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Characterization of a *Bradyrhizobium japonicum* Ferrochelatase Mutant and Isolation of the *hemH* Gene

JANA M. FRUSTACI AND MARK R. O'BRIAN*

Department of Biochemistry and Center for Advanced Molecular Biology and Immunology, 140 Farber Hall, State University of New York at Buffalo, Buffalo, New York 14214

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A Tn*5*-induced mutant of *Bradyrhizobium japonicum*, strain LORBF1, was isolated on the basis of the formation of fluorescent colonies, and stable derivatives were constructed in backgrounds of strains LO and I110. The stable mutant strains LOek4 and I110ek4 were strictly dependent upon the addition of exogenous hemin for growth in liquid culture and formed fluorescent colonies. The fluorescent compound was identified as protoporphyrin IX, the immediate precursor of protoheme. Cell extracts of strains LOek4 and I110ek4 were deficient in ferrochelatase activity, the enzyme which catalyzes the incorporation of ferrous iron into protoporphyrin IX to produce protoheme. Mutant strain I110ek4 could take up ^{55}Fe from the growth medium, but, unlike the parent strain, no significant incorporation of radiolabel into heme was found. This observation shows that heme was not synthesized in mutant strain I110ek4 and that the heme found in those cells was derived from exogenous hemin in the growth medium. The putative protein encoded by the gene disrupted in strain LORBF1 and its derivatives was homologous to ferrochelatases from eukaryotic organisms. This homology, along with the described mutant phenotype, provides strong evidence that the disrupted gene is *hemH*, that which encodes ferrochelatase. Mutant strain I110ek4 incited nodules on soybean that did not fix nitrogen, contained few viable bacteria, and did not express leghemoglobin heme or apoprotein. The data show that *B. japonicum* ferrochelatase is essential for normal nodule development.

The bacterium *Bradyrhizobium japonicum* elicits symbiotic nitrogen-fixing root nodules on soybean plants that consist of highly differentiated plant and bacterial (bacteroid) cells. Cellular hemes of both symbionts are greater in nodules than they are in the respective asymbiotic cells (1, 28), and these hemes are necessary to support the large energy requirement of nitrogen fixation. Bacterial cytochromes participate in ATP synthesis which drives nitrogen fixation, and soybean hemoglobin (leghemoglobin) facilitates the diffusion of oxygen to the respiring bacteroids. Failure to detect heme synthesis enzyme activities in the plant cytosol of root nodules led to the hypothesis that leghemoglobin heme is synthesized by the rhizobial symbiont (5, 16, 24). In addition, a *B. japonicum* mutant defective in the heme synthesis enzyme protoporphyrinogen oxidase elicits soybean nodules which contain bacteroids and express hemoglobin apoprotein but do not express hemoglobin heme (19). Subsequently, the protoporphyrinogen oxidase mutant was found to be defective in one of a family of clustered genes involved in cytochrome *c* synthesis (25), but the gene product is still unknown. Rhizobial mutants defective in δ-aminolevulinic acid (ALA) synthase, the first committed step in heme synthesis, have yielded intriguing results. ALA synthase (*hemA*) mutants of *Rhizobium* sp., *Rhizobium meliloti*, and *Azorhizobium caulinodans* elicit nodules on cowpea, alfalfa, and *Sesbania* plants, respectively, which are ineffective and lack hemoglobin (13, 23, 31). However, soybean nodules elicited by a *B. japonicum hemA* mutant contain bacteroids, fix nitrogen, and contain leghemoglobin (10), showing that *B. japonicum* ALA synthase is not essential for soybean nodule hemoglobin formation. A strong argument can be made that these mutants do not address the source of plant hemoglobin directly but rather that the different nodule phenotypes reflect differing abilities of the mutants to successfully invade their hosts and perpetuate despite the lesion in ALA synthase. Alfalfa nodules formed from *R. meliloti hemA* mutants are devoid of bacteria (6); hence, a hemoglobin-defective phenotype is expected regardless of the source of the heme prosthetic group. However, if one safely assumes that bacterial hemes are essential for a successful symbiosis, studies using *hemA* mutants show clearly that heme formation differs between *B. japonicum*-soybean nodules and the other nodule types examined. Work from this laboratory (27) shows that hemes are expressed in bacteroids, but not in cultured cells, of the *B. japonicum hemA* mutant, and we provided evidence that bacteroid heme can be synthesized from soybean-derived ALA in nodules, thereby rescuing the mutant with respect to heme synthesis and normal nodule development. Acquisition of *B. japonicum* heme synthesis mutants other than that in the *hemA* gene allows us to further address the hypothesis of interorganismic heme synthesis in soybean nodules, and isolation of the corresponding genes creates tools for studying the regulation of heme formation. In the present work, we isolated and characterized a *B. japonicum* mutant defective in ferrochelatase, the last step in heme synthesis, and isolated the *hemH* gene.

MATERIALS AND METHODS

Chemicals and reagents. All chemicals were reagent grade and were purchased from Sigma Chemical Co., St. Louis, Mo., or from J. T. Baker Chemical Co., Philipsburg, N.J. Purified Noble agar and yeast extract were obtained from Difco Laboratories, Detroit, Mich. Porphobilinogen, ALA, mesoporphyrin IX, protoporphyrin IX, and porphyrin methyl ester standards were purchased from Porphyrin Products, Logan, Utah. $^{55}FeCl_3$ (33.5 MCi/mg), [α-^{32}P]dCTP

* Corresponding author.

(3,000 Ci/mmol), and [α-^{35}S]dATP (1,185 Ci/mmol) were obtained from New England Nuclear, Boston, Mass.

Bacterial strains, media, and growth. *B. japonicum* LO (18), a Nal^r derivative of strain USDA DES122, was the parent strain from which strains LORBF1, LORBF2, LOek1, LObk2, and LOek4 were derived. *B. japonicum* MLG1 (10), I110ek1, I110bk2, and I110ek4 are derivatives of the wild-type *B. japonicum* strain I110. All strains were grown in GSY medium as described previously (8); minimal medium contained 5 mM NH_4Cl in place of the yeast extract. All mutant strains were grown in the presence of 75 μg of kanamycin per ml, and the Tn*5*-induced mutants LORBF1 and LORBF2 were grown with 75 μg of streptomycin as well. Hemin was added to autoclaved medium from a 3.8 mM stock solution of commercial hemin prepared in 0.1 N NaOH in 50% ethanol. Polyoxyethylene sorbitan monooleate (Tween 80, 0.01% [wt/vol]) was included in the medium to prevent the hemin from precipitating. The addition of Tween 80 alone did not affect the growth of cells. *Escherichia coli* strains were grown at 37°C on LB medium (15) with the appropriate antibiotics. *E. coli* SM10 (pSUP1011) was grown in the presence of kanamycin (25 μg/ml) and chloramphenicol (20 μg/ml), strain HB101 (pRK2013) was grown in the presence of kanamycin (25 μg/ml), strain HB101(pDS4101) was grown in the presence of ampicillin (50 μg/ml), and strain XL1-Blue was grown in the presence of tetracycline (20 μg/ml).

