hua's work (Hua, 1997), a third promoter P3 may exist. She found that the sequence from –378 bp to –258 bp may play a role in *hemA* expression.

The expression of the *hemA-CAT* fusion including the 5' upstream region of *hemA* from –258 to –20 (+1 the first base of the encoding region) is 76%, and the expression of *hemA-CAT* fusion including the 5'upstream region of *hemA* from –378 to –20 is 94%, compared to 100% expression of *hemA-CAT* fusion including the 5' upstream region of *hemA* from –551-bp to –20 (Figure 5 and Table 1). These results suggest there is a weak promoter or enhancer between –378 bp and –258 bp of the *hemA* upstream region; and a putative promoter P3 was proposed (Figure 4).

Table 1: Promoter strengths of various fragments of the 5' upstream region of *hemA*[1]

Plasmid expression	Size and Sequence	Percentage of CAT
pWH515-CAT[1]	515 bp (-534/-20)[2]	100%
pWH359-CAT[1]	359 bp (-378/-20)[2]	94 %
pWH239-CAT[1]	239 bp (-258/-20)[2]	76 %
pWH101-CAT[1]	101 bp (-120/-20)[2]	5 %
pWH415-CAT[1]	415 bp (-534/-119)[2]	10 %

1. This work was done by Hua; and the data is from Hua (1997, page 60).
2. The sequence is numbered with the first nucleotide of the *E. coli hemA* coding position as +1.

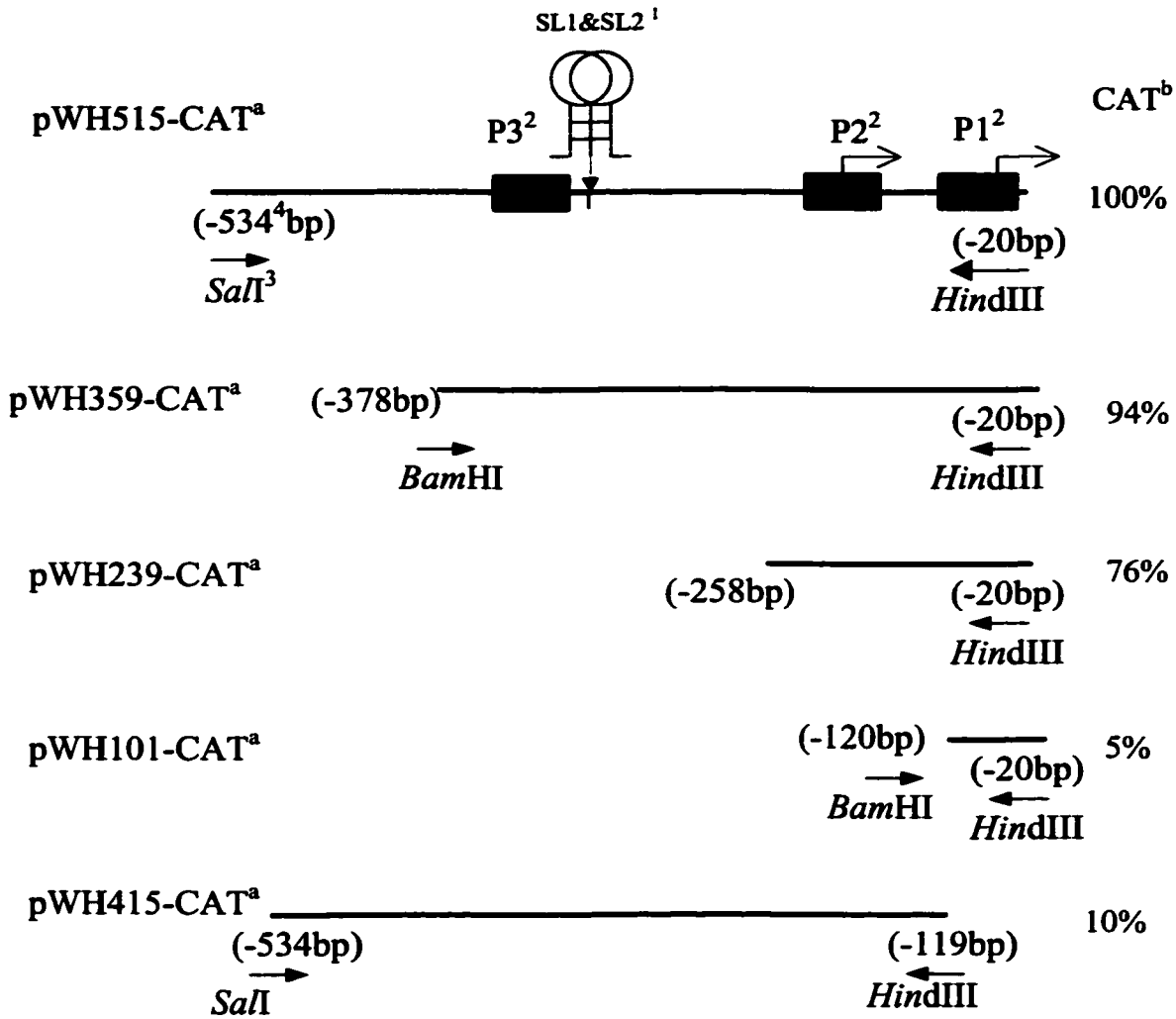


Figure 5: Various fragments of the 5' upstream region of *hemA* constructed by Hua (1997)

a. The work was done by Hua (1997, thesis page 60).

b. CAT expression (see Table 1).

1. SL1&SL2: two stem-loops. 2. Promoters—P1, P2 and putative P3.

3. The cutting sites created on the primers.

4. The first nucleotide of the *hemA* coding region is +1.

(b) The function of the two stem-loops: SL1 and SL2 in the 5' upstream region of *hemA*. There are two stem-loops immediately downstream of the putative P3 promoter called SL1 and SL2 (Figure 4 and Figure 5). The two stem-loops SL1 and SL2 are 32-bp long, located between –318 to –286 of *hemA* (Figure 4 and Figure 6). The stems have rich G-C regions. Each stem has 5 GC pairs. The loops are formed by TTTT in SL1 and TTT in SL2. The transcripts of part of the loops are similar to $tRNA^{Glu}$ in their anticodon region, which is UUC (Brun *et al.* 1990). GTS and GTR can recognize $tRNA^{Glu}$ in a sequentially specific manner (Nureki *et al.*, 1995), and they may form a complex with $tRNA^{Glu}$ (Stange-Thomann *et al.*, 1994). GTS and GTR in barley recognize different identity elements of $tRNA^{Glu}$ (Stange-Thomann *et al.*, 1994, Willows *et al.*, 1995). The bases G10, A26, U34, U35 and A37 of $tRNA^{Glu}$ are suggested as recognition elements of barley GTS. The stem bases A7-U66, U29-A41, A53-U61, and U72 may be required by barley GTR. In *Euglena gracilis* chloroplast, a point mutation in C56 on the L loop of $tRNA^{Glu}$ showed that it is an important identity element for GTR (John *et al.*, 1992; Stange-Thomman *et al.*, 1994). In *E. coli*, the major identity elements of GTS for $tRNA^{Glu}$ are located in its modified uridine 34 of the anticodon (mnm^5s^2U, 5-methylaminomethyl-2-thiouridine), acceptor and D stems with adjacent base and the variable loop (Sekine *et al.*, 1996). GTS binds to the minor groove of the D-stem helix of $tRNA^{Glu}$. So, GTS and GTR may recognize different areas of $tRNA^{Glu}$.

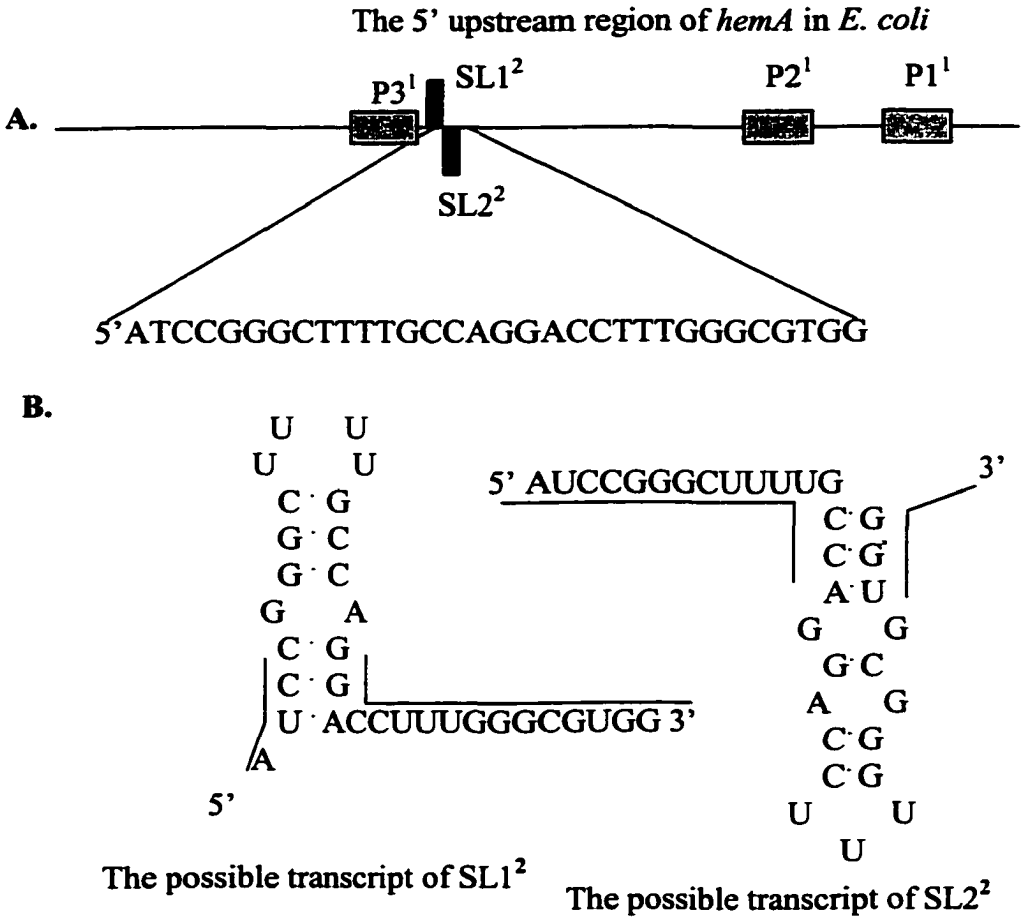


Figure 6: The possible transcript of SL1 and SL2

A: The sequence of SL1 and SL2 in the 5' upstream region of *E. coli hemA*.

B: The possible transcript of SL1 and SL2.

1. The P 1, P2 and putative P3 promoter of *hemA*.

2. SL1 and SL2: two overlapped stem-loop downstream of the putative P3 promoter of *hemA*.

Therefore, the possible involvment of SLI and SL2 in GTS and GTR recognition or some other function in *hemA* gene expression should be investigated. The two stem-loops may be involved in transcriptional or post-transcriptional regulation of *hemA* gene expression such as influencing RNA polymerase or mRNA stability.

1.2.3. Post-transcriptional regulation of GTR levels

Recent evidence suggests that major regulation of GTR is at the posttranscriptional level (Wang *et al.*, 1997). The enzymatic activity of GTR increased 10- to 25-fold during heme limitation in *S. typhimurium* and *E. coli* in a mutant strain that is unable to synthesize heme (Wang *et al.* 1997). By Western blot, it was found that the amount of the GTR protein increased dramatically. In contrast, expression from a *hemA-lacZ* operon fusion only increased less than 2 fold under the same conditions. The sequence of *hemA* in the *hemA-lacZ* operon fusion was from –693 to codon 181. The results suggested that the regulation of *hemA* could not be explained only at the transcriptional level; posttranscriptional, translational or post-translational controls are involved. Choi *et al.* (1996) and Nakayashiki *et al.* (1997) studied the effect of starvation for ALA or heme on the synthesis of porphyrins in *E. coli.* They made a *hemA-lacZ* fusion, in which the sequence of *hemA* was from –1418 to codon 83 and from –128 to codon 83 (Choi *et al.*, 1996. Nakayashiki *et al.*, 1997). They found that *lacZ* activity increased about 4-fold during ALA or heme starvation, but porphyrin accumulation increased about 10-fold (Nakayashiki *et al.*, 1997). Nakayashiki *et al.* further made a *lac-hemA* fusion by replacing the *hemA* promoter with the *lac*-promoter and studied the gene expression during heme starvation. They

found that porphyrins still accumulated significantly. Therefore, accumulation of porphyrins during heme starvation may not be a solely transcriptional effect. The results also suggested the regulation of *hemA* during starvation is at a posttranscriptional level or at the protein level. It was found that GTR stability increased during heme starvation in *S. typhimurium* (Wang *et al.*, 1997). By using pulse-chase and immunoprecipitation analysis of GTR and GTR-*lacZ*$_{1\text{-}416}$ (GTR- *lacZ* fusion protein) turnover, it was found that GTR stability increased.

Based on these experimental data, it is interesting to explore the role of the putative P3 promoter, two stem-loops, and the entire 5' upstream region of *hemA* on the expression of *hemA*. To this end, the effect of removing the stem-loops on the promoter activity of the 5' upstream region of *hemA* gene expression was investigated in this work, as well as a role for the putative P3 promoter.

1.3. Study of the regulation of *gltX* gene expression in *E. coli*

The *E. coli gltX* gene is located at 52 min of the *E. coli* linkage map (Beale 1996). The *E. coli gltX* encodes GTS, a 54-kDa protein as a monomer (Breton *et al.*, 1986; and Sanfacon *et al.*, 1983). In *E. coli,* upstream of *gltX* is the *valU* operon, whose transcripts are three $tRNA^{Val/UAC}$ and one $tRNA^{Lys/UUU}$. The structure of *gltX* was studied (Brun *et al.*, 1990). The *gltX* mRNA is monocistronic. There are three σ^{70}-dependent promoters: P1, P2 and P3 in the 5' upstream region of *gltX* (Brun *et al.*, 1990). The promoter region of *gltX* and *valU* operon are closely spaced and non-overlapping divergent (Brun *et al.*, 1990). The three primary transcripts of *gltX* from the P1, P2 and P3 promoters were detected by S1 nuclease mapping (Figure 7). The

P3 promoter is the strongest promoter, identified by a *gltX*$_{P3}$-*lacZ* protein fusion in which the P3 promoter region was inserted into a promoterless *lacZ* reporter gene (Brun *et al.*, 1990; Champagne *et al.*, 1998). Two other main transcripts that are different from the primary transcripts from the P1, P2 and P3 promoters were found by both S1 nuclease protection and primer extension. A stable stem-loop was identified behind the two main transcripts of *gltX*, but in front of the ribosome-binding site (Figure 7). The transcript of the stem-loop is a $tRNA^{Glu}$ lookalike. Two possible RNaseE cutting sites were found in front of the stem-loop. The possible RNaseE cutting sites corresponds to the two main transcripts. It was believed that primary transcripts from the P1, P2 and P3 promoters were processed by RNaseE digestion to generate the two major transcripts, which are more stable than primary transcripts. The $tRNA^{Glu}$ like- stem-loop at the 5' end of the transcripts was believed to make *gltX* mRNA more stable. The stem-loop may be involved in translational regulation of *gltX*. Since the transcripts of the stem-loop is a $tRNA^{Glu}$ lookalike, GTS or GTR may interact with *gltX* transcripts at the 5' end to regulate its expression.

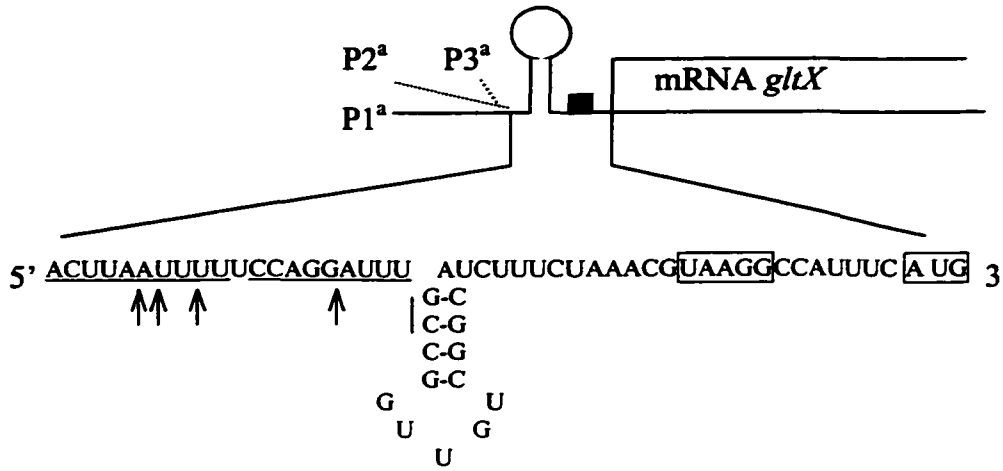


Figure 7: Illustration of the 5' end of *gltX* mRNA in *E. coli* (Brun *et al.*, 1990). Three primary transcripts are from the P1, P2 and P3 promoters. There is a $tRNA^{Glu}$ like- stem-loop at the 5' end near the ribosome binding site. In the front of stem-loop, there are two RNase E recognition sites.

a. primary transcripts from the P1, P2 and P3 promoters of *gltX*.

