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Effect of Iron Availability on Expression of the *Bradyrhizobium japonicum hemA* Gene

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Bradyrhizobium japonicum **produces Δ-aminolevulinic acid, the universal precursor of tetrapyrroles, in a reaction catalyzed by the product of the *hemA* gene. Expression of the *B. japonicum hemA* gene is affected by iron availability. Activity of a *hemA-lacZ* fusion is increased approximately threefold by iron, and RNA analysis indicates that iron regulation is at the level of mRNA accumulation. To our knowledge, this is the first example of an iron-regulated heme biosynthetic gene in prokaryotes.**

Heme-containing compounds figure prominently in the nitrogen-fixing symbioses between rhizobia and legumes. These include symbiosis-specific bacterial cytochromes and oxidases which operate in the microaerophilic environment found in nodules (20, 28) and FixL, a bacterial hemoprotein (15) which is believed to function as an oxygen sensor to regulate expression of the nitrogen fixation (*nif*) genes (10). The universal first step in the synthesis of all tetrapyrroles, including heme, is the formation of Δ-aminolevulinic acid (ALA). The soybean symbiont *Bradyrhizobium japonicum*, like other members of the α-subgroup of purple bacteria (2), produces ALA by a one-step condensation of glycine and succinyl-coenzyme A to form ALA in a reaction catalyzed by ALA synthase (ALAS), the product of the *hemA* gene. Past experiments have shown that ALAS activity in *B. japonicum* was stimulated upon addition of iron to iron-deficient cultures (30), although the level at which iron was exerting its effect was not ascertained. We have explored the molecular basis for the observed effect of iron on the expression of ALAS. In bacteria, the expression of many genes is regulated by iron. The best-studied example is found in *Escherichia coli*, in which approximately 40 genes involved in the synthesis and uptake of siderophores (ferric ion-specific ligands) are repressed under iron-replete conditions by a complex of Fur and iron (7, 24). The expression of many of the iron-regulated genes in species other than *E. coli* is controlled by Fur homologs (22, 23, 29, 33). The level of iron regulation is usually transcriptional, although there are examples of translational control (8) and of control of mRNA stability (5). In this study, we show that addition of iron to iron-deficient *B. japonicum* cells leads to an accumulation of *hemA* mRNA as well as to an increase in activity of a *hemA-lacZ* fusion. In addition, we find that the *B. japonicum hemA-lacZ* fusion is clearly expressed above background in a *fur*$^+$ *E. coli* strain relative to an isogenic *fur* strain.

Bacterial strains and growth conditions. The strains and plasmids used in this work are presented in Table 1. For iron induction studies, *B. japonicum* cultures were grown to mid-log phase in AG (31) or YEX (1) medium. The cultures were diluted 1:100 into minimal medium (18) without iron containing 0.2% xylose and grown to mid-log phase. Half the culture was transferred to a new flask and either $FeCl_3$ or ferric citrate (stock solution of 10 mM $FeCl_3$ in 2 M sodium citrate) was added to a final concentration of 10 μM Fe. After 24 h, the cultures were frozen for later assay of β-galactosidase activity (27) and determination of protein concentration (BCA protein assay [Pierce, Rockford, Ill.]) according to manufacturer's instructions. Where appropriate, cultures were supplemented with tetracycline (25 μg/ml for *E. coli* and 100 μg/ml for *B. japonicum*).

RNA analysis. Steady-state levels of *hemA* RNA were examined 24 h after addition of iron to iron-deficient cells of *B. japonicum* 110. RNA was extracted from cells grown with or without iron (9). RNA was slotted onto nylon-reinforced nitrocellulose (Minifold II slot blotter system; Schleicher and Schuell, Keene, N.H.) and cross-linked to the membrane by UV irradiation (Stratalinker; Stratagene, La Jolla, Calif.). The RNA was hybridized to the 1,211-bp *Bgl*II fragment of the *hemA* gene from pBJ110 labelled by random priming (14). Following autoradiography, mRNA abundance was quantified with a MasterScan densitometer (Scanalytics, Billerica, Mass.). The level of *hemA* mRNA in cells supplemented with iron was 12-fold higher than that in cells which received no supplement (Fig. 1). The membrane was stripped and hybridized to pBJ142, containing the *B. japonicum* gene coding for rRNA (32). rRNA, equally abundant in both growth conditions, was used as a loading control. Thus, there is regulation of *hemA* expression by iron at the level of mRNA accumulation, with iron affecting either initiation of transcription or mRNA stability.