Tn*5* mutagenesis and construction of stable mutant derivatives. A random Tn*5* mutagenesis of *B. japonicum* LO was carried out by conjugation with *E. coli* SM10(pSUP1011) as previously described (29). In our hands, strain LO yields a higher frequency of mutants than does strain I110. Tn*5*-induced mutants were selected on minimal medium plates containing 15 μM hemin, kanamycin, and streptomycin; approximately 3,500 colonies were screened for a reddish-brown appearance and fluorescence under UV light expected of colonies accumulating porphyrins. Stable mutant derivatives were constructed in backgrounds of strains LO and I110 by deleting various regions in the wild-type genome corresponding to the region mutated in the Tn*5* mutant LORBF1 and replacing the deleted DNA with a kanamycin resistance-encoding gene cassette. To do this, the *Eco*RI fragment from strain LORBF1 which contained the Tn*5* insertion and 0.4 kb of flanking genomic DNA was isolated and used as a hybridization probe to obtain a 17.0-kb *Pst*I fragment from the wild-type genome of strain LO which shared homology to it. A 6.4-kb *Eco*RV-*Hpa*I fragment was subcloned from the wild-type *Pst*I piece into the *Sca*I site of pBR322, resulting in the plasmid construct pBRHE. Deletions were made in pBRHE either by partial digestions with *Eco*RI or digestion with *Bgl*II followed by ligation with a cassette containing the *nptII* gene (see Fig. 1). The resultant plasmids were conjugated into strain LO or I110, and double recombinants were initially scored as Km^r Tc^s colonies and confirmed to be so by Southern hybridizations. In addition, I110bk2, I110ek4, LObk2, and LOek4 formed fluorescent colonies as did the original Tn*5*-induced mutant LORBF1.

DNA manipulations and sequencing. Genomic DNA from *B. japonicum* was isolated as described previously (18). Size fractionation of restricted genomic DNA was performed on a 1 to 5 M NaCl step gradient as described previously (22). Plasmid DNA preparations, restriction digests, and transformations were performed as described by Maniatis et al. (15). DNA hybridization probes were prepared by using a random primer labeling kit (Boehringer Mannheim) according to the manufacturer's instructions. Southern and colony hybridizations were performed as described by Maniatis et al. (15) by using the stringency conditions described previously (18). To determine the nucleotide sequence of the *hemH* gene, a 2.7-kb partial *Eco*RI-*Pst*I fragment (*Pst*I site from pBR322) from pBRHE was cloned into pBluescript IIKS+ (Stratagene), resulting in the plasmid construct pKSIIPE. The phenotype of the deletion mutants described above predicted that at least one end of the *hemH* gene was contained on this fragment. Unidirectional deletions were made in pKSIIPE digested with *Kpn*I and *Hin*dIII or with *Bst*XI and *Spe*I by using an Exo-Mung deletion kit (Stratagene) according to the manufacturer's directions. All plasmids prepared for sequencing were isolated from *E. coli* XL1-Blue, and double-stranded DNA sequencing was carried out by the chain termination method (26), using a Sequenase kit (version 2.0, United States Biochemicals) and [α-^{35}S]dATP. T3 and T7 primers (Promega) were used for sequencing deletions made from the *Hin*dIII and *Spe*I sites, respectively.

Purification and analysis of heme from cultured cells grown with $^{55}FeCl_3$. GSY cultures (250 ml) containing 15 μM hemin and 3.7 μM $FeCl_3$ were inoculated with approximately 5 × 10^7 cells of strain I110 or I110ek4. Fifty microcuries of $^{55}FeCl_3$ was added to growing cells at an A_{540} of 0.1 and again at an A_{540} of 0.2. Cells were grown to an A_{540} of 0.7, centrifuged, and washed in 50 mM Tris–1 mM EDTA (pH 8.0) to remove any residual hemin originating from the media. To isolate non-covalently bound heme, the cell pellets were resuspended and extracted overnight in 30 ml of ethyl acetate-acetic acid (3:1 [vol/vol]). The insoluble cell debris was removed by centrifugation, and the supernatant was extracted with 60 ml of ether; the heme-containing ether phase was washed twice successively with H_2O (50 ml), twice with 1.8 N HCl (50 ml), and again with H_2O to remove contaminating free ^{55}Fe and porphyrins. The heme-containing ether phase was evaporated in vacuo to 2.0 ml, and a dithionite-reduced minus ferricyanide-oxidized pyridine hemochromagen spectrum was determined as described previously (24) on 400 μl of the solution to quantitate the protoheme recovered ($E_{557\text{-}541}$, 20.7/mM). The remaining sample (1.6 ml) was dissolved in scintillation fluid and counted in the ^{3}H channel of a Packard Tri-Carb scintillation counter to quantitate the incorporation of ^{55}Fe into heme. Results are expressed as counts of ^{55}Fe incorporated per minute per nanomole of heme recovered.

Heme biosynthesis enzyme assays. Cell extracts and membranes were prepared as described previously (20). ALA synthase, ALA dehydratase, and porphobilinogen deaminase were assayed in cell extracts at 37°C as described previously (27). Ferrochelatase was assayed in cell extracts as described by Porra (24), using mesoporphyrin IX as the substrate. In our hands, ferrochelatase activity was linear for 40 min with 5 mg of protein assayed at 37°C. Cell extracts from strain I110ek4 contained about 1 μM protoporphyrin IX because of accumulation during growth; the addition of 1 μM protoporphyrin IX to wild-type cell extracts did not significantly inhibit ferrochelatase activity, and thus it did not interfere with the assay. Protoporphyrinogen oxidase activity was assayed spectrophotometrically in membranes at pH 8.0 and 37°C, as described by Jacobs and Jacobs (11) in the absence of glutathione and Tween 20. We found that some lots of Tween 20 interfered with the assay, and thus it was omitted.

