Iron Regulation of Gene Expression in the Bradyrhizobium Japonicum/Soybean Symbiosis

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Progress Report

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We wish to address the question of whether iron plays a regulatory role in the *Bradyrhizobium japonicum*/soybean symbiosis. Iron may be an important regulatory signal *in planta* as the bacteria must acquire iron from their plant hosts and iron-containing proteins figure prominently in all nitrogen-fixing symbioses. For example, the bacterial partner is believed to synthesize the heme moiety of leghemoglobin, which may represent as much as 25-30% of the total soluble protein in an infected plant cell. For this reason, we have focused our attention on the regulation by iron of the first step in the bacterial heme biosynthetic pathway. The enzyme which catalyzes this step, 5-aminolevulinic acid synthase, is encoded by the *hemA* gene which we had previously cloned and sequenced (Guerinot and Chelm, 1986; McClung et al., 1987).

We originally proposed five specific aims for this project. During this first funding period which began on June 1, 1991, we have concentrated on Specific Aim #1 and have made some progress on Specific Aims #3, 4 and 5. I will summarize our progress to date below. Part of the work described here was presented at the 13th North American Symbiotic Nitrogen Fixation Conference held in Banff, Alberta in August, 1991 (See attached Abstract). Dr. Harry Kurtz, a postdoctoral reserach associate, joined this project on November 1, 1991; his Curriculum vitae is attached.

Specific Aim 1. To define the *cis*-acting sequences which confer iron regulation on the *B. japonicum hemA* gene. We will also examine iron control of the *R. meliloti hemA* gene to allow a comparison between slow growing and fast growing rhizobia.

Deletion analysis of the 5' region. We have documented a 4 to 8 fold induction of a *B. japonicum hemA-lacZ* fusion by iron. We were especially interested in whether a sequence upstream of *hemA* with homology to an "iron box" as decribed for *E. coli* (de Lorenzo et al., 1987) plays any role in the observed regulation of *hemA* by iron. The "iron box" is the site at which the Fur regulatory protein binds to DNA. Fur controls the expression of siderophore (ferric specific ligands) and siderophore receptors by binding to DNA as a dimer, using Fe(II) as a corepressor (Bagg and Neilands, 1987). Iron boxes have been found upstream of iron-regulated genes in *Serratia marcescens* (Poole and Braun, 1988) and *Anacystis nidulans* (Laudenbach and Strauss, 1988), indicating that this motif may be conserved across species lines. We have now constructed and verified by DNA sequencing 10 *B. japonicum hemA-lacZ* fusions in which the 5' sequences have been progressively deleted (Figure 1). These fusions have all been constructed in *E. coli* and moved on plasmids into *B. japonicum* via electroporation. The resulting *B. japonicum* strains carrying these fusions have been assayed for induction of ß-galactosidase activity as follows: cells are first grown in iron deficient medium and the cultures are then split into two aliquots. One receives iron and the other does not. We then examine these cultures 24 hrs later to look for induction of the *hemA-lacZ* fusion. All of the deletions continue to show induction by iron, including those constructs in which the "iron box" has been deleted (Figures 1 and 2). Figure 1 presents the fold-induction for each construct and Figure 2 presents the actual ß-galactosidase activity for all the deletions tested to date. The fusion with the largest upstream deletion which we have examined still has 69 bases of untranslated leader sequence present. Our working hypothesis is that iron regulation is conferred by sequences in the 5' untranslated leader region. Thus we predict that steady state levels of *hemA* mRNA may be similiar in cells grown with and without

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sufficient iron. S1 analysis will be used to determine mRNA levels. We will also examine protein levels using western blot analysis with antibodies to ß-galactosidase. If we are indeed seeing translational control, we should be able to document that the protein levels are changing with respect to iron availability. We are also in the process of making constructs in which the 5' untranslated leader has been completely deleted.