Analysis of *hemA-lacZ* fusion in *B. japonicum*. A *Sal*I-*Bgl*II fragment from pBJ110 (18, 25) containing 345 bp of DNA upstream of *hemA* transcription start and the first 691 bp of the *hemA* gene was subcloned such that a protein fusion was made to the seventh codon of *E. coli lacZYA* (Fig. 2A). The *hemA-lacZ* fusion was subcloned into the *Bgl*II site of pRK290 to create pBJ289. *B. japonicum* 2925 was created by integration (18) of a pBR322 construct (carrying a gene conferring resistance to streptomycin and the *hemA-lacZ* fusion) into the chromosome of *B. japonicum* 110 directly upstream of the endogenous *hemA* gene (Fig. 2B). Using these translational fusions, we have documented a threefold (3.4 ± 0.6; range, two- to sevenfold) increase in β-galactosidase activity upon addition of 10 μM iron to cells carrying the fusion on a plasmid or upon addition to cells which carry the fusion integrated into the chromosome (Fig. 2C). There was no significant difference in induction values for cells which received additions of 1, 2, or 10 μM iron; however, cells supplemented with 0.5 μM iron were not fully induced compared with cells grown at higher

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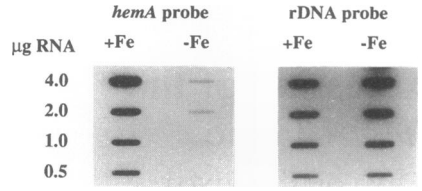


FIG. 1. Analysis of *hemA* transcript levels. *B. japonicum* 110 was grown in iron-deficient minimal medium to mid-log phase. The culture was then split, and half the culture received 10 μM $FeCl_3$. After 24 h, cells were harvested, and RNA was extracted. Samples of 4.0, 2.0, 1.0, and 0.5 μg of RNA were slotted onto nylon-reinforced nitrocellulose. The blot was hybridized to the *hemA* gene, stripped, and hybridized to the gene encoding 16S and 23S rRNA (rDNA) (32) as a control.

iron concentrations (data not shown). These results parallel those seen by monitoring the growth response of iron-deficient cells to increasing iron levels (19).

Analysis of *hemA-lacZ* fusion in *E. coli*. A region at −149 of the *hemA* upstream region (see Fig. 4B) shows similarity to an iron box, a regulatory element first described for *E. coli* which is the binding site for Fur (12). The presence of this region suggested a potential role for a Fur-like protein as a regulator of *hemA*. To determine whether expression of the *B. japonicum hemA* gene could be regulated by Fur in *E. coli*, we introduced the *B. japonicum hemA-lacZ* fusion on the low-copy-number plasmid pBJ289 into the *fur* mutant AB9001 and the isogenic *fur*$^+$ strain JP5053. These strains were then grown to an optical density at 600 nm of 0.6 at 37°C in either Luria broth or Luria broth deferrated with Chelex 100 (Bio-Rad Laboratories, Richmond, Calif.) for determination of β-galactosidase activity (27). Expression of the *hemA-lacZ* fusion did not exceed background in the *fur* mutant. In the *fur*$^+$ strain, expression of the fusion was increased above background, although it did not show an increase in response to iron (Fig. 3). Both *E. coli* strains contain an endogenous *lacZ* gene which serves as a control to show that background β-galactosidase expression does not change significantly with addition of iron.