Heme and leghemoglobin determinations. Cytochrome heme was discerned in cell extracts by a reduced-minus-oxidized absorption spectrum as previously described (8). A dithionite-reduced pyridine hemochromagen assay was per-

formed on nodule cytosol to quantitate leghemoglobin heme, using an E_{mM} of 24.5 (30). The leghemoglobin apoprotein was discerned immunologically by Western blot (immunoblot) analysis as described elsewhere (19).

Fluorescent species isolated from spent media. The spent growth medium of strain I110ek4 was used to collect the insoluble fluorescent species. A neutral methyl ester of the isolated porphyrin was prepared and analyzed by absorption spectroscopy in chloroform as described previously (30). Thin-layer chromatography of the neutral methyl ester was performed on silica plates as described elsewhere (7) with benzene-ethyl acetate-ethanol (190:20:7.5 [vol/vol/vol]) as the running solvent. Commercial porphyrin methyl esters were chromatographed with the samples.

Growth and harvest of soybeans. Soybeans (*Glycine max* cv. Essex) were grown under supplemental and natural light in a greenhouse in Buffalo, N.Y., as described previously (18). Germinated seedlings were each inoculated with approximately 10^8 cells of strain I110 or I110ek4 and grown in vermiculite containing N-free supplemental nutrients. Nodules were harvested from 31-day-old plants, crushed in buffer containing 40 mM Tris (pH 7.4) and 1 mM phenylmethylsulfonylfluoride, and passed through cheesecloth. The plant fractions were prepared as described previously (21) and assayed for leghemoglobin heme and the apoprotein. To determine viable cell counts in nodules, the nodules were weighed, surface sterilized in 95% ethanol, crushed in 40 mM Tris (pH 7.4), and plated in serial dilutions on GSY medium containing 15 μM hemin; kanamycin (75 μg/ml) was added to the medium when strain I110ek4 was plated.

Other methods. Mesoheme was synthesized from mesoporphyrin IX and $FeSO_4$ as described elsewhere (30). Protein was assayed by the method of Bradford (2). Nitrogenase activity was assayed as the reduction of acetylene to ethylene as described previously (18).

Nucleotide sequence accession number. The sequence of 1,620 bp of DNA which includes the *hemH* gene and flanking DNA shown in Fig. 5 has been deposited with GenBank under accession number M92427.

RESULTS

Isolation of porphyrin-accumulating mutants and construction of stable derivatives. *B. japonicum* is an aerobic bacterium, and thus a mutant defective in heme synthesis may be lethal unless the cells are maintained in some permissive medium. We recently reported the surprising result that a *B. japonicum* mutant which expresses no discernible heme can grow aerobically in liquid cultures (8), but that work also provided indirect evidence that a very small amount of heme may be necessary for viability nevertheless. Our strategy, therefore, was to include hemin (heme hydrochloride) in the growth medium used for the mutagenized population of cells to be screened; although uptake of hemin is likely to be inefficient, only a small amount, if any, would be needed. Approximately 3,500 Tn*5*-induced *B. japonicum* mutants were screened for colonies red to brown in color that fluoresce under UV light, which is expected of a porphyrin-accumulating mutant. Two mutant strains, LORBF1 and LORBF2, had the desired phenotype; the Tn*5* insertion within LORBF1 and LORBF2 mapped to different genetic loci as determined by Southern blot analysis. Cell extracts of strain LORBF2 had approximately 50% of the ferrochelatase activity of the wild-type strain; strain LORBF2 was not studied further in the present work. Approximately 10% of the wild-type ferrochelatase activity was observed in

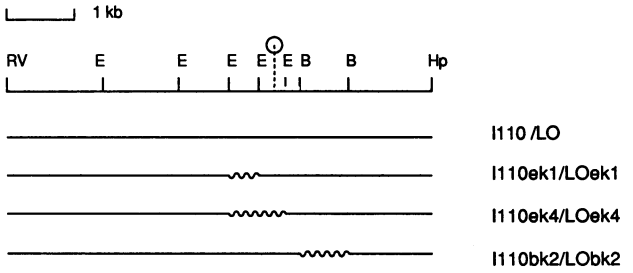

FIG. 1. Restriction map of the genetic locus mutated in strain LORBF1 and construction of deletion mutant derivatives. The wavy lines represent deleted loci, which were replaced with *nptII*-encoding cassettes. Open circle, Tn*5*. The Tn*5* transposon and *nptII* cassette are not drawn to scale. Restriction sites: RV, *Eco*RV; E, *Eco*RI; Hp, *Hpa*I; B, *Bgl*II.

LORBF1, and Southern analysis showed that 1.5 kb of the 5.4-kb transposon was deleted within the genome of LORBF1, indicating that the Tn*5* was unstable in that mutant strain. Stable genomic mutants were constructed in the vicinity of the Tn*5* insertion of LORBF1 by isolating the corresponding wild-type DNA, deleting restriction enzyme fragments, and replacing the deleted DNA with a gene cassette conferring kanamycin resistance (Fig. 1; see Materials and Methods). The deletion-containing fragments were introduced into the genomes of strain I110 or LO by homologous recombination, and the resultant mutants were assayed with respect to the fluorescent-colony phenotype, hemin auxotrophy, and ferrochelatase activity (Table 1).

Mutant strains I110ek4 and I110bk2 (and also LOek4 and LObk2) formed colonies which fluoresced under UV light, as was found for LORBF1 (Table 1). Those mutants were strict hemin auxotrophs in liquid yeast extract cultures and were almost completely defective in ferrochelatase activity (Table 1). Mutant strain I110ek1, which contains a deletion that does not include the site of the Tn*5* insertion in LORBF1, did not form fluorescent colonies, was not a hemin auxotroph, and had wild-type levels of ferrochelatase activity (Table 1). Mutant strain I110ek4 was used for the remainder of the work because the *hemA* mutant MLG1 (10) is also a I110 derivative, and it is easier to compare two mutants with the same parent strain.