The two main transcripts which are different from the primary transcripts from the P1, P2 and P3 promoter are indicated by arrows. The two RNase E recognition sites are underlined. Ribosome binding site (RBS) and start codon are boxed.

Legends: Stem loop; ■ Ribosome binding site

GTS participates in both protein synthesis and porphyrin synthesis (for review, see Freist *et al.*, 1997). The regulation mechanism is not clear. It has been suggested that expression of *gltX* was regulated by metabolism (Brun *et al.*, 1990). S1 nuclease mapping of *gltX* mRNA revealed that the amount of *gltX* mRNA present in rich medium is more than that in minimal medium. There are three FIS (factor for inversion stimulation) binding sites in the *gltX* upstream region (Champagne and Lapointe 1998). FIS plays an important role in positive regulation of stable RNA operons, like *rrnB-P1* (Rose *et al.*, 1990; Bokal, *et al.* 1997), *thrU*, and *tyrT* (Nilsson *et al.*, 1990, 1992; Lazarus and Travers, 1993) and *metY* operons (Verbeek *et al.*, 1990). FIS was also found to down-regulate the *hupB* gene and other genes (Claret and Rouviere-Yaniv, 1996; Xu and Johnson 1995). Champagne *et al.* (1998) found that FIS can down-regulate *gltX* transcription during growth acceleration.

It was found that over-expression of *hemA* in a protease-deficient strain caused a 165% increase of GTS enzymatic activity (Chen *et al.*, 1996). But it is not clear how or whether GTS is regulated in response to increase of GTR. It is also unclear if and how GTS is self-regulated. I subcloned the 5' upstream region of *gltX*, which included three promoters and three FIS binding sites, behind a promoterless CAT gene on pKK232-8 vector to investigate it. To determine how GTR influences *gltX* transcription, I cotransformed an *E. coli* strain with a plasmid harboring *hemA* and a plasmid containg the *gltX-CAT* operon fusion (5' upstream region of *gltX* inserted in front of promoterless CAT of pKK232-8 vector). I also cotransformed an *E. coli* strain with a plasmid harboring *gltX* and a plasmid containing the *gltX-CAT* operon fusion (5' upstream region of *gltX* inserted in front of promoterless CAT of

pKK232-8 vector) to determine if *gltX* expression is regulated at the transcriptional level.

1.4. Functional study of HemX

In *B. subtilis*, *hemX* is in the *hemAXDBL* operon. It has been cloned and sequenced (Petricek *et al.*, 1990). A σ^A –type promoter positioned at the -170 bp of the 5' upstream of *hemA* may be the only, or major promoter in the *hemAXDBL* operon. There are only 7 bp between *hemA* and *hemX*. Over-expression of *hemA* causes porphyrin accumulation. Expression of *hemX* down-regulates *B. subtilis'* GTR in both *B. subtilis* and *E. coli* hosts (Schröder *et al.*, 1994). The mechanism is unknown. Schröder *et al.* made both *hemA-lacZ* operon and protein fusions containing the first 34 and 300 codons respectively of *B. subtilis hemA*. The enzyme activity and antigen amount of β–galactosidase were measured in presence of HemX or absence of HemX. It was found that HemX did not directly influence the transcription or translation initiation of *B. subtilis hemA* in the presence of HemX in *E. coli* host. It was also found that HemX did not influence *B. subtilis* GTR stability in *E. coli* by determining the turnover of *B. subtilis* GTR (Schröder *et al.*, 1994).

There is 32% amino acid sequence identity between *E. coli* GTR and *B. subtilis* GTR. There is 54% amino acid sequence identity between *E. coli* HemL and *B. subtilis* HemL (NCBI BLAST: www.ncbi.nlm.nih.gov/BLAST/). There is no *hemX* gene in *E. coli*. The *hemX* gene was also found in other Gram-positive strains such as *Brevibacillus brevis*, *Bacillus sphaericus*, *Bacillus stearothermophilus* determined by sequence analysis and complementation experiments (Johansson &

Hederstedt., 1999). Their HemX are of the same size (272-276 amino acids). The HemX proteins show higher than 40% amino acid sequence identity. Johansson *et al.* (1999) found that *B. subtilis* and *Br. brevis* HemX proteins both down-regulate *B. subtilis* GTR as detected by Western blot.

In *B. subtilis*, HemX is a membrane protein (Schröder *et al.*, 1994). The expected molecular mass of HemX is 32 kDa. There are seven to eight membrane-spanning segments in hydropathy plots in sequence analysis. By using the *E. coli* minicell system, Schröder *et al.* found that 26-kDa of HemX was in the membrane fraction.

From protein domain analysis, a putative conserved cytochrome c assembly protein domain of HemX was proposed (the score 43 bit) (NCBI BLAST: www.ncbi.nlm.nih.gov/BLAST/). Our hypothesis is that HemX may be involved in heme transport, therefore allowing hemin-permeability; and the heme pool down-regulates GTR at some level. In order to determine HemX function, I inserted *B. subtilis hemX* into a modified pUC19 vector; and it was transformed into an *E. coli hemA* mutant strain which normally is hemin-impermeable and can grow on ALA but not on heme. If *hemX* renders the strain heme-permeable, then it will grow on heme.

1.5. The goals of my study

My study focused on the *hemA* promoter activity from *E. coli* by creating various parts of the *hemA* upstream region, inserted behind a promoterless CAT reporter gene in the pKK232-8 vector. The putative transcript of two overlapping stem-loops (SL1 and SL2), immediately downstream of the putative P3 promoter of

hemA, are expected to be $tRNA^{Glu}$ lookalike. The effect of the SL1 and SL2 on *hemA* promoter activity was studied by creating deletion mutations of SL1 and SL2 from the *hemA* upstream region. The generated fragment was inserted into the promoterless CAT reporter gene of pKK232-8. CAT expression of the *hemA* upstream region with the deletion of SL1 and SL2 was compared with that of the intact *hemA* upstream region.

The putative P3 promoter of *hemA* was explored, since the upstream region of *hemA* from –378 bp to-258 bp plays a role in regulation of the *hemA* promoter activity. The CAT expression was analyzed in pFL25-CAT, which contains the putative P3 promoter and SL1 and SL2.

The effect of over-expression of either GTR or GTS on the *hemA* promoter activity and *gltX* promoter activity in *E. coli* was studied by co-transforming a plasmid harboring *gltX* or *hemA*, and pKK232-8 with a promoterless CAT reporter gene and intact *hemA* upstream region or intact *gltX* upstream region inserted in the front of it into a *recA*$^-$ strain.

HemX is a membrane protein in *B. subtilis*. HemX can down-regulates *hemA* from *B. subtilis* by an unknown mechanism. *E. coli* strain is heme impermeable. Whether HemX can change heme permeability of *E. coli* was studied.

MATERIALS AND METHODS

2.1. MATERIALS

2.1.1 Chemicals and other materials

Luria-Bertani medium was purchased from Difco (Detroit, MI). Antibiotics, ALA, hemin, agarose for molecular biology analysis and AcuTaq DNA polymerase were purchased from Sigma (St. Louis, MO). The DNA markers for PCR (50 bp- 2 kb) were purchased from Sigma (St. Louis, MO). The DNA markers (λ*Hin*dIII digests, λ*Hin*dIII/*Eco*RI digests) and Tween 80 were purchased from Fisher Scientific (Pittsburgh, PA). Restriction enzymes, T4 ligase and Klenow were purchased from New England Biolabs (Beverly, MA). QIAprep spin Miniprep kit and QIAGEN plasmid Maxi kit were purchased from Qiagen (Valencia, CA). Agarose gel DNA extraction kit, Expand™ High Fidelity PCR system and CAT ELISA kit were purchased from Boehringer Mannheim (Indianapolis, IN). PLATINUM pfx DNA polymerase was purchased from Life Technologies (Rockville, MD). BioRad Protein Assay system was purchased from Bio-Rad (Hercules, CA). Zero Blunt™ TOPO (PCR cloning kit for sequencing) and TA cloning kit were purchased from Invitrogen (Carlsbad, CA). Primers A1, A2, IL1, IL2 and IR were purchased from Oligos Etc. (Wilsonville, OR). Primers A1, A2, A24, A5 and A6 were purchased from Gibco BRL, Life Technologies (Rockville, MD). Primers used in my study are listed in Table 2.

Table 2. Primers used in these studies

Primers	Sequence and Restriction Enzyme Site
A1[1]	5' TAACGCTGACCTTTATTGTCGACT 3'
	*Sal*I
A2[1]	5' AAATAATACCAAGCTTGATAGGGTTT 3'
	*Hin*dIII
IL1	5' CCTGTTCCGTTAAATGGCGTCAGAATCAGCAAGAC 3'
IL2	5' CCTGTTCCGTTAAATGGCGTCAGCATCAGCAAGAC 3'
IR	5' ATTTAACGGAACAGGCAGTGAGCACAAGAGCAG 3'
A24	5' CCAAGCTTGGTGCTCACTGCCTGTTCCGTTA 3'
	*Hin*dIII
A5	5' TTCTAAAGAAATTGTTCGTTCG 3'
A6	5' CCAAGCTTGCGTTTAGAAAGATGCCGACAACCG 3'
	*Hin*dIII

1. Primer A1 and A2 were designed by Hua (1997).

2.1.2. Bacterial strains, plasmids and fragments obtained by PCR

Strains used in the study on transcriptional regulation of *hemA* and *gltX* are listed in Table 3. Strains used in the functional study of HemX are listed in Table 4. Plasmids used in the study of transcriptional regulation of *hemA* and *gltX* are listed in Table 5. Plasmids used in the functional study of HemX are listed in Table 6. The fragments obtained by PCR are listed in Table 7.

2.1.3. Media and growth of bacteria

(a) *E. coli* strains were grown at 37°C in LB medium (Sambrook *et al.*, 1989). The following antibiotics and concentrations were used: chloramphenicol (50 μg/ml and 25 μg/ml), ampicillin (50 μg/ml or 100μg/ml), tetracycline (20 μg/ml). For medium requiring hemin, the concentration of hemin was 4 μg/ml. The stock solution of hemin contained hemin (0.02g), Tween-80 (0.5 ml), 1M Tris-pH 8.0 (0.225 ml), 10N NaOH (10 μl), dH_2O (4.275 ml). For medium requiring 5-aminolevulinic acid (ALA), the concentration was 40 μg/ml.

(b) Cultures for the chloroamphenicol acetyltransferase (CAT) assay were routinely grown at 37°C in LB medium. Strains transformed with ampicillin-resistant plasmids were selected on LB plates, supplemented with 100 μg/ml ampicillin. The bacteria transformed with tetracycline-resistant plasmids were selected on LB plates, supplemented with 20 μg/ml tetracycline. The cotransformed bacteria were selected on LB plates with both 100 μg/ml ampicillin and 20 μg/ml tetracycline.

Table 3. Bacterial strains used in study on transcriptional regulation of *hemA* and *gltX*

Strains (*E. coli.*)	Description	Reference
C600	F⁻, *thi*-1, *leu*B6, *lac*Y1, *ton*A22, *Sup*E44, λ⁻	Promega
XL-1 blue MR	Δ(*mcr*A)183, Δ(*mcr*CB-*hsd*SMR-*mrr*)173, *end*A1 *Sup*E44, *thi*-1, *rec*A1, *gyr*A96, *rel*A1, *lac*	Stratagene
Mri93 7066	*pcnB*⁻ mutant strain reduces the copy number of ColE-derived plasmids	CGSC[1]
One shot Top10	Provided with PCR4Blunt TOPO cloning kit	Qiagen
INVαF'	*end*A *rec*A1 *hsd*R17(r-k, m+k) *sup*E44⁻ *thi*-1 *gyr*A *rel*A o80 *lac*Z M15 (*lac*ZYA-*arg*F) *deo*R F'	Invitrogen
FL509C	C600/pFL509-CAT	This work
FL510C	C600/pFL510-CAT	This work
WH515C	C600/pFLWH515-CAT	This work
FL509XL	XL-1 blue MR /pFL509-CAT	This work
FL510XL	XL-1 blue MR /pFL510-CAT	This work
WH515XL	XL-1 blue MR /pFLWH515-CAT	This work
FL509M	Mri93 7066/pFL509-CAT	This work
FL510M	Mri93 7066/pFL510-CAT	This work
WH515M	Mri93 7066/pFLWH515-CAT	This work
FL509G	XL-1 blue MR/ pFL509-CAT+pFL184GLTX	This work

Table 3. Bacterial strains used in study on transcriptional regulation of *hemA* and *gltX* (Continued)

Strains (*E. coli.*)	Description	Reference
FL510G	XL-1 blue MR/ pFL510-CAT+pFL184GLTX	This work
WH515G	XL-1 blue MR/ pWH515-CAT+pFL184GLTX	This work
FL509H	XL-1 Blue MR/ pFL509-CAT +pFL184HEMA	This work
FL510H	XL-1 Blue MR/ pFL510-CAT +pFL184HEMA	This work
WH515H	XL-1 Blue MR/ pWH515-CAT +pFL184HEMA	This work
PKK232-8XL	XL-1 Blue MR/pKK232-8	This work
FL25XL	XL-1 Blue MR/pFL25-CAT	This work
GLTXXL	XL-1 Blue MR/pGLTX-CAT	This work
GLTXFL184	XL-1 Blue MR/pGLTX-CAT + pFL184	This work
GLTXFL184G	XL-1 Blue MR/pGLTX-CAT + pFL184GLTX	This work
GLTXFL184H	XL-1 Blue MR/pGLTX-CAT + pFL184HEMA	This work
GLTXUPT	One shot Top10/pGLTXUP	This work

CGSC: *E. coli* Genetic Stock Center, Yale University, New Haven, CT

Table 4. Bacterial strains used in functional study of HemX

Strains (*E. coli.*)	Description	Reference
C600	F$^-$, *thi*-1, *leu*B6, *lac*Y1, *ton*A22, *Sup*E44, λ$^-$	Promega
XL-1 Blue MR	Δ(*mcr*A)183, Δ(*mcr*CB-*hsd*SMR-*mrr*)173, *end*A1 *Sup*E44, *thi*-1, *rec*A1, *gyr*A96, *rel*A1, *lac*	Stratagene
SASX41B	*hemA*41, *rel*A, *spo*T1, *met*B1, *rrn*B$^-$, *mcr*B$^-$, Hemin-impermeable	CGSC[1]
HU227	*hemA*$^-$, Hemin-permeable	Umanoff *et al.* (1988)
HEMAXC	C600/pHEMAX	This work
HEMA2C	C600/pHEMA2	This work
HEMX21C	C600/pHEMX21	This work
HEMAX3XL	XL-1 Blue MR/pHEMAX3	This work
HEMAXS	SASX41B /pHEMAX	This work
HEMA2S	SASX41B /pHEMA2	This work
HEMX21S	SASX41B /pHEMX21	This work

Table 4. Bacterial strains used in functional study of HemX (Continued)

Strains (*E. coli.*)	Description	Reference
HEMAX3S	SASX41B /pHEMAX3	This work
PUC19H	HU227/pUC19	This work
PUC19S	SASX41B /pUC19	This work
FL184HEMAS	SASX41B /pFL184HEMA	This work
FL184HEMAXS	SASX41B /pFL184HEMA + pHEMX21	This work

1. CGSC: *E. coli* Genetic Stock Center, Yale University, New Haven, CT.

Table 5. Plasmids used in study on transcriptional regulation of *hemA* and *gltX*

Plasmids	Relevant genotype and/or phenotype	Source and Reference
pKK232-8	Amp^r, promterless CAT	Brosius (1984)
pUC19	Amp^r	NEB[1]
pACYC184	Tet^r, Cm^r, origin of replication from p15A	NEB[1]
pJL68	Amp^r , pTZ19U/ (*hemA hemM*)	Li *et al.* (1989a)
pWC69	Amp^r, pJL68 (*hemA*)	Li *et al.* (1989a)
pJCL01	Tet^r, pACYC184/ *gltX*	Li, JC. of this Lab
pCR^{TM}II	p*lacZα* ColE1 ori; fl ori	Invitrogen
pCR4Blunt-TOPO	Amp^r, Kan^r, blunt ends	Invitrogen
pFL509-CAT	Amp^r, pKK232-8/*hemA* (-SL1, -SL2)	
	(totally 478 bp: -534/-326↔-286/-20)	
	mismatch at -337(G→T), -78 (T→C)	This work
pFL510-CAT	Amp^r, pKK232-8/*hemA* (-SL1, -SL2)	
	(totally 478 bp: -534/-326↔-286/-20)	This work
pWH515-CAT	Amp^r, pKK232-8/*hemA*	
	(totally 515 bp: -534/-20)	This work
pFL25-CAT	Amp^r, pKK232-8/*hemA*	
	(totally 270bp: -534/-265)	This work