There are precedents for post-transcriptional control by iron in eukaryotes. An iron responsive element (IRE) has been identified which acts as a translational repressor or as an mRNA stabilizer (as reviewed in Klausner and Harford, 1989; Theil, 1990). The IRE is proposed to form a stem-loop structure that binds a cytoplasmic protein, termed the iron responsive element binding protein (IRE-BP). Binding of the IRE-BP is controlled by intracellular iron levels. As cellular iron becomes limiting, a greater fraction of the cell's IRE-BP is recruited into a high affinity binding state. The high affinity interaction between the IRE-BP and the IRE acts as a repressor of translation if the IRE is located in the 5' untranslated leader of an mRNA (e.g. ferritin) or as a repressor of transcript degradation if located in the 3' untranslated region of an mRNA (e.g. transferrin receptor). Most interesting from our point of view are two recent reports on the presence of an IRE in the murine and human erythroid 5-aminolevulinic acid synthase mRNAs (Dandekar et. al, 1991; Cox et al., 1991). The IRE motif occurs in the 5' untranslated leader. We do not see any homology of the *B. japonicum hemA* 5'-untranslated leader to the IRE consensus motif (CXXXXXCAGTGN where X = C,T,G or A and N = C,T or A). This does not mean that *B. japonicum hemA* mRNA does not have an IRE; prokaryotes may have a different sequence which serves the same function. The *fepB* gene in *E. coli* has a number of stem-loops in its untranslated leader which might function in iron regulation (Elkins and Earhardt, 1989; Brickman et al., 1990); *fepB* encodes a periplasmic protein required for transport of the siderophore enterobactin in *E. coli*. There is also a report that a stem-loop present at the 3' end of a *V. anguillarum* gene encoding an iron-regulated outer membrane protein may function in iron regulation (Crosa, 1989).

Gel Retardation and Footprinting Analysis. Because we have identified a region with homology to the *E. coli* "iron box" consensus sequence upstream of *hemA* and because we see some regulation of the *B. japonicum hemA* gene in *E. coli*, we wanted to determine whether the Fur system was involved in *hemA* regulation. As discussed above, Fur is a DNA binding protein known to be involved in the iron regulation of genes in *E. coli*. We have now carried out gel retardation and footprinting experiments using the *B. japonicum* promoter and purified *E. coli* Fur protein (generously provided by Joe Neilands' lab). As shown in Figure 3, we do not see a gel shift for the *hemA* fragment with Fur protein; the positive control behaved as reported in the literature. Footprinting experiments were not carried out as no potential Fur binding sites were identified in the *hemA* promoter via gel retardation.

Iron regulation of the *R. meliloti hemA* gene. We have observed a 7 to 11-fold induction of a *R. meliloti hemA-lacZ* fusion in response to iron. The *R. meliloti hemA* gene also has a region which is homologous to the *E. coli* "iron box". Using a series of plasmid constructs which have progressively larger deletions of the *R. meliloti hemA* upstream region (provided to us by Gary Ditta; Leong et al., 1985), we are in the process of moving these deletions from *E. coli* into *R. meliloti* and assaying these strains for induction by iron. It will be especially interesting to see if fast growers (*R. meliloti*) have the same type of iron regulation as slow growers (*B. japonicum*).

Specific Aim #3. To identify trans-acting factors which regulate the expression of *hemA* by iron.

Screening for regulatory mutants. We have constructed mutants of *B. japonicum* strain USDA 110 using Tn5. The *hemA lacZ* fusion has been moved into these mutagenized cells and a screen for

mutants which do not respond to iron availablity (null mutants) or which express the fusion equally well under conditions of high or low iron (constitutive mutants) is now underway. Null mutants would be indicative of an activator which responds to the presence of iron and constitutive mutants would be indicative of a repressor which is inactivated in the presence of iron.