Growth of mutant strain I110ek4 in culture. Growth of strain I110ek4 in liquid culture was strictly dependent upon the addition of exogenous hemin in yeast extract media

TABLE 1. Properties of strains I110, I110ek1, I110ek4, I110bk2, LO, LOek1, LOek4, and LObk2 grown in culture

Strain	Fluorescence of colonies under UV light	Growth dependence on exogenous hemin in liquid culture	Ferrochelatase activity[a]
I110	−	−	12.9
I110ek1	−	−	13.1
I110ek4	+	+	0.5
I110bk2	+	+	0.6
LO	−	−	9.2
LOek1	−	−	ND[b]
LOek4	+	+	0.8
LObk2	+	+	0.5

[a] Activity is expressed in nanomoles of mesoheme IX formed per hour per milligram of protein.
[b] ND, not determined.

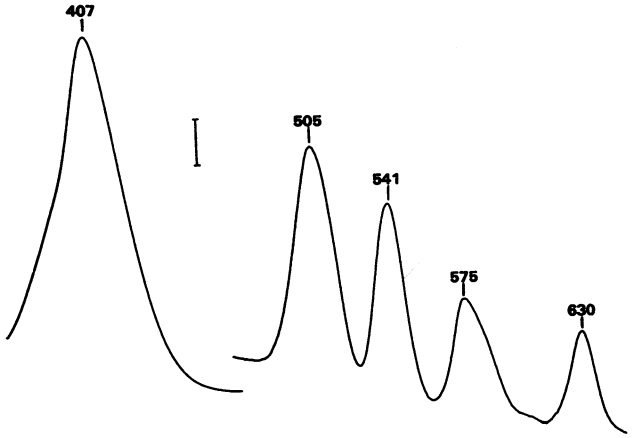

FIG. 2. Absorption spectrum of the neutral methyl ester in chloroform of insoluble protoporphyrin IX excreted by strain I110ek4 grown in liquid culture. Vertical bar, ΔA of 0.1 from 360 to 475 nm and ΔA of 0.01 from 475 to 650 nm.

(Table 1) or in minimal media (data not shown). Although 15 μM hemin was used in all experiments, a concentration of hemin as low as 1 μM was sufficient to restore growth of strain I110ek4. Neither 15 μM $FeCl_3$ nor 5 μM mesoheme could substitute for hemin in restoring growth of strain I110ek4 in liquid culture, indicating that the hemin was not functioning as an exogenous iron source. Strain I110ek4 also grew better on agar plates containing hemin, but hemin was not strictly required for growth. Differences in the growth requirements of *B. japonicum* heme synthesis mutants in liquid and on plates have been observed previously (8).

Identification of the fluorescent compound produced by strain I110ek4. Similar to the Tn*5* mutant strain LORBF1, strain I110ek4 formed reddish-brown colonies which fluoresced under UV light. In liquid culture, an insoluble fluorescent compound was excreted into the medium by strain I110ek4, which was subsequently purified. The compound was protoporphyrin IX as discerned by the absorption spectrum of the neutral methyl ester (Fig. 2) and by thin-layer chromatography. Protoporphyrin IX is the immediate precursor of protoheme, indicating that strain I110ek4 is defective in a late step of the heme biosynthetic pathway.

Heme synthesis enzyme activities. The heme auxotrophy and accumulation of protoporphyrin IX in strain I110ek4 was consistent with a deficiency in the activity of ferrochelatase, the enzyme which catalyzes the incorporation of ferrous iron into protoporphyrin IX to form protoheme. Indeed, cell extracts of strain I110ek4 contained approximately 3% of the wild-type level of ferrochelatase activity (Tables 1 and 2). Protoporphyrinogen oxidase catalyzes the oxidation of protoporphyrinogen IX to protoporphyrin IX; a lesion in this enzyme could also account for a protoporphyrin IX buildup by auto-oxidation of accumulated protoporphyrinogen IX. However, strain I110ek4 contained wild-type levels of protoporphyrinogen oxidase activity (Table 2). In addition, the heme synthesis enzyme activities of ALA synthase, ALA dehydratase, and porphobilinogen deaminase were not greatly affected by the mutation in strain I110ek4 (Table 2). The somewhat higher ALA synthase activity in strain I110ek4 may suggest that heme regulates that activity, but the differences were not great enough to make firm conclusions. Because strain I110ek4 accumulates protoporphyrin IX, the enzymatic steps necessary for its synthesis must be functional in that mutant, and thus ferrochelatase activity appears to be the only severely defective step in the heme biosynthetic pathway.

TABLE 2. Heme biosynthesis enzyme activities of strains I110 and I110ek4 grown in culture[a]

Enzyme	Activity[b]	
	I110	I110ek4
ALA synthase	2.5 ± 0.11	3.8 ± 0.20
ALA dehydratase	1.6 ± 0.06	1.4 ± 0.02
Porphobilinogen deaminase	0.22 ± 0.01	0.22 ± 0.01
Protoporphyrinogen oxidase	4.1 ± 0.16	4.1 ± 0.62
Ferrochelatase	11.2 ± 0.87	0.39 ± 0.20

[a] Cells were grown in GSY medium containing 15 μM hemin and harvested at the logarithmic phase of growth. Activities were measured in triplicate in cell extracts with the exception of protoporphyrinogen oxidase, which was assayed in membranes.

[b] Data are expressed in nanomoles of product formed per hour per milligram of protein (average ± standard deviation).

The source of heme in strain I110ek4 in cultured cells. Reduced-minus-oxidized absorption spectra of cell extracts from strains I110 and I110ek4 showed that the mutant contained approximately 20% of the cytochrome heme of the wild-type strain grown under the same conditions (Fig. 3). Because the cells were grown in the presence of 15 μM hemin, the spectra do not distinguish whether the cytochrome heme found in strain I110ek4 was derived from the media or whether synthesis occurred in the mutant strain. Two lines of evidence show that the heme is not synthesized in strain I110ek4 cells. First, the *B. japonicum* ALA synthase mutant MLG1 was grown in the presence and absence of exogenous hemin to determine whether hemin entered the cells. Strain MLG1 cells grown in yeast extract medium culture have been previously shown to contain no detectable cytochrome heme (8); however, cytochrome heme was detected in cell extracts of MLG1 grown in the presence of