Table 5. Plasmids used in study on transcriptional regulation of *hemA* and *gltX* (Continued)

Plasmids	Relevant phenotype	Source and Reference
pGLTX-CAT	Amp^r, pKK232-8/*gltX*	
	(totally 195 bp: -207/-13)	This work
pFL184	Tet^r, pACYC184 / ΔCm^r	This work
pFL184HEMA	pFL184/*hemA*	This work
pFL184GLTX	pFL184/*gltX*	This work
pFL509	Amp^r, $pCR^{TM}II$/*hemA*	This work
	(totally 501 bp: -551/-326↔-286/-10)	
	mismatch at -337(G→T), -78 (T→C)	This work
pFL24	Amp^r, pUC19/*hemA*	
	(totally 270bp: -534/-265)	This work
pGLTXUP	Amp^r, Kan^r	
	pCR4Blunt-TOPO/*gltX*	
	(totally 195 bp: -207/-13)	This work

1. NEB: New England Biolabs.

Table 6. Plasmids used in functional study of HemX

Plasmids	Description	Source and Ref.
pUC19	Amp^r	NEB[1]
pUC19(*SalI*)	Amp^r, pUC19/*SalI*	This work
pHEMAX	Cm^r, pHP13/*hemAX* (*B. subtilis*)	Schröder *et al.* (1994)
pHEMA2	Cm^r, 1.4kb-*hemAX*/Δ*hemX* (*B. subtilis*)	Schröder *et al.* (1994)
pHEMAX3	Amp^r, pUC19(*SalI*)/*hemAX* (*B. subtilis*)	This work
pHEMX21	Amp^r, pHEMAX3/Δ*hemA* (*B. subtilis*)	This work
pACYC184	Tet^r, Cm^r, origin of replication from p15A	New England Biolabs
pFL184	Tet^r, pACYC184 /Δ Cm^r	This work
pFL184HEMA	pFL184 /*hemA* (*E. coli.*)	This work

1. NEB: New England Biolabs.

Table 7: Fragments of 5' upstream region of *hemA* constructed by PCR

Name	FP[2]	BP[3]	Templates	Size and Sequence[1]
FL509	PCR1a:[5] A1	IL1	pJL68	226bp (-326bp to –551bp)
	PCR1b:[6] IR	A2	pJL68	275bp (-286bp to –10bp)
	PCR2: [7] A1	A2	PCR products from PCR1a& PCR1b	501bp (-551bp to –326bp, -286bp to –10bp), ΔSL1&SL2[4] and point mutation in putative P3 at -337bp (G→T), -78bp(T→C)
FL510	PCR1a: A1	IL2	pJL68	275bp (-286bp to –10bp)
	PCR1b: IR	A2	pJL68	501bp (-551bp to –326bp)
	PCR2: A1	A2	PCR products from PCR1a& PCR1b	501bp (-551bp to –326bp, -286bp to –10bp), ΔSL1&SL2[4]
WH515[a]	A1	A2	pJL68	542bp (-10bp to –551bp)
FL24	A1	A24	pWH515-CAT	288bp (-264bp to –551bp)
GLTXUP	A5	A6	*E. coli.* genomic DNA	195bp (-13bp to –207bp)

(Continue)

Table 7: (Continued)

1. The first nucleotide of the *hemA* coding region is +1.

2. FR: Forward primer.

3. BP: Backward primer.

4. ΔSL1&SL2 deletion of two overlapping stem-loops.

5. PCR1a: Amplication of left side fragment in the first round PCR.

6. PCR1b: Amplication of right side fragment in the first round PCR

7. PCR2: Amplication of the whole fragment in the second round PCR.

a. WH515 was made by Hua in our lab (1997). See detail in the text.

(c) Cultures for functional study of HemX were routinely grown at 37°C in LB medium. C600 strains and XL-1 Blue MR strains transformed with ampicillin-resistant plasmids were selected on LB plates supplemented with 100 μg/ml ampicillin. C600 strain and XL-1 Blue MR strain transformed with chloramphenicol-resistant plasmids were selected on LB plates, supplemented with 50 μg/ml chloramphenicol. SASX41B strains transformed with ampicillin-resistant plasmids were selected on LB plates supplemented with 50 μg/ml ampicillin. SASX41B strains transformed with chloramphenicol-resistant plasmids were selected on LB plates, supplemented with 50 μg/ml or 25 μg/ml chloramphenicol. Hemin (4 μg/ml) and ALA (40 μg/ml) were added when necessary.

2.2. METHODS

2.2.1. DNA techniques

(a) Isolation and preparation of DNA. Large- and small-scale preparations of plasmid DNA were done by using QIAprep spin Miniprep kit and QIAGEN plasmid Maxi kit, following the instructions of the suppliers. Calf intestinal phosphatase (CIP) treatment was used to prevent self-ligation. Klenow treatment was used to create blunt ends. Purification of DNA from agarose gels was done by using agarose gel DNA extraction kit, following the instructions of the suppliers. DNA concentration was determined by DNA/Protein/Enzyme Analyzer (Shimadzu, Model Biospec-1601).

(b) Ligation and transformation. Ligations were performed at 16°C for 16 hours in a refrigerated circulating bath (Hoefer Scientific, RCB300). The ratio of the insert to vector was 3:1. For blunt-end ligation, T4 ligase (2000 unit/μl) was used. 1μl 13% PEG was added to 20μl ligation mixtures to increase the ligation efficiency. All transformations were done by electroporation unless the kit was provided with chemical competent cells. Competent cells were prepared by the following procedure (Sharma and Schimke, 1996). A fresh overnight culture was transferred into a non-salt medium with appropriate antibiotics and or ALA when necessary, and grown at 37°C to log phase ($O.D._{600}$ 0.5). After 5 min on ice, the cells were spun down at 4°C at 3000rpm. The pellets were washed with ice-cold water twice and 10% glycerol once. They were suspended into 10% glycerol to $10^8 \sim 10^9$ cells/ml and stored at

–70°C. 100ng to 200ng plasmid DNA was added into 40μl competent cells. Electroporation was performed in an *E. coli* Pulser (Bio-Rad, Model 450). The time constant was 5 (MSEC), and voltage was set at 1.8 (KV).

(c) Cloning. A TA cloning kit was used to clone the PCR product for FL509. The instructions in the kit were followed. Zero blunt™ TOPO (PCR cloning kit for sequencing) was used to clone the PCR product of the GLTXUP fragment. The instructions in the kit were followed. Zero blunt™TOPO (PCR cloning kit for sequencing) and TA cloning vector were used for directly cloning from the PCR product and further sequencing.

(d) PCR. PCR reactions were performed in a TECHNE PCR instrument (Techne, Model FGENO5TP). Expand™ High Fidelity PCR system or AcuTaq polymerase,or PLATINUM pfx DNA polymerase with its kit was used for the PCR reaction. The basic instructions in the kits were followed.

2.2.2. Protein determination

The bacterial pellets from the culture at different conditions were collected. They were washed twice with cold PBS buffer and lysed by sonication in Ultrasonic processor (MISONIX, Model XL 2015). The pulse™ duty cycle was 50% 1 sec. The cell debris was removed from the protein. The protein standard IgG in the BioRad Protein Assay kit was used to determine the protein concentration. The absorbance was measured at 595nm. The instructions in the kit were followed.

2.2.3. CAT ELISA assay

The CAT ELISA assay kit was used to measure CAT reporter gene expression in different constructs. The instructions in the CAT ELISA assay kit for microplate procedures were followed. CAT protein was determined as absorbance at 405nm absorbance in a Microplate reader (Bio-Rad, Model 450). Each CAT assay was done in duplicate. Each experiment was repeated two or three times.

2.2.4. Sequencing

The FL510 fragment was partly sequenced. Fragments FL509, FL24 and GLTXUP were completely sequenced. The insert of pFL509 was sequenced at the New York Blood Center. It was sequenced with the A1 forward and A2 backward primers. After subcloning into pKK232-8, the insert of pFL509-CAT was sequenced at the New York Blood Center, New York. It was sequenced by using pKK232-8 backward primer (5' CGGTCTGGTTATAGGTACATTGAGC 3'). The insert of pFL510-CAT was sequenced by Robert J. Donnelly, Molecular Resource Facility, New Jersey Medical School, UMDNJ, Newark, New Jersey, by using the A2 backward primer. The insert of pFL24 was sequenced at the sequence facility center of Rockefeller University, New York, by using the M13 forward (5' GTAAAACGACGGCCA 3') and the backward primers (5'CAGGAAACAGCTATGAC 3'). The insert of pGLTXUP was sequenced at the sequence facility center of Rockefeller University by using M13 forward (5' GTAAAACGACGGCCA 3') and backward primers (5'CAGGAAACAGCTATGAC 3').

2.2.5. Amplification of FL509, FL510, FL24 and GLTXUP fragments by PCR (Table 7, Figure 8 and Figure 9)

FL509 and FL510 fragments (Table 7 and Figure 9) were generated based on PCR-assisted deletion mutagenesis methods with some modifications (Link *et al.*, 1997 and Herlitze *et al.*, 1990). Expand™ High Fidelity PCR system was used for the PCR reaction. The instructions in the kit were followed. Two rounds of PCR were used to finish the deletion mutagenesis. In the first round of PCR, the PCR program was 94°C 1 min, 54°C 2 min, 72°C 2 min for 25 cycles and 7 min delay at 72°C. During the second round of PCR, The PCR program was 94°C 1 min, 50°C 3 min, 72°C 2 min for 25 cycles, and 7 min delay at 72°C.

The FL24 fragment was generated by PCR, using AcuTaq DNA polymerase. The product instructions were followed. The PCR program was 94°C 1 min, 50°C 1 min and 30 sec, 72°C 1 min for 25 cycles, and 10 min delay at 70°C.

The 5' upstream region of *gltX* was amplified by PCR from *E. coli* C600 genomic DNA, using PLATINUM pfx DNA polymerase. The instructions in the kit were followed. The PCR program was 94°C 2 min as a hot start, followed by 25 cycles 94°C 1 min, 63.9°C 2 min, and 68°C 2 min. There was a 10 min delay at 68°C. The details for each construction are in the results.

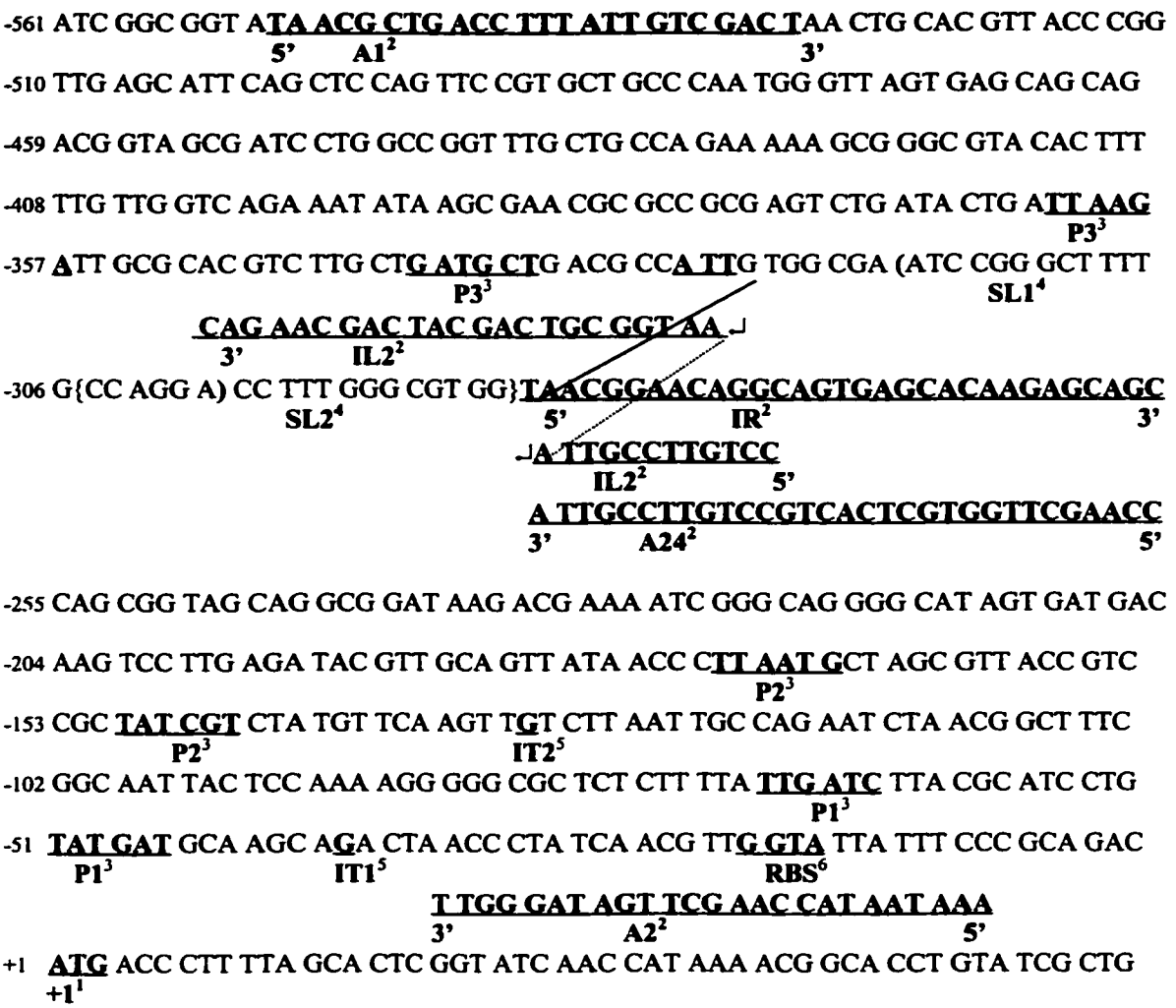


Figure 8: Site-directed deletion of the two overlapping stem-loops of *hemA*

1. The first nucleotide of the *hemA* coding position is numbered as +1.
2. The position and sequences of primers A1, A2, IL2, IR and A24 are indicated.
3. P1, P2 and putative P3 promoters of *hemA* are underlined.
4. SL1 and SL2: overlapping stem-loops.
5. IT1 and IT2: transcription initiation sites of *hemA*.
6. RBS: ribosome binding site.