Effect of heme on expression of *hemA*. It is possible that the 4 to 8 fold induction of ß-galactosidase activity which we have observed for the *B. japonicum hemA-lacZ* fusion in the presence of iron is not a direct effect of iron *per se* but rather is an effect of some iron-containing compound. One possible candidate is heme itself. Cells starved for iron are presumably heme deficient. Of course, it is more likely that heme would be acting as a negative regulator of *hemA* expression rather than as an activator. That is, heme would feedback inhibit its own biosynthetic pathway. To try to address the role of heme itself, we are using a *hemA* mutant of *B. japonicum*, MLG1 (Guerinot and Chelm, 1986) into which we have moved the *hemA-lacZ* fusion. We were originally planning to grow cells of MLG1 carrying the *hemA-lacZ* fusion in medium with ALA and then transfer the cells to minimal media with or without added iron. The expression of the fusion would then be monitored over time. However, Frustaci et al. (1991) have recently reported that MLG1 can grow without ALA supplementation in certain media which contain yeast extract. Remarkably, such cells appear to have no measurable heme. Therefore we will also try growing the cells as described by Frustaci et al. (1991) and determine if the fusion is expressed and if so, if its expression is responsive to addition of iron.

Specific Aim #4. To identify new loci which are transcriptionally responsive to changes in iron availability.

We are searching for genes which are transcriptionally responsive to alterations in iron availability. This should yield two classes of genes: those which have increased expression in the presence of iron and those which have increased expression in the absence of iron. We are using transposon mutagenesis where the transposon carries an easily screenable marker for transcriptional activity at the point of insertion. Dr. Harry Kurtz, a postdoctoral research associate who joined the lab in November, has just begun an analysis of Tn*phoA* mutants of *B. japonicum*. Tn*phoA* creates fusions between target genes and the *E. coli phoA* gene encoding alkaline phosphatase (Manoil and Beckwith, 1985). In-frame fusions encode hybrid proteins with the amino terminal portion of a target gene fused to the carboxyl terminal portion of alkaline phosphatase. Because the alkaline phosphatase encoded by the *phoA* gene of Tn*phoA* lacks the amino terminal sequences necessary for its own secretion and because the cytoplasmic precursor of alkaline phosphatase has no enzyme activity, the Tn*phoA* system will generate PhoA$^+$ fusions only when the transposon is inserted into a gene encoding a secreted or membrane spanning protein. We hope that the transposon mutants generated via Tn*phoA* will be by necessity enriched for mutants with membrane defects and hence a good source of potential iron uptake mutants. Such mutants will presumably show more PhoA activity in the absence of iron as iron uptake systems are expressed when iron is limiting.

Specific Aim #5. To examine the effects of mutations in various known regulatory genes for their effect on the expression of *hemA*.

The *fixLJ* genes belong to a family of two component regulatory systems, where FixL would be the sensor and FixJ would be a DNA binding protein. *fixLJ* were originally identified in *R. meliloti* and were shown to be involved in oxygen control of *nif* and *fix* genes (David et al., 1988). In *B. japonicum*, *fixLJ*-like genes also control symbiotically important genes but, unlike the case in *R. meliloti*, they do not appear to control *nifA*-dependent genes (Anthamatten and Hennecke, 1991). We have obtained *fixL* and *fixJ* mutants of *B. japonicum* (generously provided by Hauke Hennecke's lab) and have moved the *hemA-lacZ* fusion into these strains on plasmids via electroporation. Although we have not yet examined oxygen control of *hemA* expression, we have

looked to see if *fixLJ* could possibly be involved in iron regulation of *hemA*. It appears that *fixL* but not *fixJ* is required for the iron induction we observe with the *hemA-lacZ* fusion (Table 1). It is possible that FixL may be acting with a different regulator to effect iron control. Hopefully, our search for iron regulatory proteins will shed light on this.

Table 1. Expression of a *B. japonicum hemA-lacZ* fusion (pBJ289) in a *B. japonicum fixL* or *fixJ* mutant background.

Strain	-Fe	+Fe	Induction Ratio
pBJ289/110 (wild type)	645	2710	4.2
pBJ289/7403 (*fixL* mutant)	1149	1046	1.1
pBJ289/7361 (*fixJ* mutant)	375	1059	2.8

Second Year of Funding

During the second year of funding, we will continue to work on the aims as outlined in the original proposal. If we do have some type of translational control of *hemA* expression, we will design further experiments to address this issue.