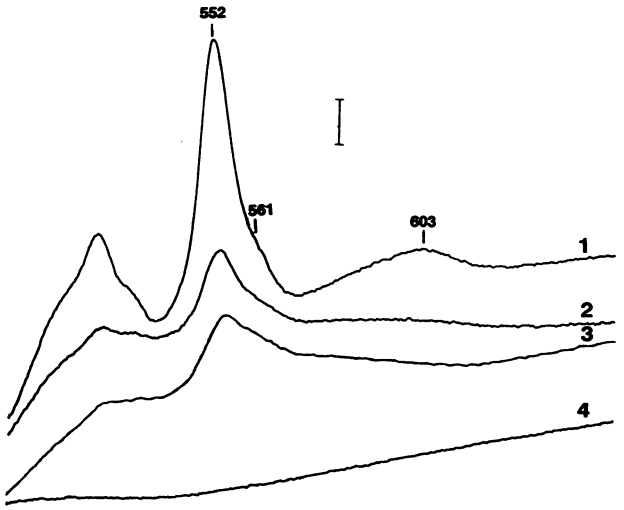

FIG. 3. Dithionite-reduced minus ferricyanide-oxidized cytochrome spectra of cell extracts of strains I110, I110ek4, and MLG1. Scans 1, 2, and 3 are spectra from strains I110, I110ek4, and MLG1, respectively, grown in GSY medium containing 15 μM hemin. Scan 4 is a spectrum of strain MLG1 grown in GSY medium without hemin. The protein concentration in all samples was 2 mg/ml. The vertical bar represents a ΔA of 0.004.

TABLE 3. ^{55}Fe incorporation into heme by strains I110 and I110ek4 grown in culture[a]

Strain and trial	Total ^{55}Fe incorporated into cells (cpm)	Total heme recovered (nmol)	Sp act of purified heme (cpm/nmol)
I110			
Trial 1	5.0×10^7	21.9	2,888
Trial 2	5.8×10^7	27.1	3,956
I110ek4			
Trial 1	4.0×10^7	4.7	158
Trial 2	5.3×10^7	8.1	47

[a] $^{55}FeCl_3$ and unlabeled hemin were added directly to the GSY medium during growth of strains I110 and I110ek4. Cells were grown to an A_{540} of 0.7, and an aliquot was removed to determine the total incorporation of ^{55}Fe into cells. Heme was extracted from the cells as described in the text, and the ^{55}Fe incorporation was determined within the heme.

15 μM exogenous hemin (Fig. 3), indicating that hemin in the medium can enter *B. japonicum* cells. In particular, the presence of mesoheme, the *c*-type cytochrome heme synthesized from protoheme (peak at 552 nm), shows conclusively that hemin entered the cells and was subsequently modified. The second set of experiments addressed whether strain I110ek4 was able to synthesize heme in growing cultured cells. The mutant strain was grown in the presence of $^{55}FeCl_3$ and unlabeled hemin, and then non-covalently bound heme was purified from the cells to determine whether it was labeled with ^{55}Fe. Although ^{55}Fe was taken up by strain I110ek4 cells, no significant ^{55}Fe incorporation was found in the heme extracted from those cells (Table 3). The heme extracted from the medium had a similar specific activity (188 cpm/nmol of heme) as that recovered from strain I110ek4 (103 cpm/nmol of heme), and thus the radioactivity recovered in the heme from the strain I110ek4 was comparable to background levels. However, heme extracted from strain I110 cells contained significant radiolabel (3,444 cpm/nmol of heme), as expected of a cell with normal heme synthesis enzyme activities. These results show that strain I110ek4 behaves as a ferrochelatase mutant in whole cells since ^{55}Fe enters cells grown in culture but is not incorporated into heme. We conclude that strain I110ek4 cannot synthesize heme and that the cytochrome heme discerned in strain I110ek4 originated from exogenous hemin in the media. This conclusion is consistent with the defective ferrochelatase activity and the hemin auxotrophy of the mutant.

Identification of the gene essential for heme synthesis. The nucleotide sequence of the wild-type genomic region corresponding to that mutagenized in strain LORBF1 was determined (Fig. 4). The open reading frame mutated in strain LORBF1 is 1,032 kb in size, and the predicted sequence of the encoded peptide contains 344 amino acids (Fig. 5). While this work was in progress, the ferrochelatase genes from humans, mice, and *Saccharomyces cerevisiae* were obtained (3, 9, 12, 17, 32), and we found that the eukaryotic ferrochelatases shared considerable homology with the putative bacterial amino acid sequence obtained in the present work. The homology was somewhat higher with the human enzyme (Fig. 6) than with the mouse or yeast proteins; the former shared 27% identity and 49% similarity (as defined as identical plus conservative amino acid substitutions) with the predicted amino acid sequence from *B. japonicum*. The putative bacterial ferrochelatase was 26 and 22% identical (50 and 45% similar) to the mouse and yeast ferrochelatases, respectively. The homology of the putative *B. japonicum*

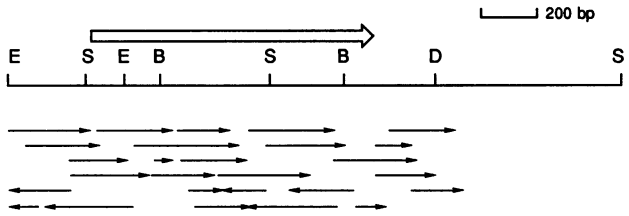

FIG. 4. Strategy used for sequencing the *B. japonicum hemH* gene. The arrows show the direction and extent of sequencing for each subclone. Restriction sites: E, *Eco*RI; S, *Sal*I; B, *Bgl*II; D, *Dde*I. The 344-amino-acid open reading frame is depicted by the open arrow.

protein with the eukaryotic ferrochelatases, along with the ferrochelatase deficiency of *B. japonicum* mutants disrupted in the described gene, strongly implies that the gene encodes ferrochelatase. We propose that the *B. japonicum* gene identified in the present work be designated *hemH*, which is

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GAATTCGGCGAGCAGCACCGCGAAGGCGAAGAACGGAAAGGTCTCGATGCCGTTCTGGTG  60
GGCGCCGAGCGCGCTGCGCGATGGCGTCCTCATAGAAGGCGGGATCGCGCGGCGGGAATT 120
GTCGAACCCGCGAAACCGGATCCATTTGATCGAGGCGATCGTCGCAAGGTAGAGCAGCAG 180
CGCTCCGAATACGCACCATTCCGCGAGTGTCATCTTCCCTCCCCCGCTTGGGTGCGACCC 240
TAGCGAAACCGGGCTCATCTTGACAAGTTCGGTGCAACGCGCGACGCAACTGATCATGTC 300
                                                      M  S  -