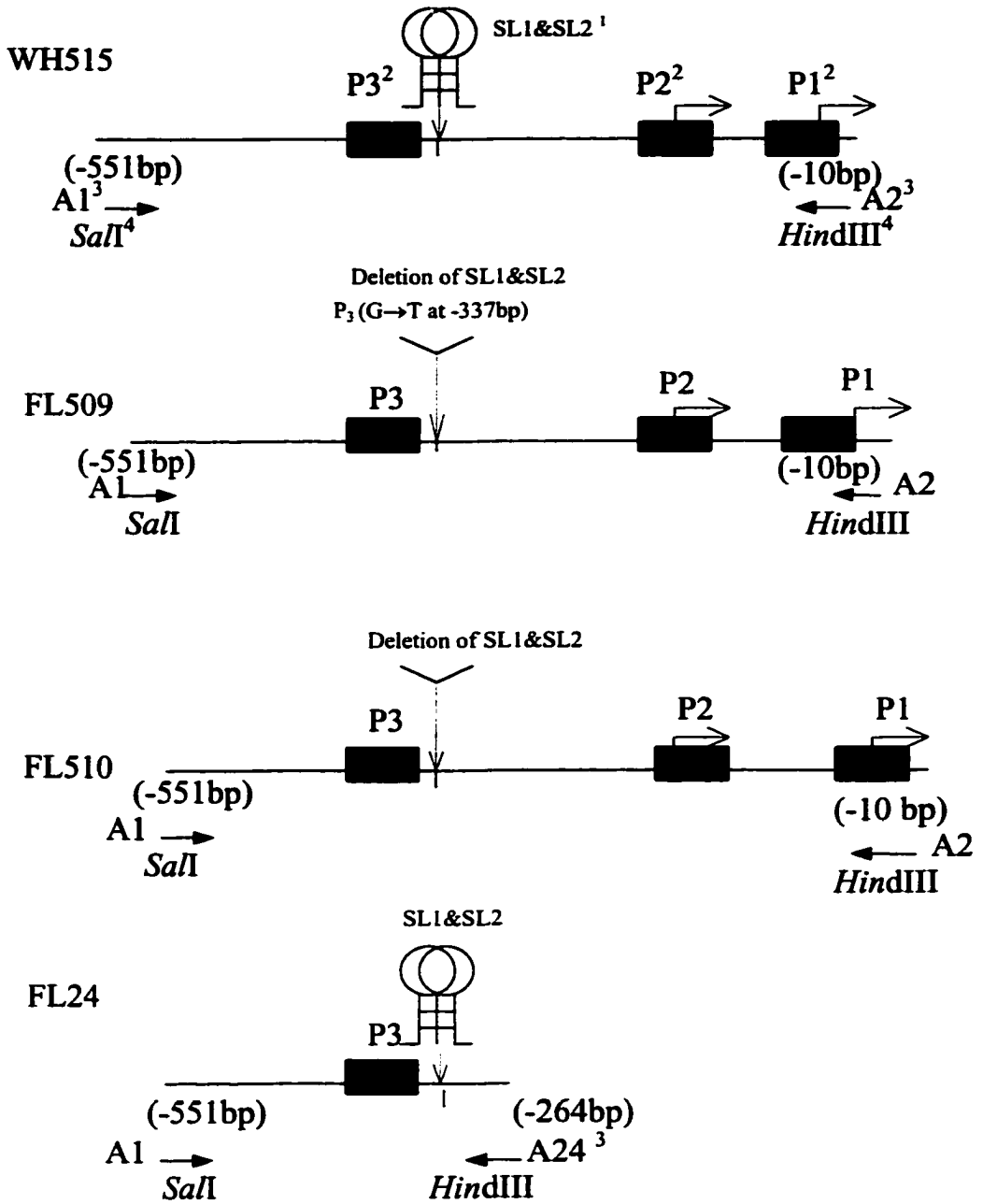


Figure 9: Fragments of the 5' upstream region of *hemA*

-WH515, FL509, FL510 and FL24

1. SL1&SL2: two stem-loops. 2. Promoters—P1, P2 and putative P3.

3. A1, A2 and A24 are external primers used to amplify the fragments.

4. The cutting sites created on the primers.

2.2.6. Construction of pFL509-CAT, pFL510-CAT, pFL25-CAT, and pGLTX-CAT operon fusions

The DNA fragments generated by PCR — FL510, FL24 and GLTXUP (Table 7) were run on a 1% agarose gel. The appropriate bands were cut from the gel and further purified. FL24 was cloned into pUC19 for sequencing. The FL510 and FL24 were digested by appropriate restriction enzymes and ligated into vector pKK232-8 in front of the CAT reporter gene. The PCR product of FL509 was cloned into pCR™II vector for sequencing. The GLTXUP fragment was cloned into pCR4Blunt-TOPO vector for sequencing. Fragment GLTXUP and FL509 were further subcloned into vector pKK232-8 in front of the CAT reporter gene. Plasmid DNA was isolated. The resulting plasmids were transformed into competent strains (Table 3) by electroporation; and CAT expression was determined. The detailed steps are described in Results.

2.2.7. Subcloning *B. subtilis hemX*

pHEMAX has a 3.6-kb fragment containing the *hemA* and *hemX* genes, inserted between *Bam*HI and *Eco*RI sites of the pHP13 vector. In order to study HemX function, *hemX* was subcloned.

The *hemX* coding region begins 7 bp after the stop codon of *hemA* in the *hemAXCDBL* operon of *B. subtilis*. There are two *Sal*I cutting sites in the *hemA* coding region (Figure 10). A 654-bp fragment of the *hemA* coding region was deleted between the two *Sal*I cutting sites. Since there is another *Sal*I cutting site on the multiple cloning site of the pHP13 vector, which is located in front of the 3.6-kb

hemAX insert, the 3.6-kb *hemAX* fragment was inserted in a modified pUC19, in which the *Sal*I cutting site had been deleted (Figure 11).

First, pUC19 was cut by *Sal*I and treated by Klenow fragment to fill the ends. Then blunt ended-ligation removed the *Sal*I site from the pUC19, yielding pUC19 (*Sal* I).

Then, pHEMAX (pHP13) was digested by *Eco*RI and *Bam*HI (Figure 10 and Figure 11). A 3600-bp *hemAX* insert of pHEMAX and a 4900-bp pHP13 vector were seen on the gel. pUC19 (*Sal* I) was digested by *Eco*R I and *Bam*H I. It was ligated to the 3600-bp insert from pHEMAX and transformed into XL-1 Blue MR. A colony which gave the correct results after restriction enzyme analysis was isolated. The plasmid was named pHEMAX3 (Figure 11). Finally, the pHEMAX3 was digested by *Sal* I and self-ligated (Figure 10 and Figure 11). SASX41B was transformed with the self-ligation products, and grown on LB with ampicillin and ALA. The colonies, which needed ALA, were selected; and plasmid DNA was isolated. The plasmid pHEMX21 showed the correct construct by restriction enzyme analysis.

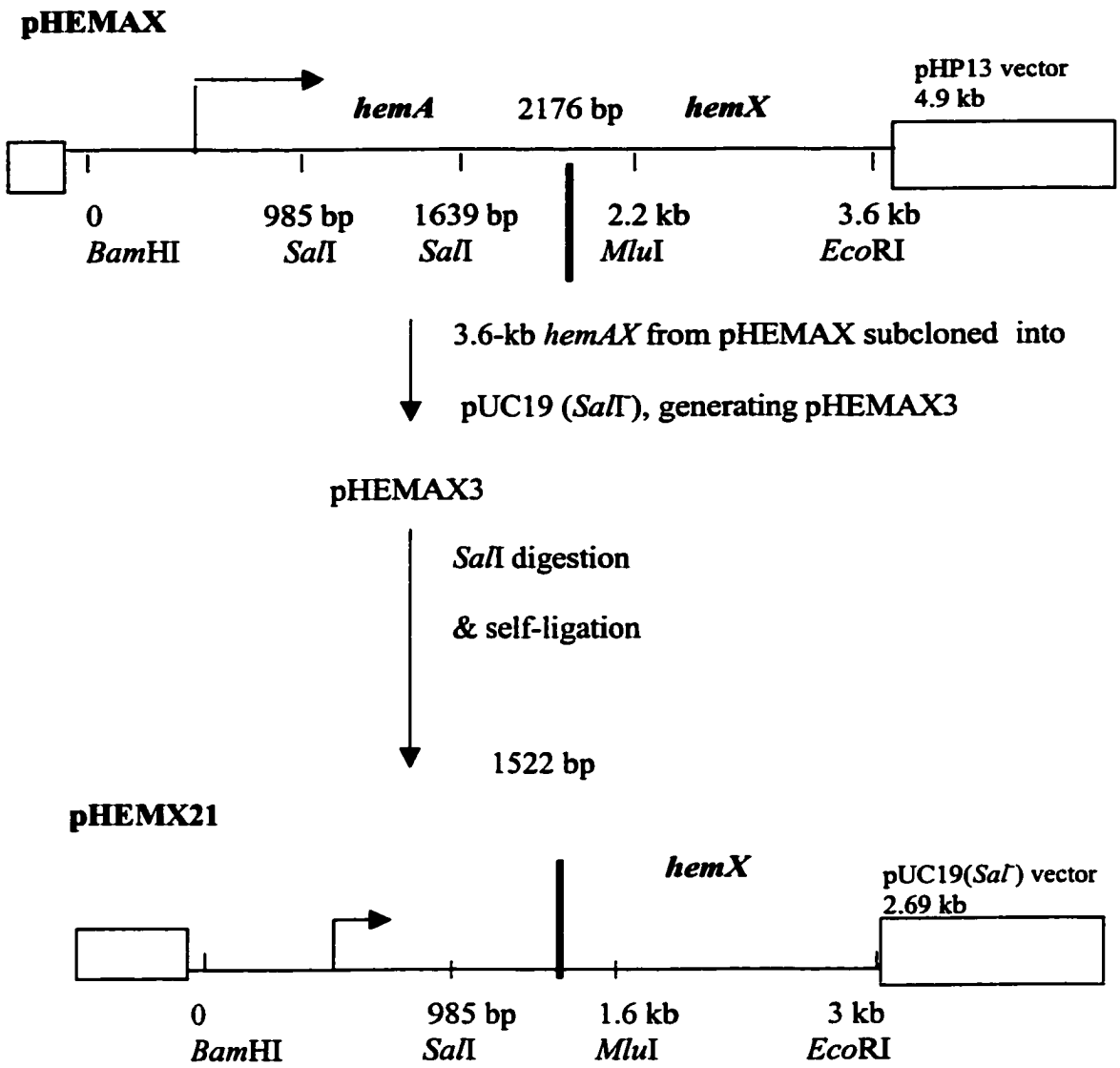


Figure 10: Strategy for subcloning *hemX*

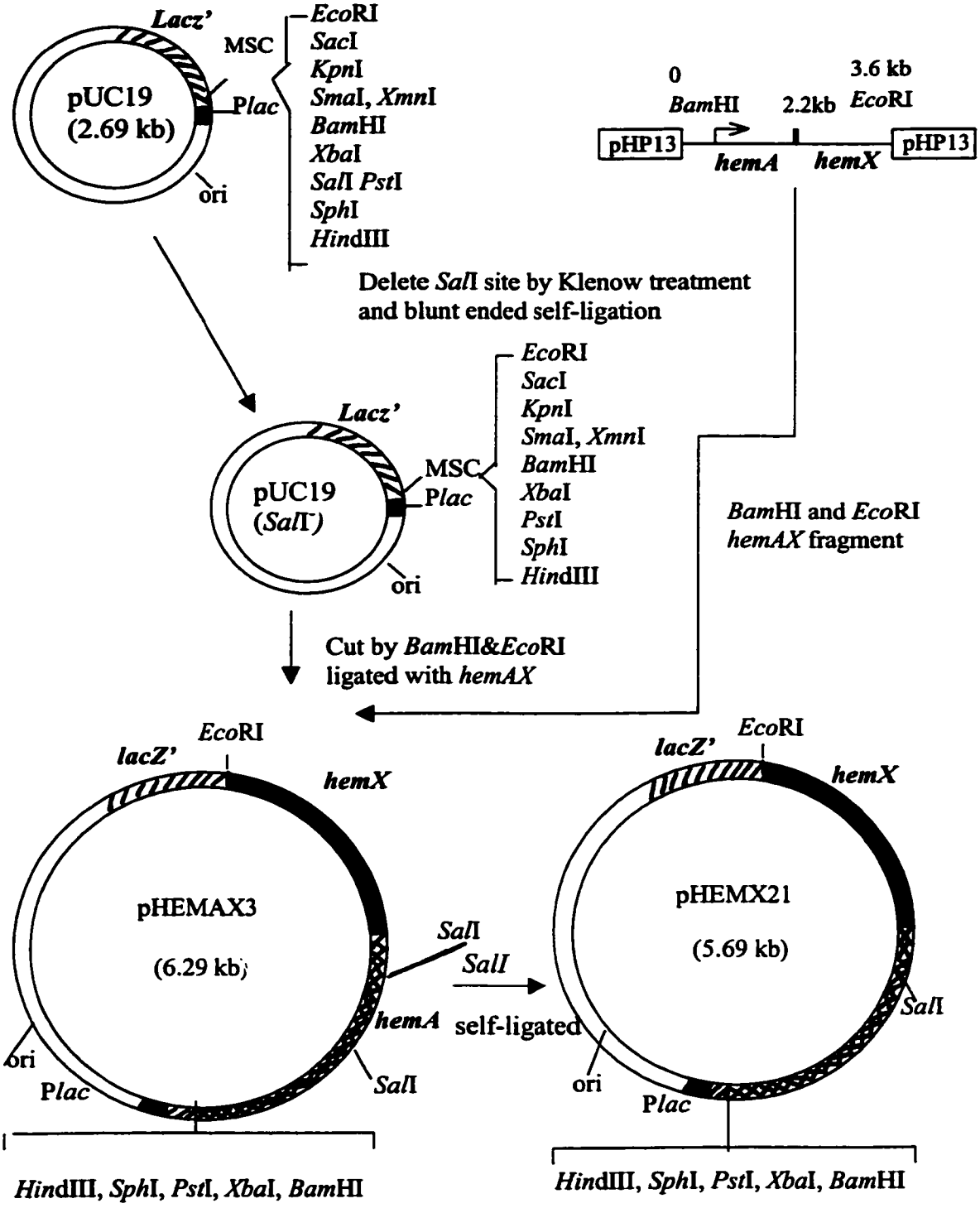


Figure 11: Construction of pHEMAX3 and pHEMX21

RESULTS

3.1. Analysis of the role of the 5' upstream region of *hemA* in its expression in *E. coli.*

3.1.1. Construction of pFL510-CAT and pFL509-CAT operon fusion (Figure 14)

In order to know whether SL1 and SL2 are involved in the regulation of *hemA* gene expression, PCR-assisted deletion was used to delete SL1 and SL2 from the 5' upstream region of *hemA* to give FL510, which was then cloned into pKK232-8 in front of a CAT reporter vector (Figure 12, Figure 13, Figure 14, and Table 7).

In the first round of PCR, pJL68 was used as a template. The fragment, upstream of SL1 and SL2, was amplified by using primer A1 (5' TAA CGC TGA CCT TTA TTG TCG GAC T 3'), and primer IL2 (5' CCT GTT CCG TTA AAT GGC GTC AGC ATC AGC AAG AC 3'). The fragment, downstream of SL1 and SL2, was amplified by using primerA2 (5' AAA TAA TAC CAA GCT TGA TAG GGT TT 3'), and primer IR (5' ATT TAA CGG AAC AGG CAG TGA GCA CAA GAG CAG C 3').

During the second round of PCR, the fragments, generated from the first time PCR, were annealed at 3' complementary tails to create a new template with a deletion of SL1 and SL2. They were further amplified by primer A1 and primer A2. The final PCR products were purified on a 1% agarose gel. They were digested by *Sal*I and *Hin*dIII, and ligated into the same polylinker site of the vector pKK232-8 to create pFL510-CAT.

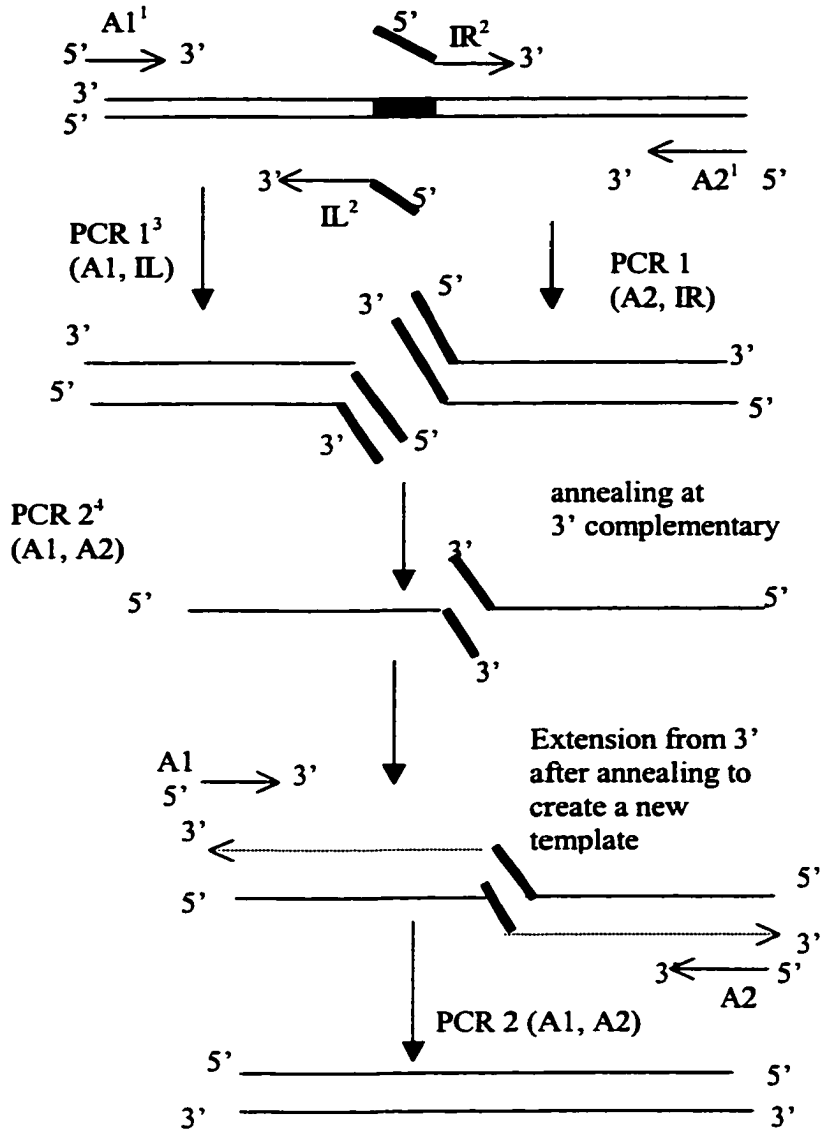


Figure 12: Mechanism of PCR assistant deletion mutations

1. A1 and A2 are two external primers. 2. IL: left internal primer; IR: right internal primer; IL and IR are complementary at 5' end.