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Figure 1. Deletion series of the *Bradyrhizobium japonicum* *hemA* promoter

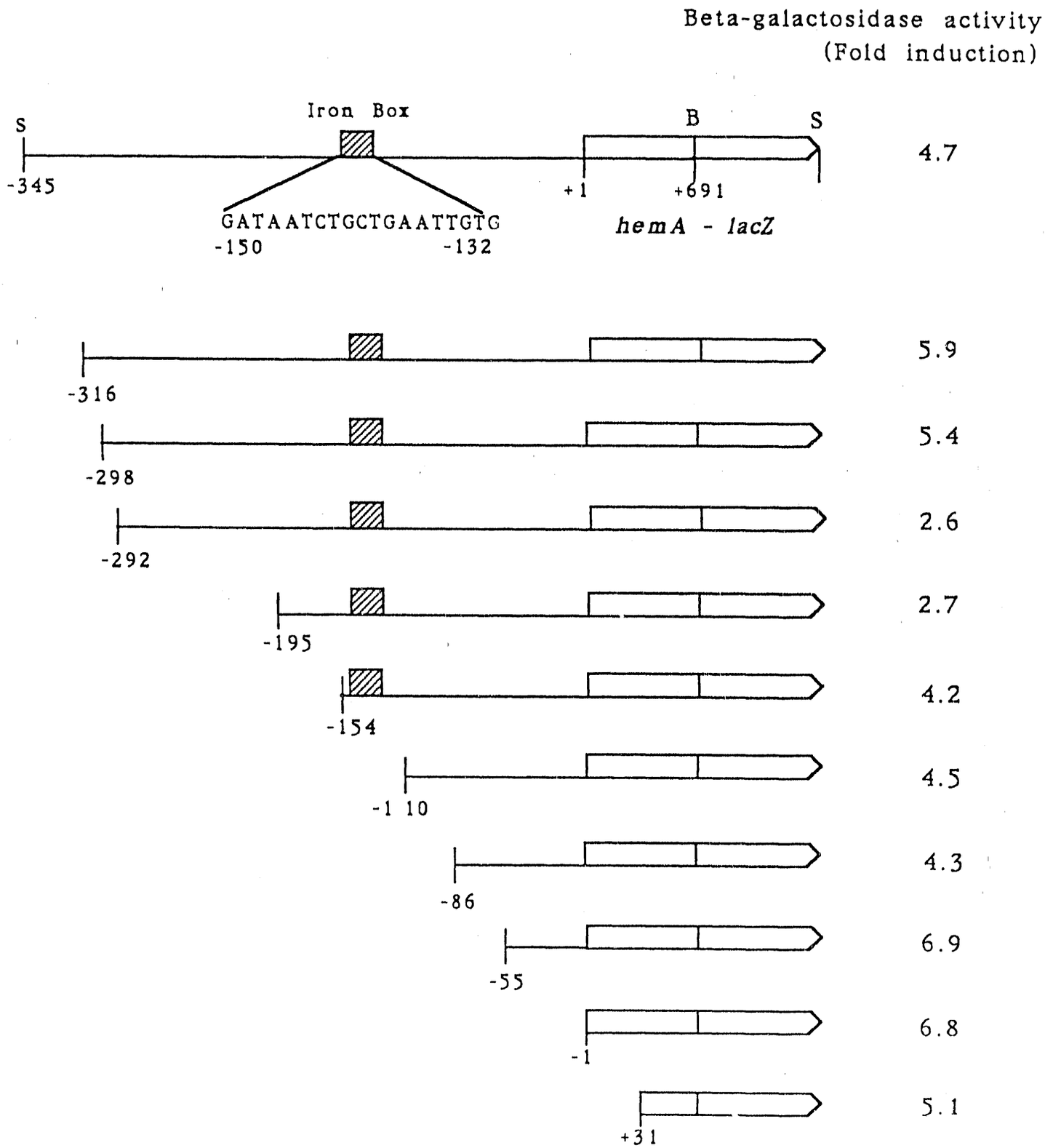