GACCGCCGCTCCCAACGAGACCACACAGCCAACCGTCCGGTCCGGCCAGAAGCGGGTCGG 360
 T  A  A  P  N  E  T  T  Q  P  T  V  R  S  G  Q  K  R  V  G  -

CGTGCTCCTGGTCAATCTCGGCACGCCCGATACGGCCGATGCGCCCGGGGTGCGGGTCTA 420
 V  L  L  V  N  L  G  T  P  D  T  A  D  A  P  G  V  R  V  Y  -

TCTCAAGGAATTCCTCTCGGACGCCCGGGTCATCGAGGACCAGGGCCTGGTCTGGAAGGT 480
 L  K  E  F  L  S  D  A  R  V  I  E  D  Q  G  L  V  W  K  V  -

GGTGCTGAACGGGATCATCCTGCGCCAGCGTCCCCGCAGCAAGGCGCTCGACTACCAGAA 540
 V  L  N  G  I  I  L  R  Q  R  P  R  S  K  A  L  D  Y  Q  K  -

GATCTGGAACAACGAGAAGAACGAGTCGCCGCTGAAGACCATCACGCGGTCGCAAAGCGC 600
 I  W  N  N  E  K  N  E  S  P  L  K  T  I  T  R  S  Q  S  A  -

AAAGCTCGCCGCCGCGCTGTCGGATCGCGATCATGTCGTGGTGGACTGGGCGATGCGCTA 660
 K  L  A  A  A  L  S  D  R  D  H  V  V  V  D  W  A  M  R  Y  -

CGGCAATCCCTCGATCAAGTCGGGCATCGACGCGCTGATCGGAGGGATGCGACCGCATCT 720
 G  N  P  S  I  K  S  G  I  D  A  L  I  G  G  M  R  P  H  L  -

CGCGGTCCCGCTTTATCCGCAATATTCCGCCTCGACCTCGGCGACCGTCTGCGACGAGGT 780
 A  V  P  L  Y  P  Q  Y  S  A  S  T  S  A  T  V  C  D  E  V  -

GTTCCGCGTGCTCGCCCGCCTGCGTGCGCAGCCGACGCTGCGCGTGACGCCGCCGTACTA 840
 F  R  V  L  A  R  L  R  A  Q  P  T  L  R  V  T  P  P  Y  Y  -

CGAGGACGAGGCCTATATCGAGGCGCTCGCCGTCTCGATCGAGACGCATCTGGCAACCCT 900
 E  D  E  A  Y  I  E  A  L  A  V  S  I  E  T  H  L  A  T  L  -

GCCGTTCAAGCCGGAGCTGATCGTCGCCTCCTTCCACGGCATGCCGAAATCCTATGTCGA 960
 P  F  K  P  E  L  I  V  A  S  F  H  G  M  P  K  S  Y  V  D  -

CAAGGGCGATCCCTATCAGGAGCATTGCATCGCCACGACCGAGGCGCTGCGCGCCGCTCG 1020
 K  G  D  P  Y  Q  E  H  C  I  A  T  T  E  A  L  R  A  A  R  -

GCGGCTGGACGCATCAAAATTGCTGCTGACCTTCCAGTCGCGCTTCGGCAATGACGAGTG 1080
 R  L  D  A  S  K  L  L  L  T  F  Q  S  R  F  G  N  D  E  W  -

GCTCCAGCCCTACACCGACAAGACGATGGAGCGGCTCGCGAAAGAGGGCGTGCGCCGCAT 1140
 L  Q  P  Y  T  D  K  T  M  E  R  L  A  K  E  G  V  R  R  I  -

CGCGGTGGTGACGCCGGGTTTTGCCGCCGACTGCCTGGAGACGCTGGAGGAGATCGCGCA 1200
 A  V  V  T  P  G  F  A  A  D  C  L  E  T  L  E  E  I  A  Q  -

GGAGAATGCCGAGATCTTCAAGCACAATGGCGGCGAGACGTTTTCCGCGATCCCCTGCCT 1260
 E  N  A  E  I  F  K  H  N  G  G  E  T  F  S  A  I  P  C  L  -

CAACGACAGCGAACCCGGCATGGACGTGATCCGCACCCTGGTGCTGCGCGAGCTCCAGGG 1320
 N  D  S  E  P  G  M  D  V  I  R  T  L  V  L  R  E  L  Q  G  -