3. First round of PCR. 4. Second round of PCR.

The fragment needed to be deleted

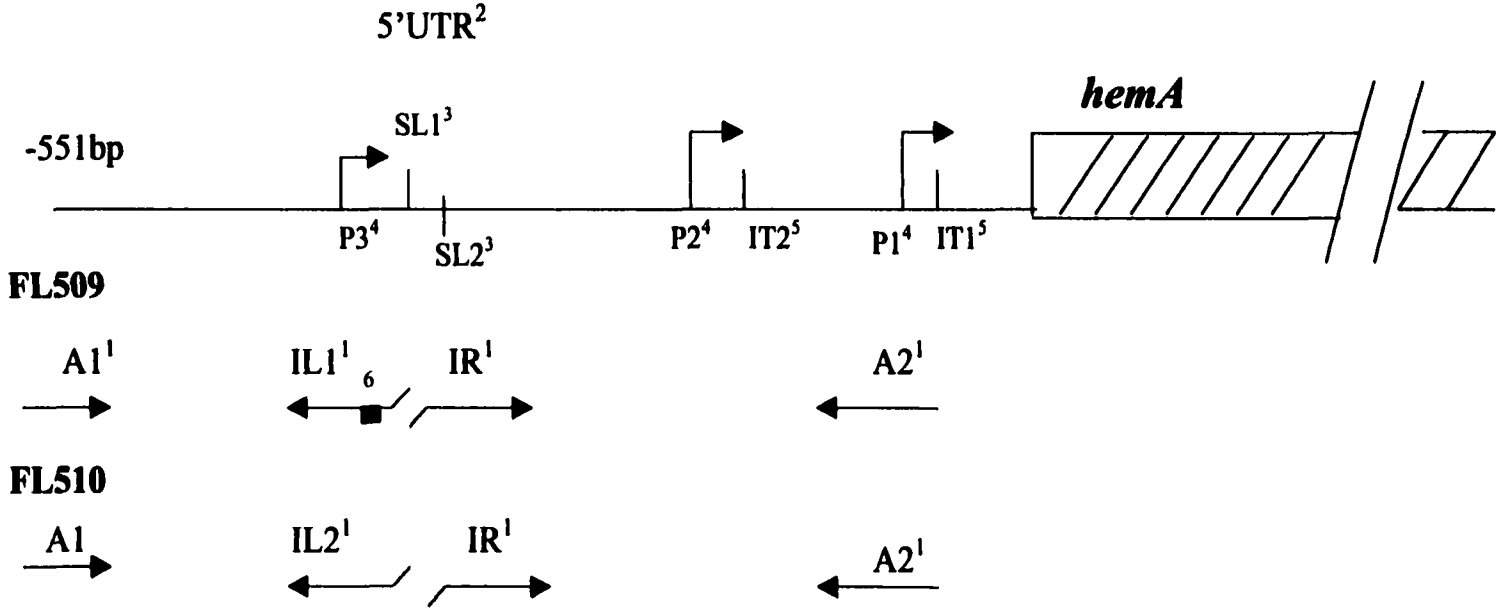


Figure 13: Creation of FL509 and FL510 fragments by PCR assistant deletion mutations.

1. Primers. 2. The *hemA* upstream region. 3. Stem loops. 4. Promoters of *hemA*. 5. Initiation of transcription sites.

6. A point mutation on IL1 primer.

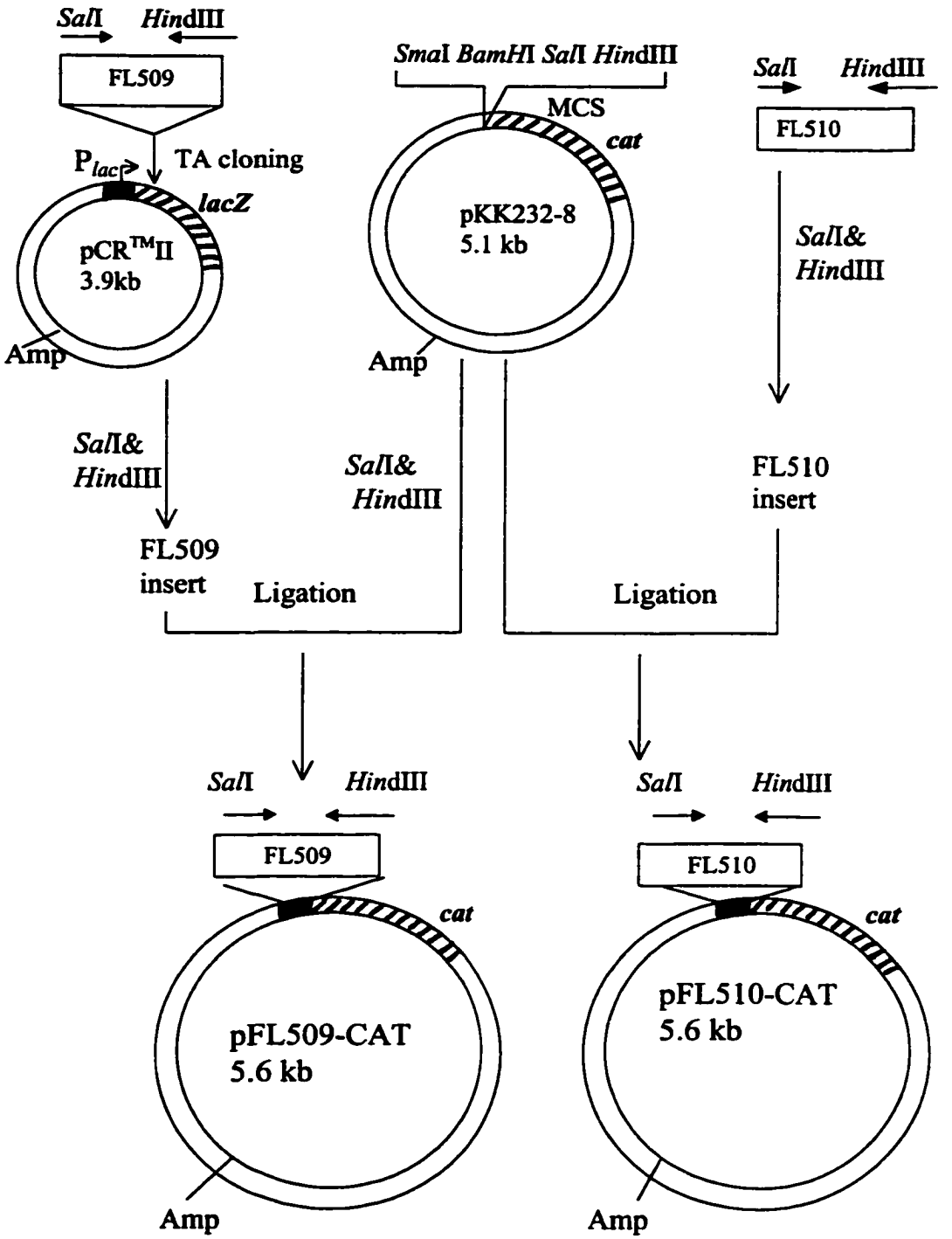


Figure 14: Steps in construction of pFL509-CAT and pFL510-CAT (see text for the explanation)

Figure 14: (Continued)

FL509 fragment: the *hemA* upstream region from -551 to -10 with the deletion of SL1 and SL2, and mutations in the putative P3 promoter at -337 bp (G→T), and in -78bp (T →C).

FL510 fragment: the *hemA* upstream region from -551 to -10 with the deletion of SL1 and SL2.

FL509 was constructed in the same way as FL510, except in the first round of PCR, I used primer IL1 (5' CCT GTT CCG TTA AAT GGC GTC AGA ATC CAG CAA GAC 3'), instead of IL2. A single base change underlined in IL1 creates a point mutation on the putative P3 promoter. As a result, FL509 has the 5' upstream region of *hemA* with deletion of SL1 and SL2, and a point mutation at –337 on the putative P3 promoter. The FL509 fragment was ligated into pCR™II vector and transformed into INVαF' chemically competent cells. Transformants were selected by blue-white screening. Restriction enzyme analysis confirmed the size of the insert. The plasmids with the insert (pFL509) were sent for sequencing. pFL509 was digested by *Sal*I and *Hin*dIII. The insert was purified from a 1% agarose gel and ligated into the same polylinker site of the vector pKK232-8 to create pFL509-CAT. The pWH515-CAT, which has the intact *hemA* 5' upstream region of *hemA* in pKK232-8 in front of the promoterless CAT reporter gene, was used as a positive control.

3.1.2. The structure of the FL509 fragment.

The first base of the *hemA* coding region is numbered as +1. The FL509 fragment corresponds to -551 to –10 of *hemA* upstream region with a 39-bp deletion from –324 to –286, and a point mutation in –337 (G→T) in putative P3 promoter of *hemA* and another at –78 (T→C) (Figure 9 and Figure 14 and Table 7). Both strands of Fl509 in pFL509 were sequenced. The fragment of the sequenced area was from –505 to –17 of the 5' upstream region of *hemA*. One complementary strand of FL509 in pFL509-CATwas partially sequenced again. The fragment of the sequenced area was from –27 to –429 of the 5' upstream region of *hemA*. In the –337 position, there

is a point mutation from G to T. In –78 position, there is another point mutation from T to C. SL1 and SL2 from –325 to –286, totaling 39 base pairs, is completely deleted. The deleted sequence is GTG GCG AAT CCG GGC TTT TGC CAG GAC CTT TGG GCG TGG.

3.1.3. The structure of the FL510 fragment

The FL510 fragment corresponds to -551 to –10 of the *hemA* upstream region with a 39-bp deletion from –324 to –286 (Figure 8 and Figure 9 and Table 7). One complementary strand of FL510 corresponding the 5' upstream region of *hemA* from –60 to –429 was sequenced. The SL1 and SL2 from –324 to –286, totaling 39 base pairs, are completely deleted. The deleted sequence is GTG GCG AAT CCG GGC TTT TGC CAG GAC CTT TGG GCG TGG.

3.1.4. The role of the two stem-loops (SL1 and SL2) in *hemA* gene expression (Figure 15).

pFL509-CAT, pFL510-CAT and pWH515-CAT were transformed into Mri93 7066 strain by electroporation. Mri93 7066 is a *pcnB*⁻ mutant strain. The *pcnB*⁻ mutation reduces the copy number of ColE-derived plasmids. The CAT expression by pFL509-CAT, pFL510-CAT and pWH515-CAT in overnight culture in MRi93 7066 strain is shown in Figure 15. The CAT reporter gene expression of pFL509-CAT, pFL510-CAT and pWH515-CAT in XL-1 Blue MR is shown in Figure 16. Comparison of the data from Figure 15 and Figure 16, *pcnB*⁻ strain reduced the CAT expression by 10-fold. It suggested that the ratio of copy number for low copy

plasmid to medium copy is about 1 to 10. So, the regulation factors were not seriously diluted in the experiment shown in Figure 15.

For pFL510-CAT and pWH515-CAT in C600 (data not shown), XL-1 Blue MR (Figure 16) and *pcnB*⁻ strain- MRi93 7066 (Figure 15), the amount of CAT expression per total protein is similar in overnight culture. The CAT expression in log phase was also studied. Similar results were obtained. Because the CAT expression was low in the log phase, the results were not very accurate. The results suggest that SL1 and SL2 do not play a significant role in transcriptional regulation of the *hemA* gene expression under normal conditions. In contrast, for pFL509-CAT in C600, *pcnB*⁻ strain (Figure 15) and XL-1 Blue MR (Figure 16), the CAT amount per total protein increases two fold in overnight culture, compared with pWH515-CAT in the strains. Whether the increase of CAT expression by pFL509-CAT in these strains is due to the point mutation in the putative P3 promoter or the other mutation has not been determined. This experiment was repeated three times under similar conditions.

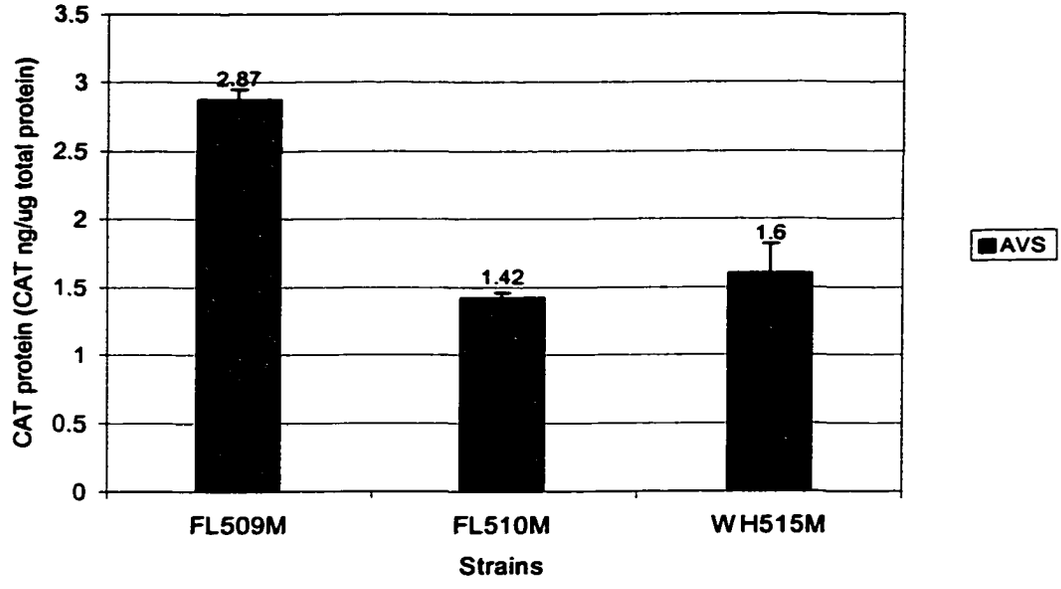


Figure 15: The effect of deletion of two stem-loops and of two point mutations on the promoter activity of the 5' *hemA* upstream region

The results are expressed as CAT protein (CAT ng/ug total protein). The data bar shows the average of duplicates in CAT assays. Standard deviations are shown in the Y error bar. AVS: The average CAT in overnight culture. This experiment was repeated three times under similar conditions with similar results. Strains – FL509M, FL510M and WH515M are described in Table 3. Fl509M: pFL509-CAT (deletion of SL1&SL2 and a point mutation in putative P3 promoter) was transformed into *pcnB*⁻ strain. FL510M: pFL510-CAT (deletion of SL1&SL2) was transformed into *pcnB*⁻ strain. WH515M: positive control also in *pcnB*⁻ strain.

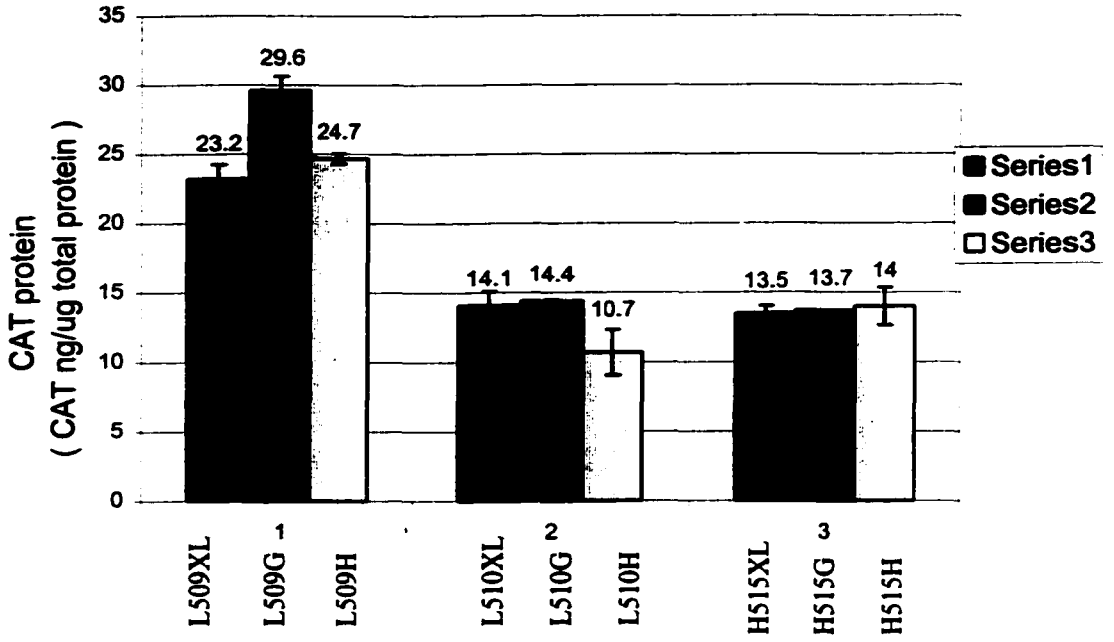


Figure 16: The effect of over-expression of *gltX* or *hemA* on the promoter activity of the 5' upstream region of *hemA*

The results are expressed as CAT protein (CAT ng/ug total protein). The data bar shows the average of duplicates in CAT assays. Standard deviations are shown in the Y error bar. Series 1: no cotransformation in the strains. Series 2: cotransformed a plasmid harboring *gltX*. Series 3: cotransformed a plasmid harboring *hemA*. This experiment was repeated three times under similar conditions. Similar results were obtained. Strains – FL509XL, FL510XL and WH515XL are described in Table 3. Fl509XL: pFL509-CAT (deletion of SL1&SL2 and a point mutation in putative P3 promoter) was transformed into XL-1 Blue MR strain. FL510M: pFL510-CAT (deletion of SL1&SL2) was transformed into XL-1 Blue MR strain. WH515XL: positive control. FL509G, FL510G and WH515G (see Table 3): over-expression of *gltX* in the strains. FL509, FL519 and WH515 (see Table 3): over-expression of *hemA* in the strains.