Figure 2. Effect of iron on *hemA-lacZ* activity

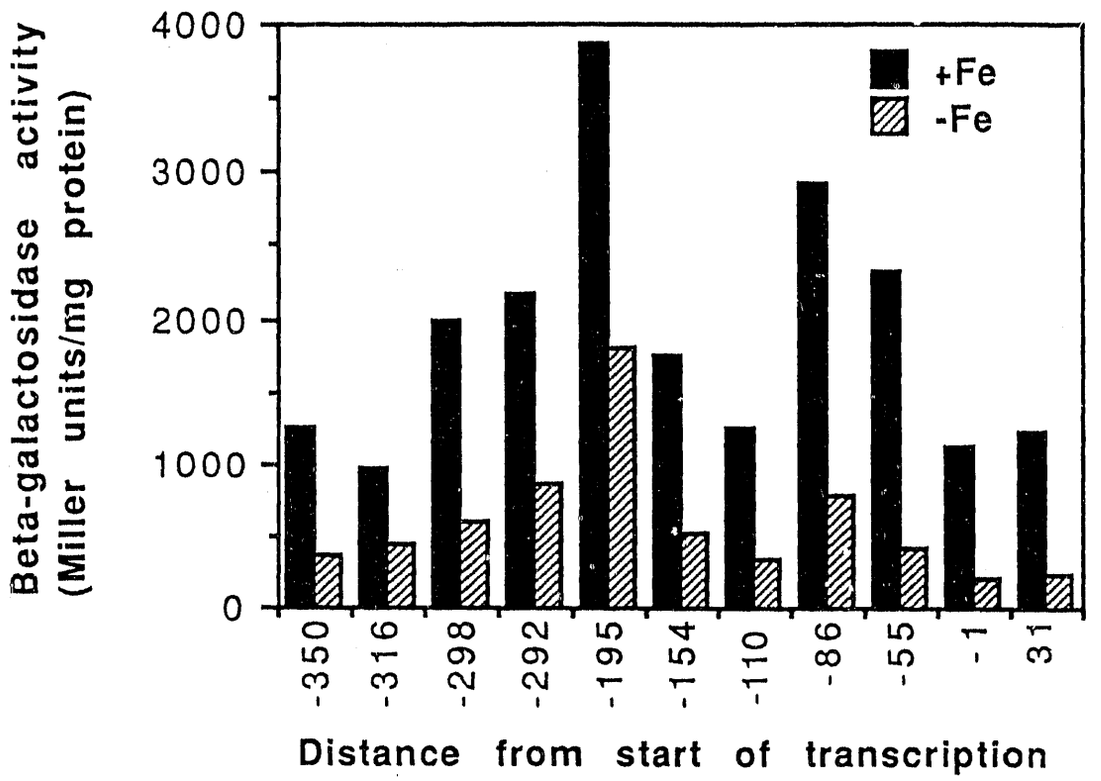
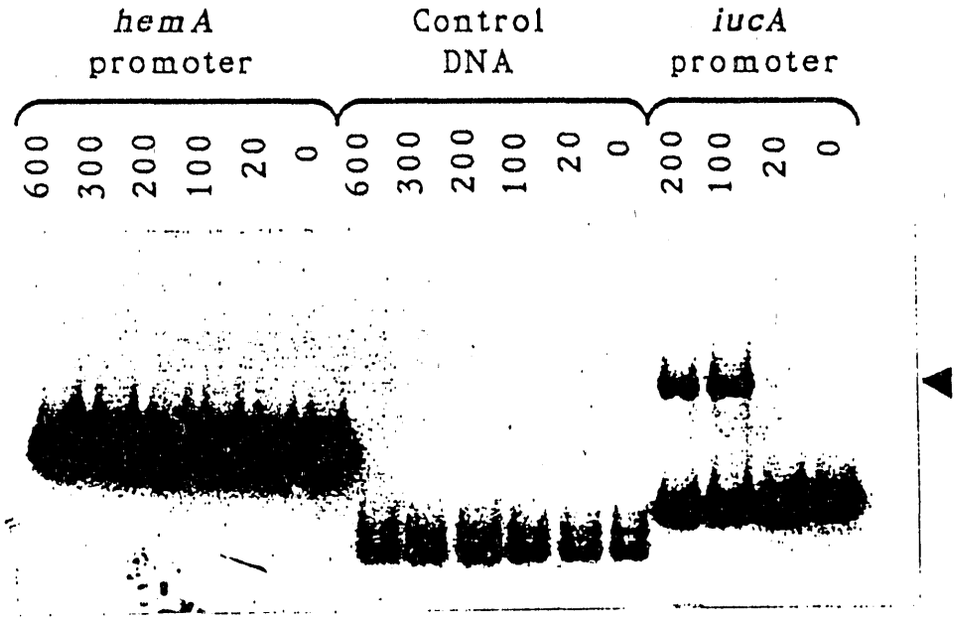