TABLE 1. Strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
E. coli		
AB9001	*fur* derivative of JP5053	J. B. Neilands
JP5053	*argHI metBI nagAI rpsLI55 rpoB* F$^-$	J. B. Neilands
B. japonicum		
110	Small-colony derivative of *B. japonicum* USDA 3I1b110	18
2925	*B. japonicum hemA-lacZ* chromosomal insertion	John Somerville
Plasmids		
pABN40	*E. coli iucA*	4
pBJ110	*B. japonicum hemA*	18
pBJ142	*B. japonicum* rRNA gene operon	32
pBJ289	*B. japonicum hemA-lacZ* in pRK290[a], Tet^r	John Somerville
pBluescriptSK−		Stratagene

[a] Described in reference 13.

It is important to note that the levels of expression in *E. coli* were only 1/10 of those seen when the fusion was carried by *B. japonicum*, suggesting that *E. coli* Fur is a poor substitute for the endogenous *B. japonicum* regulator of *hemA*. Similar results have been seen with the iron-regulated *fatA* gene of *Vibrio anguillarum*, which showed a slight response to Fur when expressed in *E. coli* (36), and with the *toxPO* operon of *Corynebacterium diphtheriae*, which showed no response to Fur (6). Interestingly, *toxPO*, which is regulated by the Fur-like DtxR, does have a sequence similar to the *E. coli* iron box in its upstream region, although binding studies indicate that sequences important for binding of DtxR are different from those for Fur (34, 35).

Mobility shift with Fur. To determine whether purified *E. coli* Fur protein binds the *B. japonicum hemA* iron box, a

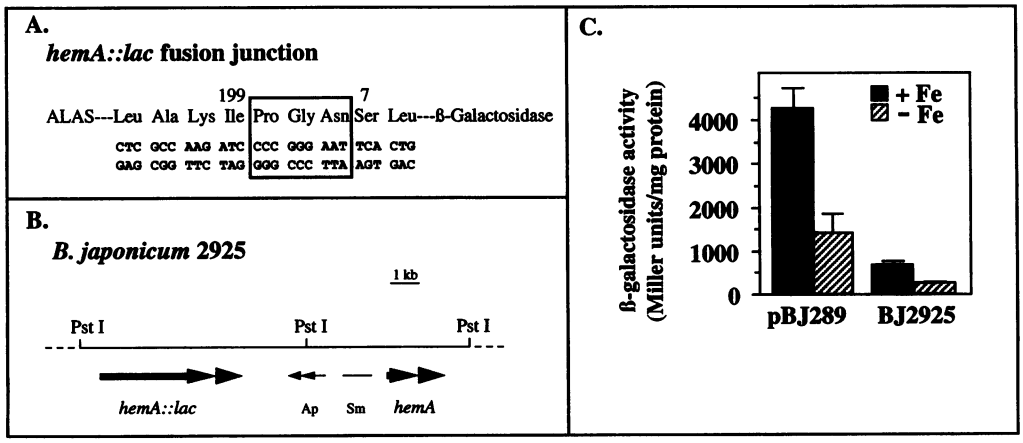


FIG. 2. *B. japonicum hemA-lacZ* fusion. (A) Junction, containing *Sma*I and *Eco*RI sites, created in construction of the *hemA-lacZ* fusion. The 199th amino acid of HemA is followed by three amino acids created during the subcloning procedure and then followed by the 7th amino acid in LacZ. (B) Physical map of the genomic region of *B. japonicum* 2925, which carries the *hemA-lacZ* fusion integrated into the chromosome immediately upstream of the endogenous *hemA* gene. Arrows, direction of transcription. (C) Expression of the *B. japonicum hemA-lacZ* fusion in response to iron. *B. japonicum* 110 containing plasmid pBJ289 and *B. japonicum* 2925 were grown in iron-deficient minimal medium to mid-log phase. The cultures were then split, and half the cultures were supplemented with 10 μM iron for 24 h. The data are averages of at least three independent trials.