3.1.5. The effect of expression of GTS or GTR on regulation of the 5' upstream region of *hemA* (Figure 16)

In order to know whether GTS and GTR regulate the 5' upstream region of *hemA*, the effects of *gltX* or *hemA* over-expression on operon fusion — pFL509-CAT, pFL510-CAT and pWH515-CAT were studied. I cotransformed XL-1 Blue MR with pFL184HEMA, containing the complete *hemA* upstream region and the entire coding region, and pFL509-CAT or pFL510-CAT, or pWH515-CAT respectively. I also cotransformed XL-1 Blue MR with pFL184GLTX, containing the strongest promoter of *gltX*—P3 and the entire coding region, and pFL509-CAT, or pFL510-CAT, or pWH515-CAT respectively.

In order to make *gltX* or *hemA* gene coexist with pKK232-8 based vectors and study the CAT expression, the modified pACYC184 vector was used to subclone *hemA* and *gltX* respectively. The pACYC184 was digested with *Bsa*A1, and was resolved on a 1% agarose gel. A 2831-bp fragment was cut and purified. The 2831-bp fragment was self-ligated to create pFL184 vector, which does not carry the chloramphenicol resistance gene. The pFL184 vector carries the origin of replication from p15A, which can co-exist with a plasmid that carries a ColE1 origin. Because pFL184 does not carry the chloramphenicol-resistantce gene, it makes the CAT assay more reliable (Figure 17).

The *gltX* fragment was generated by *Eco*RI restriction enzyme digestion of pJCL01, which carries the main *gltX* promoter-P3 and the entire GTS coding region. A 2000-bp fragment was purified from a 1% agarose gel. It was further treated by

DNA polymerase, large (Klenow) fragment and dNTP to create a blunt end *gltX* fragment (Figure 17).

The *hemA* fragment, which contains the complete 5' upstream region of *hemA* and its coding region, was generated from WC69 by *Eco*RI and *Hin*dIII restriction enzyme digestion. After Klenow treatment, a blunt end *hemA* fragment was prepared (Figure 17).

The pFL184 vector was digested with *Xmn*I. The 5' phosphate groups of DNA were removed by calf intestinal alkaline phosphatase. Blunt ended *hemA* and *gltX* were ligated into pFL184 respectively to generate pFL184HEMA and pFL184GLTX. The expression of *hemA* in pFL184hemA and *gltX* in pFL184gltX used their own promoters. The pFL184HEMA and pFL184GLTX were transformed into XL-1 Blue MR respectively (Figure 17).

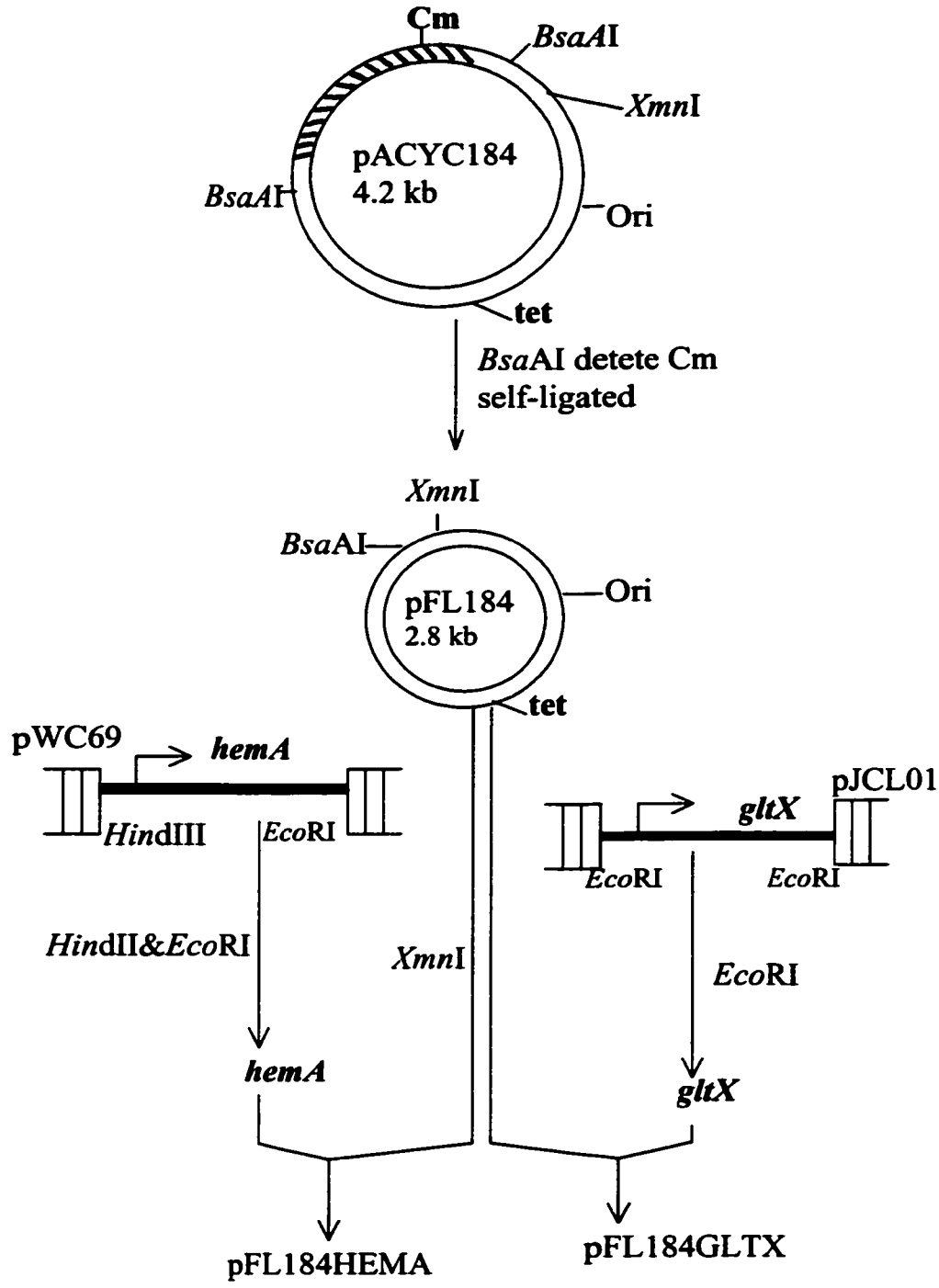


Figure 17: Construction of pFL184GLTX and pFL184HEMA

(See text for explanation)

Three sets of experiments were designed to determine the effect of over-expression of *hemA* or *gltX* on regulation of promoter activity of the *hemA* upstream region:

1) pFL509–CAT cotransformed with pFL184GLTX and pFL509-CAT cotransformed with pFL184HEMA into XL-1 Blue MR.
2) pFL510-CAT cotransformed with pFL184GLTX, and pFL510-CAT cotransformed with pFL184HEMA into XL-1 Blue MR.
3) pWH515-CAT cotransformed with pFL184GLTX and pWH515-CAT cotransformed with pFL184HEMA into XL-1 Blue MR.

E. coli XL-1Blue MR transformed strains were grown under ampicillin (100 μg/ml) and tetracycline (20 μg/ml) selection. The amount of CAT expression per total protein in overnight culture was determined (Figure 16). The results suggested that over-expression of *hemA* and *gltX* do not influence *hemA* transcription under the experimental conditions used. This experiment was repeated three times under similar conditions.

3.2. The putative P3 promoter

3.2.1. Construction of pFL25-CAT operon fusion

In order to test the putative P3 promoter activity, pWH515-CAT was used as a template. For PCR, primers A1 and primer 24 (5' CCA AGC TTG GTG CTC ACT GCC TGT TCC GTT A 3') were used to amplify the putative P3 promoter region, which does not have P1 and P2 of *hemA* (Table 7 and Figure 9). A 288-bp fragment — FL24, corresponding to –551 to –264 in the *hemA* upstream region, was amplified by PCR and purified on a 1% agarose gel. FL24 was digested by *Sal*I and *Hin*dIII, and ligated into the same polylinker site of pUC19, creating pFL24 for sequencing; and in front of the promoterless CAT gene in pKK232-8, creating pFL25-CAT for detecting the putative P3 promoter by a CAT ELISA assay.

3.2.2. The structure of the FL24 fragment

The FL24 fragment is 288 bp, corresponding to the fragment from –264 to –551 of the 5'upstream region of *hemA* (The first base of the *hemA* coding region is numbered as +1). Both strands of the FL24 fragments were completely sequenced. The sequence is completely correct.

3.2.3. The putative P3 promoter

pFL25-CAT was transformed into XL-1 Blue MR. CAT expression of pFL25-CAT was compared with empty plasmid pKK232-8 in XL-1 Blue MR. There was a two to three-fold increase of CAT expression by pFL25-CAT, compared with pKK232-8, although the amount of CAT produced is much less than for constructs

containing the intact 5' upstream region of *hemA* (Figure 18). P3 may be a weak promoter of *hemA* activated under specific stress conditions. This experiment was done three times under the same conditions. Figure 18 shows the mean of the three repeats and their standard deviation.

All the data obtained in Figure 15 and Figure 16, indicated that a point mutation on the putative P3 promoter of *hemA* or and the other point mutation downstream of the P2 promoter may lead to a two-fold increase of CAT expression. The result in Figure 18 suggested there was a very weak P3 promoter activity–about 100 times weaker than that of P1, P2 and putative P3 together. A point mutation on putative P3 promoter may change the strength of the putative P3 promoter. We do not know that FL24 may have a weak promoter activity; or the expression of CAT driven by FL24 is due to the random sequence insertion in front of the promoterless CAT gene in pKK232-8.

The putative P3 promoter may be a σ^{24} – dependent promoter (Figure 4). In order to look for the putative σ^{24} – dependent P3 promoter in response to heat stress, pFL25-CAT and pKK232-8 were transformed into a low copy plasmid strain (*pcnB-*) and heat for 1 hour at 43°C. Heat shock did not induce the CAT expression in pFL25-CAT. In low copy plasmid strain, the putative P3 promoter activity was not detected both at normal condition and heat stress condition. This experiment was done once (data not shown).

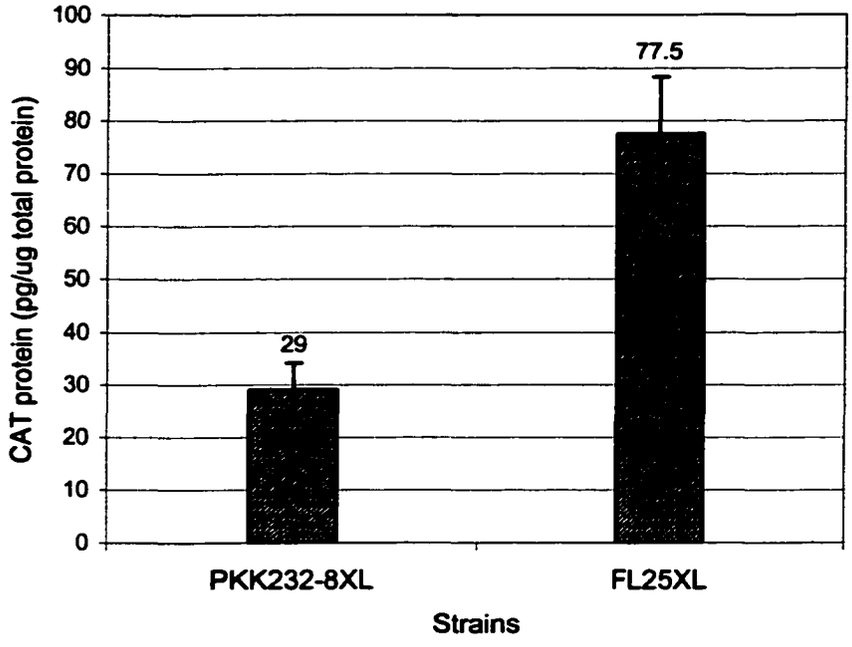


Figure 18: Detection of the putative P3 promoter activity in the 5' *hemA* upstream region

The results are expressed as CAT protein (CAT pg/ug total protein). The data bar shows the average of three repeats of CAT assays. Standard deviations are shown in the Y error bar. PKK232-8XL: pKK232-8 transformed into XL-1 Blue MR as a control; FL25XL: pFL25-CAT (containing the putative P3 promoter, and SL1 and SL2 without P1 and P2) transformed into XL-1 Blue MR.

3.3. Transcriptional regulation of *gltX* by over-expression of *hemA* or *gltX*

3.3.1. Construction of pGLTX-CAT operon fusion (Figure 20)

The 5' upstream region of *gltX* was amplified by PCR from *E. coli* C600 genomic DNA. A5 and A6 were used as forward and backward primers respectively (A5: 5' TTC TAA AGA AAT TGT TCG TTC G 3', and A6: 5' CCA AGC TTG CGT TTA GAA AGA TGC CGA CAA CCG 3') (Table 7 and Figure 19). *E. coli* genomic DNA was used as the template. The PCR products were resolved on a 1% agarose gel. A 200-bp band was isolated on a 1% agarose gel. After gel purification, the 200-bp fragment was ligated into PCR4Blunt-TOPO vector and transformed into one shot TOP10 chemically competent cells. Transformants were selected by blue-white screening. The instructions in the TOPO cloning kit were followed. Restriction enzyme analysis confirmed the size and oritation of the insert in the plasmid, named pGLTXUP. The GLTXUP fragment in pGLTXUP was sequenced. pGLTXUP was further digested with *Pme*I and *Hin*dIII. The 200-bp fragment was purified on a 1% agarose gel. The pKK232-8 vector was digested with *Sma*I and *Hin*dIII. The 200-bp GLTXUP fragment was ligated into the *Sma*I and *Hin*dIII sites of pKK232-8 to create the pGLTX-CAT operon fusion. Restriction enzyme analysis confirmed the size of the fusion insert (data not shown).