Figure 3. Gel retardation assays with purified *E. coli* Fur protein and the *B. japonicum hemA* promoter. A. ^{32}P end-labeled fragments were incubated with different nanomolar concentrations of purified *E. coli* Fur protein. A 300 bp fragment from -344 to -44 of the *hemA* promoter did not form a complex with Fur protein nor did a 222 bp control fragment of SK- which has no homology to the iron box consensus sequence. A 250 bp fragment containing the *E. coli iucA* promoter does form a complex with Fur protein. B. The consensus sequence for the *E. coli* iron box is shown along with the iron box from the *E. coli iucA* gene and the presumptive iron box from the *B. japonicum hemA* gene.

A.



B.

E. coli iron box consensus	GATAATGATAATCATTATC
E. coli iucA iron box	GATAATGAGAATCATTATT
B. japonicum hemA iron box	GATAATCTGCTGAATTGTG

G29P

Iron Regulation of *hemA* in *Bradyrhizobium japonicum*.

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Our research focuses on understanding the role of iron in the nitrogen-fixing symbiosis between *Bradyrhizobium japonicum* and soybean. We are approaching this study by examining genes which are expressed under iron deficient growth conditions or, conversely, which are expressed under iron sufficient growth conditions. Expression of the *B. japonicum hem*A gene, which encodes 5-aminolevulinic acid synthase, the first and rate limiting enzyme in the heme biosynthetic pathway, is enhanced 4 to 11 fold in the presence of iron. In *E. coli*, a *B. japonicum hemA-lacZ* fusion appears to be positively regulated by Fur, a DNA -binding regulatory protein. However, Fur has been characterized in *E. coli* as a negative regulator, specifically of siderophore biosynthesis. Siderophores are extracellularly secreted ferric iron sequestering molecules which are synthesized under low iron conditions. A role for Fur as a positive regulator of heme synthesis in *B. japonicum* is intriguing. There is already evidence for Fur-like regulatory proteins in other bacterial species. Fur has been shown to negatively regulate *Vibrio cholerae* hemolysin expression and the *Corynebacterium diptheriae tox* promotor in *E. coli* and a *fur* homolog has been cloned from *Yersinia pestis*. The *B. japonicum hemA* promotor region, which contains a region of homology (centered at -152) to the *E. coli* consensus Fur DNA binding sequence ("iron box"), is currently under investigation using a series of *hemA-lacZ* promotor deletions spanning from approximately -317 to the start of transcription. We have shown that purified *E. coli* Fur protein does not bind the *B. japonicum hemA* "iron box" in gel shift or footprint assays; efforts are being made to clone the *B. japonicum fur* homolog. Experiments are also underway in which we are examining the expression of our *hemA-lacZ* fusion in bacteroids to define the importance of *hemA* in symbiotic heme synthesis. By using the promotor constructs, we can also define which *cis* elements are required for nodule expression, which may or may not be the same as those required for expression in the free-living state.

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Kurtz, Jr., H. D. and J. Smit (1992) Analysis of a *Caulobacter crescentus* gene cluster involved in attachment of the holdfast to the cell. **Journal of Bacteriology 174:** 687-694

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