CTGGATATAGTGAGCCTGGATCTGATGGTTCGCCCCTTGAACCGCCGCGCGTGTCTGGCG 1380
 W  I  *

CTTCTTGGGACGATCGTCGTGCTGCCGGCTTCGCGACAGGCGCACGAGGCGAATGCGACG 1440
CGGCGGCTCGGCGTGCTCTCGGTCACGGCCGCCGACGATGCGATCGGGCAGACGCGCAGC 1500
GCCATCCTGGTCGAGGCGCTCGCCGCCCACGGCTGGAAGGAGCACGGCAATCTCAGGATC 1560
GACTGGCGCCAGGCCGGGCGACAGGGCCGGGATCGCGGGCTCGCCGACG             1620
```

FIG. 5. Nucleotide sequence of the *B. japonicum hemH* gene and the deduced amino acid sequence of ferrochelatase. The nucleotide sequence of the 344-amino-acid open reading frame is shown with approximately 300 bp of flanking DNA on each end. The nucleotides are numbered starting at the *Eco*RI site. *, stop codon.

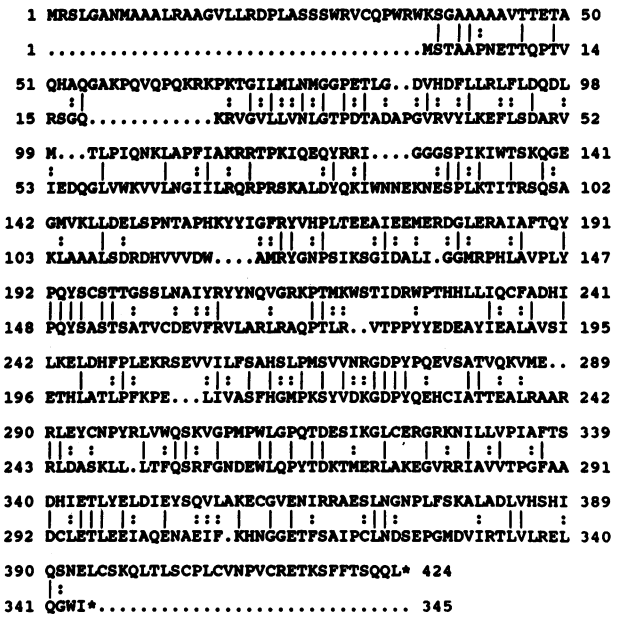

FIG. 6. Comparison of the putative amino acid sequences of the ferrochelatases from humans (top) and *B. japonicum* (bottom). Solid vertical line denotes identity, and dotted line denotes conservative amino acid substitutions as described by Lipman and Pearson (14).

consistent with the nomenclature of bacterial heme synthesis genes.

Symbiosis of strain I110ek4 and soybean plants. Strain I110ek4 incited small, poorly developed nodules on soybean plants that contained few viable bacteria and which could not fix nitrogen (Table 4). The general appearance of 28-day-old plants inoculated with strain I110ek4 was identical to that of the uninoculated plant controls (data not shown). We were unable to detect heme in the plant cytosol of nodules incited by strain I110ek4, indicating the absence of leghemoglobin heme, whereas the nodules induced by strain I110 contained copious amounts of heme (Table 4). In addition, no leghemoglobin apoprotein was detected immunologically in plant cytosol of nodules elicited by mutant strain I110ek4 (Table 4), but it was easily detected in nodules formed from the parent strain. Thus, unlike ALA synthase (10), *B. japonicum* ferrochelatase is essential for normal nodule development on soybean plants.

DISCUSSION

In the present work, we describe a *B. japonicum* mutant that is defective in ferrochelatase activity, and thus it is incapable of catalyzing the last step of the heme biosynthetic pathway. Consistent with this defect, the mutant, strain I110ek4, was a strict hemin auxotroph in liquid cultures, it accumulated the heme precursor protoporphyrin IX, and growing cultures could not incorporate ^{55}Fe into heme. The putative protein encoded by the gene disrupted in the ferrochelatase mutant is homologous to eukaryotic ferrochelatases; this homology, along with the described phenotype of strain I110ek4, strongly suggests that the gene isolated herein is *hemH*, that which encodes ferrochelatase.

TABLE 4. Symbiotic properties of soybean nodules incited by strains I110 and I110ek4

Strain	Nitrogenase activity[a]	Viable cell count[b]	Hemoglobin heme[c]	Presence of apoleghemoglobin[d]
I110	10.2 ± 0.6	9.2×10^{10}	145 ± 12	+
I110ek4	0	1.5×10^{4}	0	−

[a] Activity is expressed in micromoles of ethylene formed per hour per gram of nodules (average ± standard deviation).
[b] CFU per gram of nodule (fresh weight).
[c] Nanomoles of heme per gram of nodule (fresh weight).
[d] Determined immunologically by cross-reactivity of soybean nodule extracts with antileghemoglobin antibodies as described in the text.

The hemin auxotrophy of mutant strain I110ek4 is interesting in the context of our previous observation that the *B. japonicum* ALA synthase mutant MLG1 grows well aerobically in yeast extract liquid culture despite the absence of discernible heme (8). Those studies also showed that the ALA analog levulinic acid inhibits growth of strain MLG1, implying that ALA, and hence heme, may be present and necessary in a very small quantity for the viability of *B. japonicum*. The hemin auxotrophy of strain I110ek4 supports this view, and thus far it provides the best evidence that *B. japonicum* requires heme, but the amount needed in culture is below the level of direct detection. We reiterate that data which support a heme requirement for *B. japonicum* do not mitigate our previous conclusion that the overwhelming majority of heme normally expressed by that bacterium is not necessary for aerobic growth and respiration in culture (8) and that the energetics of *B. japonicum* remain an enigma in that regard. Because exogenous hemin is sufficient to allow growth of the ferrochelatase mutant I110ek4 (Table 1) but not of the ALA synthase mutant in liquid minimal media (8), ALA is likely to be required for something in addition to heme synthesis. Vitamin B_{12} formation requires ALA for synthesis of the corrin ring (33), and sulfite reductase, which contains a corrinoid prosthetic group (33), is necessary for the synthesis of sulfur amino acids. We recently found that the *B. japonicum* ALA synthase mutant grows, albeit poorly, in defined media when hemin, cysteine, and vitamin B_{12} are all present (4), but we are unable to find conditions for normal growth of that mutant in liquid minimal media unless ALA is added.

B. japonicum ferrochelatase is necessary for normal nodule development; soybean nodules elicited by mutant strain I110ek4 are abnormal in appearance, do not fix nitrogen, contain few viable bacteria, and do not express leghemoglobin heme or the apoprotein (Table 4). The conclusion that *B. japonicum* ferrochelatase is essential for normal nodule development is not a trivial one in light of the observation that an ALA synthase mutant of *B. japonicum* incites nitrogen-fixing nodules on soybean plants which contain leghemoglobin (10). Previously, we provided evidence that the ALA synthase mutant is rescued symbiotically because it can take up ALA formed by the plant host and synthesize heme from it (27). The present work implies that a ferrochelatase mutant of *B. japonicum* cannot be rescued by soybean plants and is therefore consistent with our previous conclusion that the host provides the bacterial endosymbiont with an early heme precursor (27). Mutant strain I110ek4 also differs from a *B. japonicum* heme synthesis mutant defective in protoporphyrinogen oxidase activity (strain LO505) in that nodules incited by the latter contain bacteria and express leghemoglobin apoprotein but do not express the heme moiety (19). Ramseier et al. (25) showed that strain LO505 is mutated in one of a cluster of genes involved in cytochrome *c* synthesis, but the functions of these genes are not known.