-256 GCTGTGTGGA GTCGCATTAT AGGGAGAGTT CAAAATGAGT

-216 CAACGCATTT TCTAAAGAAA TTGTTCGTTC GTCGTAAATT

5' → Primer A5[2] 3'

-176 TAAGCAAGAT GATCGCAAAA CAGACCGTGT TGCGCAATTT

P1[5] (-35)

-136 GTCAACGAAA ACAATAATGC GTAAGGTAGA AACCCGAACT

P2 (-35) P1 (-10) P2 (-10)

-96 ACATTGAGGA ATCAGGCGGG AGTGATAGAA TATCGCCCAC

P3 (-35) P3 (-10)

-56 TTAATTTTTC CAGGATTTGC CGGTTGTCGG CATCTTTCTA

3' GCCAACAGCCGTAGAAAGAT

Primer A6[3] ←

-16 AACGTAAGGC CATTTCATGA AAATCAAAAC TCGCTTCGCG

RBS[4] +1

TTGCGTTCGAACC 5'

+24 CCAAGCCCAA CAGGCTATCT

Figure 19: The *gltX-ValU* upstream region. (Brun, *et al.*, 1990)

1. The sequence is numbered with the first nucleotide of the *gltX* coding position as +1.

2. A5 is the forward primer used to amplify the *gltX* upstream region.

2. A6 is the backward primer used to amplify the *gltX* upstream region.

4. RBS: *gltX* ribosome-binding site is boxed.

5. Three promoters —— P1, P2 and P3 are underlined

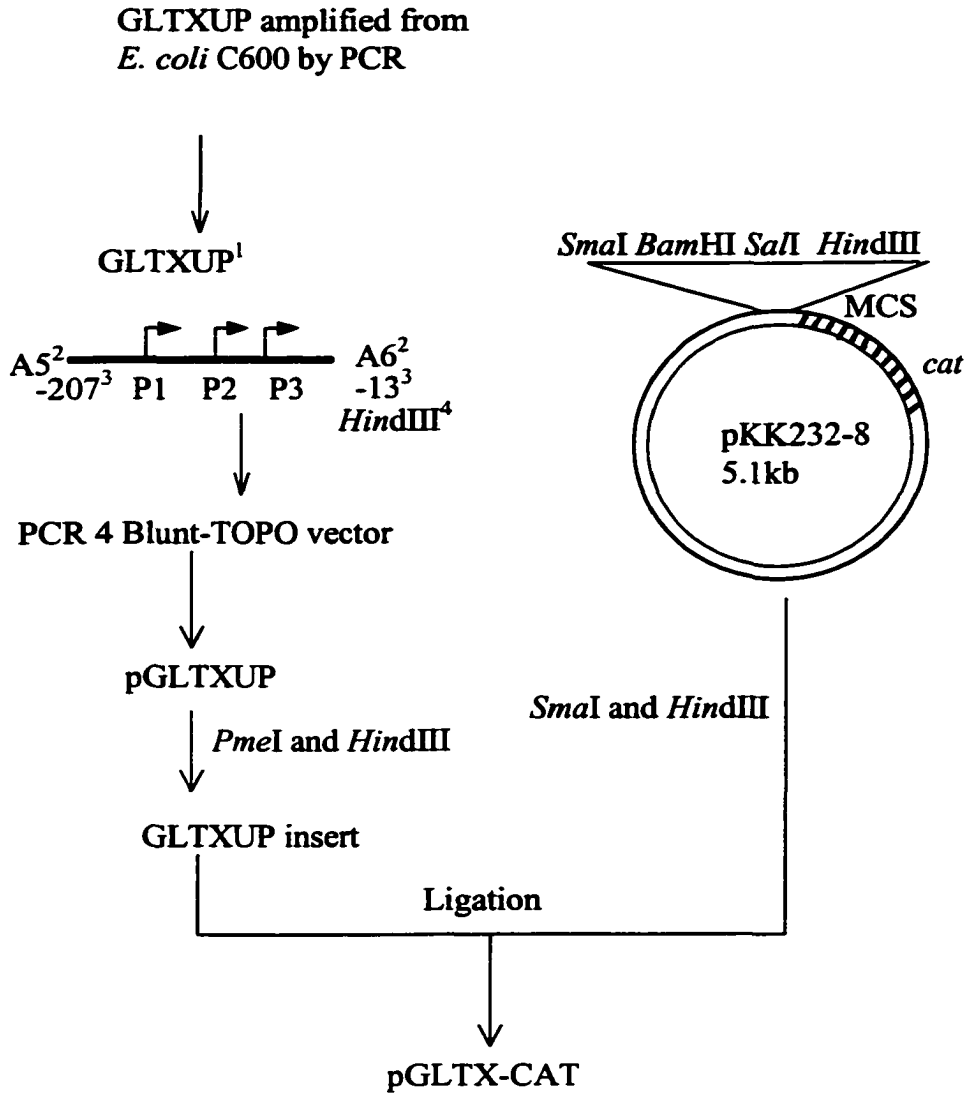


Figure 20: Construction of pGLTX-CAT (see text for explanation)

1. The fragment of the 5' upstream region of *E. coli gltX* generated by PCR.
2. A5 and A6 primers were used to amplify GLTXUP.
3. GLTXUP- the PCR product is from –207 to –13 of the *gltX* upstream region. The first base of *gtlX* coding region is +1.
4. *Hin*dIII cutting site is in A6 primer.

3.3.2. The structure of the GLTXUP fragment

The GLTXUP fragment is 195 bp, corresponding to the fragment from –13 to –207 of the 5'upstream region of *gltX* (The first base of the *gltX* coding region is numbered as +1). The structure of GLTXUP was confirmed by sequencing both strands.

3.3.3. The effect of GTS and GTR on regulation of *gltX* upstream region (Figure 21)

The pGLTX-CAT operon fusion was cotransformed with pFL184, pFL184HEMA and pFL184GLTX respectively into XL-1Blue MR. The resulting three bacterial strains were grown under ampicillin (100 μg/ml) and tetracycline (20 μg/ml) selection, at 37°C overnight with weak shaking (100-150 rpm). The amount of CAT protein per total protein was determined by CAT ELISA assay. The results suggest that over-expression of *hemA* or *gltX* does not influence *gltX* transcription under our experimental conditions (Figure 21). This experiment was repeated twice under similar conditions.

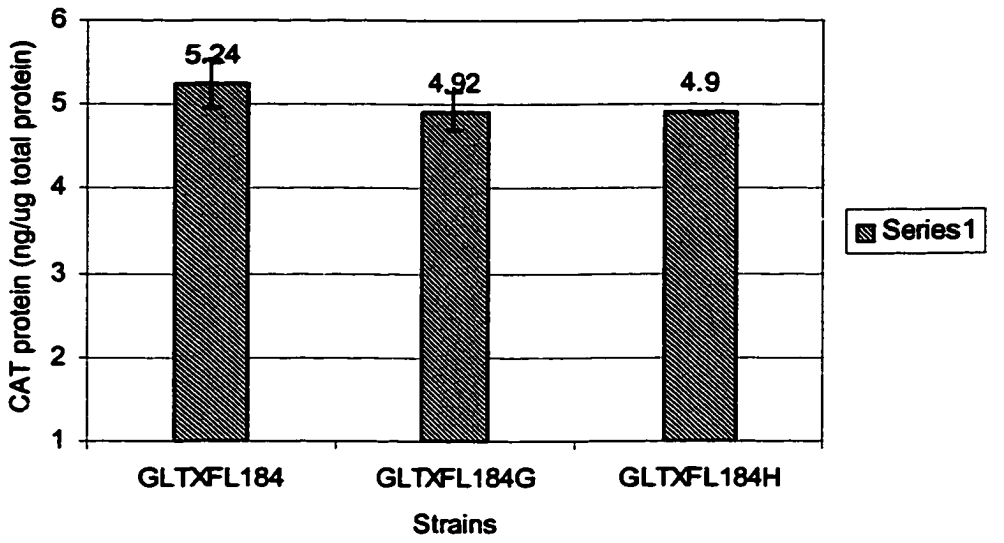


Figure 21: The effect of over-expression of *gltX* or *hemA* on promoter activity of the 5' upstream region of *gltX*

The results are expressed as CAT protein (CAT ng/ug total protein). The data bar shows the average of duplicates in CAT assays. Standard deviations are shown in the Y error bar. Series 1: The average CAT in overnight culture. This experiment was repeated twice under similar conditions. Similar results were obtained. Strains – GLTXFL184, GLTXFL184G and GLTXFL184H are described in Table 3. GLTXFL184: control; GLTXFL184G: over-expression of *gltX* in the strains; GLTXFL184H: over-expression of *hemA* in the strains.

3.4. The effect of expression of HemX from *B. subtilis* on heme permeability of *E. coli*

3.4.1. Construction of pHEMAX3 and pHEMX21

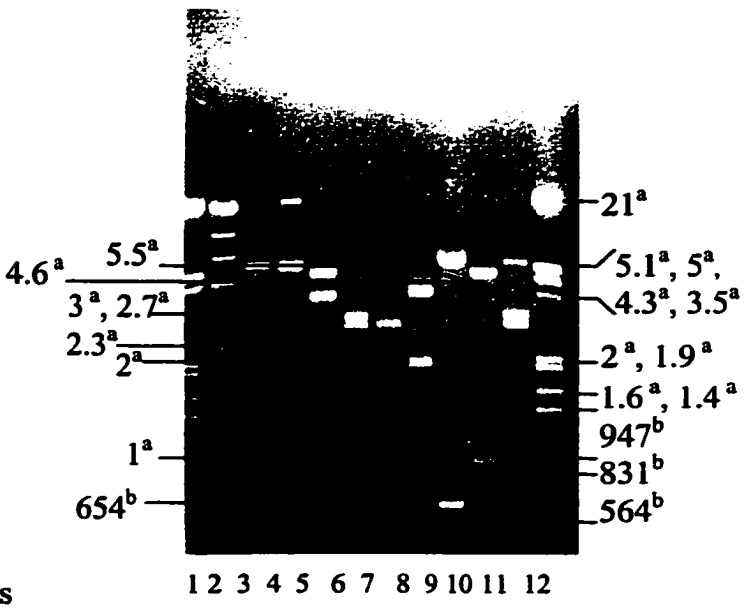
The restriction enzyme cutting sites of *hemAX* fragment of *B. subtilis* are shown on Figure 10. The *hemAX* fragment was subcloned into a modified pUC19 (*SalI*$^-$) vector at *Bam*HI-*Eco*RI cutting sites, creating pHEMAX3. pHEMAX3 was digested with *SalI* to delete 654-bp of the *hemA* coding region and self-ligated to give pHEMX21.

Restriction enzyme analysis of pHEMX21 was done after construction. The expected results can be calculated from Figure 10 and Figure 11. The expected bands after restriction enzyme digestion of pHEMX21 are listed in Table 8. The restriction enzyme digestion analysis of pHEMX21 is shown in Figure 22. The results are summarized in Figure 22.

From restriction enzyme analysis results, the structure of pHEMX21 was confirmed. It suggested that pHEMAX3 and pHEMX21 were correctly constructed and located in the same orientation in front of the *lac* promoter in modified pUC19 (Figure 22). pHEMA2 (*B. subtilis hemA*), pHEMAX (*B. subtilis hemA+hemX*), pHEMX21 (*B. subtilis hemX*) and pHEMAX3 (*B. subtilis hemA+hemX*), were transformed into SASX41B strain by electroporation (Table 4). After transformation, HEMX21S and HEMAX3S were cultured in LB media and LB plates, supplemented with 100 μg/ml ampicillin and 40 μg/ml ALA to facilitate their growth.

Table 8: The calculated fragments by restriction enzyme analysis of pHEMAX, pHEMA2 and pHEMX21.

alculated ragments	*Sal*I	*Eco*RI& *Bam*HI	*Eco*RI& *Sal*I	*Xba*I& *Eco*RI	*Bam*HI& *Sal*I
HEMX21	5.7 kb	3 kb 2.69kb	3.69kb 2 kb	2.69 kb 3 kb	4.69 kb 1 kb
HEMAX3	5.7 kb 654 bp	2.69kb 3.6 kb			
UC19 *Sal*I⁻)	NO cutting Site	2.69kb			
HEMAX	654 bp 1 kb 6.9kb	3.6 kb 4.9kb			
HEMA2	654 bp 1 kb 5.4 kb				



Lane 1: λ*Hin*dIII/EcoRI markers

Lane 2: λ*Hin*dIII marker

Lane 3: pHEMX21 cut by *Sal*I

Lane 4: undigested pHEMX2

Lane 5: pHEMAX cut by *Eco*RI & *Bam*HI

Lane 6: pHEMX21 cut by *Eco*RI & *Bam*HI

Lane 7: pUC19 cut by *Eco*RI&*Bam*HI

Lane 8: pHEMX21 cut by *Eco*RI & *Sal*I

Lane 9: pHEMAX3 cut by *Sal*I

Lane 10: pHEMX21 cut by *Bam*HI & *Sal*I

Lane 11: pHEMX21 cut by *Eco*RI & *Xba*I

Lane 12: λ*Hin*dIII/*Eco*RI markers

Figure 22: Restriction enzyme digestion analysis of pHEMX21 (Continue)

Figure 22: (Continued)

The molecular sizes of *Hin*dIII-digested λ are 23kb, 9.4kb, 6.6kb, 4.4kb, 2.3kb, 2.0kb and 564bp. The molecular sizes of *Hin*dIII/*Eco*RI-digested λ are 21kb, 5.1kb, 5kb, 4.3kb, 3.5kb, 2.0kb, 1.9kb, 1.6kb, 1.4kb, 947bp, 31bp and 564bp. In lane 9, pHEMAX3 was digested with *Sal*I. A 5.7-kb and and the 654-bp fragment from the *hemA* coding region are on the gel. In lane 3, pHEMX21 was linearized with *Sal*I. A 5.7-kb band is on the gel. In lane 6, pHEMX21 was digested with *Eco*RI and *Bam*HI. Two bandsare 2.69-kb pUC19 vector and 3-kb *hemX* fragment with its in-frame promoter. In lane 7, a single band is the linearized pUC19 (2.69 kb). In lane 8, pHEMX21 was cut with *Eco*RI and *Sal*I. Two bands on the gel are 3.6 kb and 2 kb. In lane 10, pHEMX21 was digested with *Bam*HI and *Sal*I. Two bands on the gel are 4.7 kb and 1 kb. In lane 11, pHEMX21 was digested with *Xba*I and *Eco*RI. Two fragments are 3.0 kb and 2.7 kb. A 5.7-kb fragment on the gel is due to incomplete digestion.

a. Unit is kb. b. Unit is bp.

3.4.2. Complementation of SASX41B mutant by pHEMA2, pHEMAX and pHEMAX3

I further tested whether pHEMA2, pHEMAX and pHEMAX3 can complement the SASX41B mutant. HEMA2S (Table 4) grew on LB with chloramphenicol (50 μg/ml and 25 μg/ml) at 37°C overnight. The colonies were relatively small, compared with FL184HEMAS. This suggested that the *B. subtilis hemA* complements SASX41B less efficiently than the *E.coli hemA*. This is most likely due to 32% amino acid sequence identity of GTR between *E. coli* and *B. subtilis*.

HEMAXS (Table 4) grew on LB with chloramphenicol (50 μg/ml and 25 μg/ml) at 37°C overnight. The colonies were very tiny. After more than two days incubation, they reached the normal colony size. HEMAX3S grew on LB with ampicillin (50 μg/ml) at 37°C overnight. The colonies were very small too. It took more than two days incubation, before they reached normal size. The results show both *B. subtilis hemAX* or *hemA* can complement the SASX41B mutant, but *hemAX* was much less efficiently than the *B. subtilis hemA* alone in *E.coli*. This suggested that *hemX* down-regulated GTR even in the *E. coli* host.

3.4.3. Down-regulating *B. subtilis* GTR in *E. coli* by HemX.

Porphyrin accumulation in HEMAXC and HEMA2C was checked, after growth on LB plates with chloramphenicol (50 μg/ml and 25 μg/ml) at 37°C for three days. HEMA2C became fluorescent, but HEMAXC did not. These results suggested that HemX down-regulated *B. subtilis* GTR. After pFL184HEMA and pHEMX21

were cotransformed into SASX41B (Table 4), porphyrin accumulation was examined in both FL184HEMAS and FL184HEMAXS. FL184HEMAS and FL184HEMAXS both became fluorescent, suggesting *hemX* expression was not as effective in suppressing GTR from *E. coli.*

3.4.4. Determining the hemin permeability of HEMX21S

pHEMX21 was transformed into SASX41B to give HEMX21S. HEMX21S, transformant, was not hemin permeable because it did not grow on LB plate with ampicillin (50 μg/ml) and hemin (4 μg/ml) at 37°C overnight. PUC19H — pUC19 transformed into HU227 (hemin permeable strain), and PUC19S — pUC19 transformed into SASX41B (hemin-impermeable strain) were positive and negative controls respectively (Table 4). PUC19H grew well on LB plate with ampicillin (50 μg/ml) and hemin (4 μg/ml) at 37°C overnight. PUC19S and HEMX21S did not grow on the same plate under the same conditions. Therefore it appears that HEMX21S is hemin-impermeable. It suggests that *hemX* in *B. subtilis* down-regulates *B. subtilis* GTR without changing the hemin permeability. However, HemX may function as a receptor since HemX is an 8 integral membrane protein. Since HemX has a putative conserved cytochrome c assembly protein domain, it is not known whether HemX is involved in cytochrome c biogenesis. We also do not know whether HemX is involved in activation of protease action for GTR to down-regulate GTR at the post-translational level.

DISCUSSION:

4.1. The gene regulation of *hemA*

4.1.1. The effect of the two stem-loops on the promoter activities of the *hemA* upstream region

It was found that GTS and GTR may recognize the different parts of $tRNA^{Glu}$ and form a complex with $tRNA^{Glu}$ (Jahn, 1992b; Stange-Thomann *et al.*, 1994; Willows *et al.*, 1995). Since the putative transcripts of SL1 and SL2 look like $tRNA^{Glu}$, we also assume that either GTS or GTR, or both may form a complex with the putative transcripts of SL1 and SL2 to regulate *hemA* gene expression. After deletion of SL1 and SL2 by PCR, we do not find any effect on the transcriptional regulation of *hemA* by the CAT assay. The study was done with both low copy plasmids and high copy plasmids. We co-transformed XL-1 Blue MR with pWH515-CAT and a plasmid harboring *gltX* or *hemA* respectively, and compared the CAT expression with the strain harboring pWH515-CAT alone. The CAT expression did not change. It suggested that over-expression of GTS or GTR does not change *hemA* promoter activity.

Our data indicates that SL1 and SL2 do not play a significant role in regulation of the *hemA* transcription under normal conditions.

4.1.2. A putative P3 promoter

After deletion of P1 and P2, we studied the putative P3 promoter activity of *hemA*. The putative P3 promoter activity is very weak, only accounting for 1% of promoter activities of the *hemA* upstream region in medium copy plasmids. We do

not know if this weak activity is due to the random sequence insertion in front of the CAT promoterless vector, or a promoter activity. Dr. Hua (1997) suggested that the putative P3 promoter is either a σ^{70}-dependent promoter or, and σ^{32}-dependent promoter (Figure 4 and Table 9).