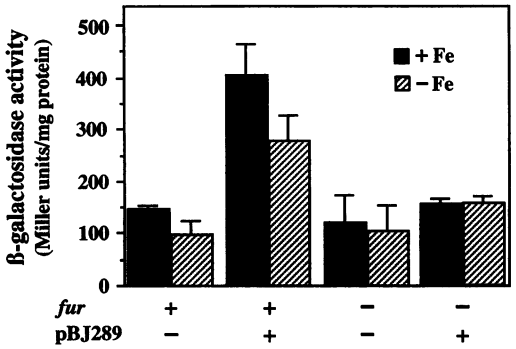
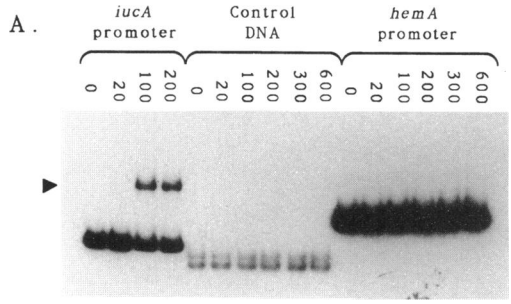


FIG. 3. Effect of a *fur* mutation on expression of the *B. japonicum hemA-lacZ* fusion in *E. coli*. A *fur* wild-type strain, JP5053, and its *fur* mutant derivative AB9001 were grown with and without plasmid pBJ289 in iron-deficient and iron-sufficient media to an optical density at 600 nm of 0.6. β-Galactosidase activity was then measured. Each value is the average of two independent trials.

mobility shift assay was employed (11). A 300-bp fragment from −344 to −44 of the *hemA* upstream region (*Eco*RI to *Bam*HI) including the putative iron box did not form a complex with purified *E. coli* Fur protein, nor did a 222-bp negative control fragment of SK− which has no homology to the iron box consensus sequence (Fig. 4A). A 250-bp *Eco*RI-*Pvu*II fragment of pABN40 containing the *E. coli iucA* promoter sequence did form a complex with Fur (4). As shown in Fig. 4B, the *iucA* iron box matches at 17 of 19 positions to the *E. coli* iron box consensus sequence. The region of DNA upstream of the *B. japonicum hemA* gene contains an iron box which matches at 10 of 19 positions to the consensus binding site; the matches include the six most highly conserved nucleotides (Fig. 4B) (17).

When one considers that synthesis of ALA is the first step in the heme biosynthetic pathway and that regulation at the first



B.

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

FIG. 4. (A) Mobility shift assay. ^{32}P-labelled DNA fragments were incubated with the indicated nanomolar concentrations of purified *E. coli* Fur protein. DNA fragments used: (i) a 250-bp positive control fragment of pABN40 containing the *E. coli iucA* iron box (7), (ii) a 222-bp negative control fragment of pBluescriptSK− which has no homology to the *E. coli* iron box consensus sequence, and (iii) a 300-bp fragment from −344 to −44 of the *B. japonicum hemA* gene which includes the presumptive iron box. Arrowhead, *iucA*-Fur complex. (B) Comparison of iron box regions from *E. coli iucA* (4) and *B. japonicum hemA* (25) with the *E. coli* iron box consensus sequence (7, 12, 16).

step would prevent synthesis of heme precursors in a low-iron environment as well as allow for increased production when iron is plentiful, it is not surprising that expression of *hemA* would be affected by iron availability. To our knowledge, this is the first example of an iron-regulated heme biosynthetic gene in prokaryotes. Interestingly, the gene encoding the mammalian erythroid ALAS is regulated by iron (3, 26). Whereas the effect of iron on *hemA* transcription appears to be at the level of mRNA abundance in *B. japonicum*, regulation of the erythroid ALAS gene is at the level of translation, via an iron-responsive element, IRE, a stem-loop structure located in the 5′ untranslated region of the gene (3, 26). Although the 5′ untranslated leader of the *B. japonicum hemA* gene is predicted to form a stem-loop structure (data not shown), there is no homology to the iron-responsive element consensus motif (21); in addition, the data presented here do not suggest that there is translational control of *hemA* by iron.

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