Because nodules elicited by mutant strain I110ek4 contain

few viable bacteria, it is probable that leghemoglobin is one of numerous late proteins missing in those nodules; hence, we do not believe that the present work directly addresses the source of the hemoglobin prosthetic group. However, acquisition of the *B. japonicum hemH* gene provides a tool for addressing this question in future.

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REFERENCES

1. **Appleby, C. A.** 1984. Leghemoglobin and *Rhizobium* respiration. Annu. Rev. Plant Physiol. **35**:443–478.
2. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
3. **Brenner, D. A., and F. Frasier.** 1991. Cloning of murine ferrochelatase. Proc. Natl. Acad. Sci. USA **88**:849–853.
4. **Chauhan, S., and M. R. O'Brian.** Unpublished data.
5. **Cutting, J. A., and H. M. Schulman.** 1969. The site of heme synthesis in soybean root nodules. Biochim. Biophys. Acta **192**:486–493.
6. **Dickstein, R., D. C. Scheirer, W. H. Fowle, and F. M. Ausubel.** 1991. Nodules elicited by *Rhizobium meliloti* heme mutants are arrested at an early stage of development. Mol. Gen. Genet. **230**:423–432.
7. **Döss, M., and B. Ulshofer.** 1971. Porphyrin stability as a function of the number of carboxylic acid side chains. Biochem. Biophys. Acta **237**:356–360.
8. **Frustaci, J., I. Sangwan, and M. R. O'Brian.** 1991. Aerobic growth and respiration of a δ-aminolevulinic acid synthase (*hemA*) mutant of *Bradyrhizobium japonicum*. J. Bacteriol. **173**:1145–1150.
9. **Gokhman, I., and A. Zamir.** 1990. The nucleotide sequence of the ferrochelatase and $tRNA^{val}$ gene region from *Saccharomyces cerevisiae*. Nucleic Acids Res. **18**:6130.
10. **Guerinot, M. L., and B. K. Chelm.** 1986. Bacterial δ-aminolevulinic acid synthase activity is not essential for leghemoglobin formation in the soybean/*Bradyrhizobium japonicum* symbiosis. Proc. Natl. Acad. Sci. USA **83**:1837–1841.
11. **Jacobs, N. J., and J. M. Jacobs.** 1982. Assay for enzymatic protoporphyrinogen oxidation, a late step in heme synthesis. Enzyme **28**:206–219.
12. **Labbe-Bois, R.** 1990. The ferrochelatase from *Saccharomyces cerevisiae*. Sequence, disruption, and expression of the structural gene *HEM15*. J. Biol. Chem. **265**:7278–7283.
13. **Leong, S. A., G. S. Ditta, and D. R. Helinski.** 1982. Heme biosynthesis in *Rhizobium*. Identification of a cloned gene coding for δ-aminolevulinic acid synthetase from *Rhizobium meliloti*. J. Biol. Chem. **257**:8724–8730.
14. **Lipman, D. J., and W. R. Pearson.** 1985. Rapid and sensitive protein similarity searches. Science **227**:1435–1441.
15. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Nadler, K. D., and Y. J. Avissar.** 1977. Heme synthesis in soybean root nodules. 1. On the role of bacteroid δ-aminolevulinic acid synthase and δ-aminolevulinic acid dehydratase in the synthesis of the heme of leghemoglobin. Plant Physiol. **60**:433–436.
17. **Nakahashi, Y., S. Taketani, M. Okuda, K. Inoue, and R. Tokunaga.** 1990. Molecular cloning and sequence analysis of cDNA encoding human ferrochelatase. Biochem. Biophys. Res. Commun. **173**:748–755.
18. **O'Brian, M. R., P. M. Kirshbom, and R. J. Maier.** 1987. Tn*5*-induced cytochrome mutants of *Bradyrhizobium japonicum*: effects of the mutations on cells grown symbiotically and in culture. J. Bacteriol. **169**:1089–1094.
19. **O'Brian, M. R., P. M. Kirshbom, and R. J. Maier.** 1987. Bacterial heme synthesis is required for expression of the leghemoglobin holoprotein but not the apoprotein in soybean root nodules. Proc. Natl. Acad. Sci. USA **84**:8390–8393.
20. **O'Brian, M. R., and R. J. Maier.** 1982. Electron transport components involved in hydrogen oxidation in free-living *Rhizobium japonicum*. J. Bacteriol. **152**:422–430.
21. **O'Brian, M. R., and R. J. Maier.** 1983. Involvement of cytochromes and a flavoprotein in hydrogen oxidation in *Rhizobium japonicum* bacteroids. J. Bacteriol. **155**:481–487.
22. **O'Brian, M. R., and R. J. Maier.** 1987. Isolation of a cytochrome aa_3 gene from *Bradyrhizobium japonicum*. Proc. Natl. Acad. Sci. USA **84**:3219–3223.
23. **Pawlowski, K., J. Schell, and F. J. de Bruijn.** 1988. Construction and characterization of heme biosynthesis (*hemA*) mutants of *Azorhizobium caulinodans* ORS571; *hemA* is essential for nodulation and symbiotic nitrogen-fixation, p. 380. *In* F. Bothe, J. de Bruijn, and W. E. Newton (ed.), Nitrogen-fixation: hundred years after. Gustav Fischer, New York.
24. **Porra, R. J.** 1975. A rapid spectrophotometric assay for ferrochelatase activity in preparations containing much endogenous hemoglobin and its application to soybean root-nodule preparations. Anal. Biochem. **68**:289–298.
25. **Ramseier, T. M., H. V. Winteler, and H. Hennecke.** 1991. Discovery and sequence analysis of bacterial genes involved in the biogenesis of c-type cytochromes. J. Biol. Chem. **266**:7793–7803.
26. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
27. **Sangwan, I., and M. R. O'Brian.** 1991. Evidence for an interorganismic heme biosynthetic pathway in symbiotic soybean root nodules. Science **251**:1220–1222.
28. **Sangwan, I., and M. R. O'Brian.** 1992. Characterization of δ-aminolevulinic acid formation in soybean root nodules. Plant Physiol. **98**:1074–1079.
29. **Simon, R., U. Priefer, and A. Puhler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Biotechnology **1**:784–790.
30. **Smith, K. M.** 1975. Porphyrins and metalloporphyrins. Elsevier Scientific, Amsterdam.
31. **Stanley, J., D. N. Dowling, and W. J. Broughton.** 1988. Cloning of *hemA* from *Rhizobium* sp. NGR234 and symbiotic phenotype of a gene-directed mutant in diverse legume genera. Mol. Gen. Genet. **215**:32–37.
32. **Taketani, S., Y. Nakahashi, T. Osumi, and R. Tokunaga.** 1990. Molecular cloning, sequencing, and expression of mouse ferrochelatase. J. Biol. Chem. **265**:19377–19380.
33. **Warren, M. J., and A. I. Scott.** 1990. Tetrapyrrole assembly and modification into the ligands of biologically functional cofactors. Trends Biochem. Sci. **15**:486–491.