The putative σ^{70}-dependent P3 promoter has 4 bases matched to the consensus sequence in –35 (P3-TTAAGA/consensus sequence-TTGACA), and 3 bases matched in –10 (P3-GATGCT/consensus sequence-TATAAT) (Figure 4). A point muation in pFL509-CAT changes the –10 region from P3-GATGCT to P3-GATTCT. In –10 region, adenine at –11 and thymine at –7 are most conserved and critical for RNA polymerase binding (Xu *et al.*, 2001). Dr. Xu *et al.* found that adenine at –9 is also critical for RNA polymerase binding. In pFL509-CAT, we changed G→T at –9. We do not know that this point mutation combined the mismatches at –12 and –8 at the putative σ^{70}-dependent P3 promoter may create a strong promoter. It is not clear that increase of CAT expression of pFL509-CAT is due to the point mutation at the putative σ^{70}-dependent P3 promoter. The other point mutation downstream of the P2 promoter of *hemA* at the -78 position creates a stronger G-C stem in pFL509-CAT (Figure 4). The possible change of the secondary structure of mRNA of *hemA*, leading to greater stabilization of the mRNA and increase the half-life of mRNA is remained to be determined.

Table 9: The sequence of the putative P3 promoter of *E. coli hemA* in –10 and –35 regions[1]

The putative P3 promoter of *hemA*	-10 regions	-35 regions
σ^{70}-dependent P3 promoter of *hemA*	GATGCT	TTAAGA
Consensus sequence of σ70-dependent promoter	TATAAT	TTGACA
σ^{32}-dependent P3 promoter of *hemA*	CGCCAT	ATTGCGC
Consensus sequence of σ^{32}-dependent promoter	CCCCAT	CTTGAAA
σ^{24}-dependent P3 promoter of *hemA*	TAAGCGA	ACACTT
Consensus sequence of σ^{24}-dependent promoter	TCTGA	GAACTT

1. The location of the putative P3 promoter *in E. coli hemA* is in Figure 4.

σ^{24} and σ^{32} are members of a sigma family that responds to stresses, such as heat shock and other environmental stresses (for review, see Gross 1996). The *E. coli* σ^{24} regulon is induced in response to a periplasmic stress. The *E. coli* σ^{32} regulon is induced in response to a cytoplasmic stress (Raivio *et. al.* 2001). The consensus sequence of σ^{32} –dependent promoters is CTTGAAA in –35 regions, and CCCCAT in –10 regions of the promoters (Gross, 1996). Based on the current consensus sequence of σ^{32} –dependent promoter, there are 3 bases matched in –35 region; and 5 out of 6 bases matched in –10 region between the putative σ^{32} –dependent promoter of *hemA* and the consensus sequence of σ^{32} –dependent promoters (Figure 4).

The genes which σ^{24} controls transcription include the expression of its own genes, σ^{32}, periplasmic protease (HtrA/DegP) and periplasmic peptidyl prolyl isomerase (FkpA), which functions as a folding envelope protein, etc (Dartigalongue *et al.*, 2001). The authors made a sequence alignment of σ^{24}-dependent promoters. After calculating the frequency of the homologous sequence, the consensus sequence corresponding to the –10 region of promoters is TCTGA, and –35 region of promoters is GAACTT. The space between –10 and –35 regions of promoters is 12 to 19 bps. The space between –10 region of promoters and initiation site is 3 to 14 bps. The putative σ^{24} –dependent promoter sequence of *hemA* was searched. The sequence AGCGA in corresponding –10 region and ACACTT in corresponding –35 region of σ^{24}-dependent P3 promoter in the *hemA* upstream region were found (Figure 4 and Table 9). Totally, 6 bases out of 11 bases are matched to the consensus sequence of σ^{24}-dependent promoters in –10 and –35 regions. The space between –10region and –35 region is 17 bp. In order to further determine the putative σ^{24}-dependent P3

promoter in the *hemA* upstream region, primer extension analysis can be used to directly detect the possible transcripts. The cells can be shifted to 50°C for 5 to 10 min from 30°C to induce σ^{24} expression. Complementary oligonucleotides based on the *hemA* sequence can be used as primers. Avian myeloblastosis virus reverse transcriptase can be used for strand extension.

4.1.3. Future study in regulation of *hemA*.

Evidence suggested that GTR and GTS may form a complex with $tRNA^{Glu}$ (Jahn, 1992b). Over-expression of $tRNA^{Glu}$ and both GTS and GTR can be used to detect their interaction and determine their regulation of *hemA*.

Recent evidence suggests that regulation of *hemA* at the transcriptional level is very slight. During heme starvation, the expression of *LacZ* increases very slightly in a *hemA-lacZ* operon fusion (Choi *et al.*, 1996; Nakayashiki *et al.*, 1997). By contrast, the porphyrins accumulate dramatically. The same results were observed in *E. coli* and *S. typhimurium* by Wang *et al.* (1997). It suggested that regulation of *hemA* is at the post-transcriptional level.

Immunological detection of GTR protein by Western blot during the heme starvation created by *hemL*, *hemB*, *hemE* and *hemH* mutants showed a greatly increased amount of GTR protein in *S. typhimurium* (Wang *et al.*, 1997). When supplemented with heme, the amount of GTR decreased again. The GTR enzymatic activity increased 20- to 25-fold in the heme starvation created by *hemE* and *hemB* mutants and 15-folds in a *hemH* mutant. Increase of GTR during heme starvation was also observed in *E. coli* by the same method. The regulation of *hemA* by heme is

conserved in *E. coli* and *S. typhimurium*. Wang *et al.* (1999a) used pulse-chase analysis to study GTR turn-over during heme starvation created by *hemL* deletion in S. *typhimurium*. It was found that GTR turnover decreased 15- fold during heme starvation. Both *lon* and *clpP* proteases were required for GTR turnover in this study. The findings suggested that heme down-regulates *hemA* expression. Heme regulates *hemA* expression at the post-translational level through conditional proteolysis of GTR. It was also found that the HemA1-18-LacZ fusion did not respond to heme starvation. The HemA1-416-LacZ fusion responded to heme starvation. It also suggested that the first 18 amino acids at the N-terminal end of GTR are not enough in regulation of *hemA* during heme starvation.

The N-terminal end of GTR serves as the tag for proteolysis regulated by heme in *S. typhimurium*. Wang *et al.* (1999b) created a mutant GTR (HemA[KK]) with positive charge close to the N-terminus by inserting two-lysine residues between the 2nd- and 3d- amino acid position of wild-type GTR (HemA [WT]) by site-directed mutagenesis. Pulse-chase analysis was used to determine the turnover of HemA[KK] and HemA [WT] during heme starvation. It was found that the turnover of HemA[KK] decreased more than 15 times compared to that of HemA [WT]. So HemA[KK] is stabilized against proteolysis regulated by heme. The N-terminal end of GTR is important for proteolysis regulated by heme.

Lon and clpAP protease activity was involved in regulation of GTR stability (Wang *et al.*, 1999a; Wang *et al.*, 1999b). The regulation of σ^{32}, a heat shock transcription factor, and UmuD involved in regulation of the SOS response to damaged DNA are also involved in proteolysis. Lon is a single chain enzyme; and

the clpAP family proteases are double chain enzymes (Wawrzynow *et al.*, 1996). They share homologous substrate-recognition domain-sensor and substrate-discrimination domains (SSD domain) (Simth *et al.*, 1999). Simth *et al.* (1999) analyzed the target-recognition of the SSD domain. They found that the SSD domain functions in energy-dependent substrate-recognition. 25 amino acids in the N-terminal region of UmuD can be specifically recognized and bound by SSD domain of Lon. ClpYQ degrades σ^{32} at the C-terminal end. ClpX and ClpXP degrade Arc-ssrA at the C-terminal end. The SSD domains of Lon and Clp proteases can specifically recognize protein targets. So, regulation of GTR shares the similar mechanism with that of σ^{32} and UmuD. It will be interesting to study how cellular heme change regulates specific protease activity. In *E. coli* and *S. typhimurium,* a heme binding-site in GTR was not found, although, heme was co-precipitated from GTR in plants (Vothknecht *et al.*, 1996). Computer analysis of barley predicted a heme-binding site (Brody *et al.*, 1999). In *S. typhimurium,* the first 18 amino acids at the N-terminus of GTR did not response to heme starvation although it is sufficient to be the target for Lon digestion (Wang *et al.*, 1999b). More coding sequence of the GTR is required for responding to cellular heme changes. The specific region of GTR responding to heme has not been studied in *E. coli* and S. *typhimurium.* If heme can bind to GTR, it is possible to change the GTR conformation due to the high hydrophobic character of heme and thus induce the targeting proteolysis. If heme does not directly bind to GTR, heme may signal other chaperons to change the GTR conformation and regulate GTR proteolysis.

In summary, based on current studies, the regulation of *hemA* should focus on the post-transcriptional level, especially the post-translational level.

4.2. The gene regulation of *gltX* in *E. coli*

4.2.1. The effect of over-expression of *hemA* or *gltX* on promoter activity of *gltX* in *E. coli*.

We do not find that over-expression of *hemA* or *gltX* regulates the promoter activity of *gltX* in *E. coli*. The regulation of *gltX* is complicated since it is involved in protein synthesis.

In *E. coli*, there are three FIS binding sites in the *gltX* upstream region. FIS down-regulates itself about two- fold during growth acceleration, but not in steady-state. The *valU* promoter region is required for the regulation (Champagne and Lapointe, 1998). Champagne *et al.* (1998) suggested that FIS may only modulate the expression of *gltX*.

4.2.2. Future study

Whether and how GTS is auto-regulated is not clear in *E. coli*. The auto-regulation of *E. coli* threonyl-tRNA synthetase is at the translational level. The leading transcript before the ribosome binding-site serves as a translational operator (Springer *et al.*, 1986). The translational operator has four domains — ribosome binding domain (domain 1), recognition domain (stem-loop) (domain 2), a linker which links domain 2 and domain 4 (domain 3) and a binding domain (another stem-loop) which binds threonyl-tRNA synthetase (domain 4). Domain 2 and 4 are similar

to the anticodon of $tRNA^{thr}$. The binding of threonyl-tRNA synthetase to domain 2 and 4 represses the translation from the domain 1 (Brunel *et al.*, 1995. and Romby, *et al.*, 1996). In the *gltX* upstream region, there is a stem-loop in the front of the ribosome- binding site (Figure 7). The transcript of the stem-loop is similar to the anticodon and loops of $tRNA^{Glu}$, which serve as a possible binding-site for GTS or GTR (Brun *et al.*, 1990). It is unclear that regulation of *gltX* expression shares the similar mechanism with the regulation of threonyl-tRNA synthetase at the translational level. We only studied over-expression of GTR or GTS on regulation of promoter activity of *gltX*. pGLTXUP-CAT uses the ribosome binding-site of CAT. We can construct a protein fusion by inserting the *gltX* upstream region with its own ribosome binding-site and the amino acids of the coding sequence in front of a protein fusion vector. Regulation at the translation initiation level can be studied. Site-directed mutagenesis can be used to study the specific domains involved in translational regulation.

4.3. Function of HemX

HemX is a membrane protein in *B. subtilis*. HemX down-regulates the *B. subtlis hemA* expression. We do not know the mechanism. We do not find that HemX changes heme permeability in *E. coli*. Maybe the system we used can not detect the change or HemX does not change heme permeability. Computer analysis predicted that HemX has a cytochrome c assembly protein domain. We do not know whether HemX is involved in cytochrome c biogenesis.

Heme is a highly hydrophobic molecule. Hemin can interact with the phospholipid bilayer (Cannon *et al.*, 1984). Gram-negative bacteria, such as wild-type *E. coli and S. typhimurum* , are impermeable to exogenous heme because of the outer-membrane barrier (Sasarman *et al.*, 1970; Sasarman *et al.*, 1968; McConville&Charles., 1975 and 1979; Janzer *et al.*, 1981).

In Gram-negative bacteria such as *Shigella dysenteriae* (Mills *et al.*, 1997), *Yersinia enterocolitica* (Stojiljkoviv &Hantke., 1992), *Yersinia pestis* (Thompson *et al.*, 1999), many pathogenic *E. coli* strains (Torres *et al.*, 1997; Nagy *et al.* 2001), *Vibrio cholerae* (Henderson and Payne 1993 and 1994), and other pathogenic *Vibrio* (Litwin and Byrne, 1998; O'Malley, 1999), *Haemophilus ducreyi* (Thamas *et al.*, 1998), *Haemophilus influenzae* type b (Cope *et al.*, 1995), and *Pseudomonas aeruginosa* (Vasil *et al.*, 1999; Ochsner *et al.*, 2000) etc, translocation of heme across the outer-membrane is dependent on expression of heme receptor in the outer-membrane.

The heme-utilization gene, *chuA*, was isolated from enterohaemorrhagic *E. coli* O157:H7 strain (Torres *et al.*, 1997). ChuA encoded by *chuA* is a 69kDa outer membrane protein. Expression of *chuA* in a wild-type *E. coli* strain can make the strain use hemin (Torres *et al.*, 1997). Both *chuA* and *ton* are required to make *E. coli* permeable to hemin. Hemin receptor HemR from *Y. enterocolitica* is a 78-kDa outer-membrane protein. Cells that have lost the HemR receptor and had muted *tonB* gene were unable to take up hemin (Stojiljkoviv &Hantke., 1992). ShuA, a 70-kDa outer-membrane protein encoded by *shuA* was identified as a heme receptor in *S. dysenteriae* (Mills *et al.*, 1997). *chuA* is highly homologous to *shuA*, less homologous

to *hemR* (Stojiljkovic & Hantke, 1992; Torres & Payne, 1997). *chuA, hemR*, and *shuA* share the conserved Fur-box and are Ton-dependent. Further translocation of heme across cytoplasmic membrane is through an ABC transporter-Ton complex (Stojiljkovic &Hantke, 1994; Lee, 1995). The cytoplasmic membrane is leaky to heme (Stojiljkovic &Hantke, 1994).

hemX encoding HemX in the *B. subtilis hemAXCDBL* operon (Petricek *et al.*, 1990), is not present in *E. coli.* By using an *E. coli* minicell system, HemX was found in the membrane fraction of *E. coli.* By using rocket immunoelectrophoresis, it was found that *B. subtilis* HemX down-regulates *B. subtilis* GTR in *B. subtilis* and a heterogeneous strain, *E. coli.* There is no change of GTR turn-over when HemX down-regulates *B. subtilis* GTR (Schröder *et al.*, 1994). *hemA-lacZ* fusions did not reveal regulation on its major promoter or on translation (Schröder *et al.*, 1994). We transformed a plasmid harboring *hemX* into an *E. coli* strain which is heme impermeable. Over-expression of *hemX* did not change *E. coli* into a heme-permeable strain. It is because HemX is probably not a heme recepter. HemX is only found in Gram-positive bacteria, and located in the cytoplasmic membrane (Johansson & Hederstedt, 1999). HemX is not in the outer-membrane of Gram-negative bacteria, in which the receptor for heme is required for heme-permeability. It may be reasonable to expect that HemX can not change *E. coli* to be permeable to heme.

Computer analysis predicted that HemX is a 7- transmembrane protein, and has a putative cytochrome c assembly domain. Cytochrome c assembly in Gram-positive bacteria is outside the cytoplasmic membrane. Cytochrome c assembly in Gram-negative bacteria is in the periplasm. Cytochrome c biogenesis needs heme and

apocytochrome transported separately to their functional site to be assembled. There are two systems involved in cytochrome c assembly (for review, see Goldman & Kranz, 2001). There are four genes in system II, *ccsA, ccsB, ccsX* and *ccdA*. *ccsA* encodes a 6 transmembrane- domain protein and a periplasmic WWD domain, which has the conserved tryptophan-rich region and key histidinyl residues (Goldman *et al.*, 1998). The function of the highly conserved WWD domain is not completely understood. The authors suggested that the WWD domain may be involved in maintaining the heme in proper oritation for transportation to apocytochrome c mixed disulfide at cytoplasmic membrane in order to synthesize cytochrome c. Plants and some Gram-positive bacteria such as *B. subtilis* use system II. There is no ABC transporter in system II. There are 9 genes in system I. HelC encoded by *helC* (*ccmC*) in *helABCD* (*ccmABCD*) encoding a ABC transporter has the WWD domain, and plays a central role in heme transport. Gram-negative bacteria like *E. coli* use system I. HemX has the putative cytochrome c assembly domain and shows 30% amino acid sequence identity with CcsA from green plant *Mesostigma viride* (www.ncbi.nlm.nih.gov/BLAST). There is no WWD domain in *B. subtilis* HemX. The function of HemX is still unknown.

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