Bacterial δ -aminolevulinic acid synthase activity is not essential for leghemoglobin formation in the soybean/*Bradyrhizobium japonicum* symbiosis

(heme biosynthesis/nitrogen fixation)

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Communicated by Anton Lang, November 14, 1985

ABSTRACT Previous studies of legume nodules have indicated that formation of the heme moiety of leghemoglobin is a function of the bacterial symbiont. We now show that a *hemA* mutant of *Bradyrhizobium japonicum* that cannot carry out the first step in heme biosynthesis forms fully effective nodules on soybeans. The bacterial mutant strain was constructed by first isolating the wild-type *hemA* gene encoding δ -aminolevulinic acid synthase (EC 2.3.1.37) from a cosmid library, using a fragment of the *Rhizobium meliloti hemA* gene as a hybridization probe. A deletion of the *hemA* gene region, generated *in vitro*, then was used to construct the analogous chromosomal mutation by gene-directed mutagenesis. The mutant strain had no δ -aminolevulinic acid synthase activity and was unable to grow in minimal medium unless δ -aminolevulinic acid was added. Despite its auxotrophy, the mutant strain incited nodules that appeared normal, contained heme, and were capable of high levels of acetylene reduction. These results rule out bacterial δ -aminolevulinic acid synthase activity as the exclusive source of δ -aminolevulinic acid for heme formation in soybean nodules.

Bacteria of the genera *Rhizobium* and *Bradyrhizobium* are able to form nitrogen-fixing associations with leguminous plants. Establishment of the symbiosis begins with recognition and invasion of the roots of the appropriate legume host by rhizobia, followed by the proliferation and differentiation of both host and bacterial cells to form highly specialized structures called root nodules. It is within these root nodules that conditions are established for bacterial nitrogen fixation and for host plant assimilation of the nitrogen fixed. Metabolite exchange between the host plant and its microsymbiont is essential in these symbioses, in which the photosynthetic carbon-fixation capability of plants is combined with the bacterial ability to reduce atmospheric N_2 . The myoglobin-like hemoprotein leghemoglobin is only found in nodules; neither partner is able to produce it when grown separately. All known nitrogen-fixing symbioses involving rhizobia require the presence of a hemoglobin to function effectively (for review, see ref. 1). It is thought that leghemoglobin allows the delivery of a high flux of oxygen at a very low tension, thus permitting the bacteria to carry out oxidative metabolism while protecting the oxygen-sensitive nitrogenase from inactivation. The apoprotein of leghemoglobin is encoded in the plant genome, as indicated both by product chemistry and by analyses of isolated genes (1). In contrast, the heme moiety (Lb-heme) is thought to be synthesized by the bacteria. The tetrapyrrole biosynthetic pathway, which leads to the formation of corrins, bilins, and chlorophyll as well as to that of heme, is operational in plants (2), and the possibility that

heme precursors are transferred between the symbiotic partners has not been excluded.

Previous studies of Lb-heme biosynthesis in nodules demonstrated that the bacterial fraction from nodules was more active in incorporating specific precursors into heme *in vitro* than was the plant fraction, suggesting that the bacteria were the site of nodule Lb-heme synthesis (3, 4). Consistent with this interpretation were reports that δ -aminolevulinic acid (ALA) synthase [succinyl-CoA:glycine (C-succinyltransferase (decarboxylating), EC 2.3.1.37) (5) and ferrochelatase (6) were both associated predominantly with the bacterial fraction in root nodule extracts. In addition, Nadler and Avissar (5) showed parallel increases in bacterial ALA synthase activity and the heme content of developing soybean nodules. Not all available data support the idea that the bacteria carry out the complete synthesis of Lb-heme. For example, ALA dehydratase has been found in both the plant and bacterial fractions of nodules (5, 7). Godfrey *et al.* (7) suggested that in the nodule there is a coordinate effort to produce heme. Such a coordination occurs in animal cells, where different intermediates in heme biosynthesis are produced in either the cytoplasm or the mitochondria (8).

Rhizobial strains with mutations in the heme biosynthetic pathway might be useful in clarifying which partner carries out the various steps of Lb-heme formation in nodules. Many rhizobial mutants have been found that form white nodules, and some of these nodules have been shown to lack heme (9, 10). However, none of these mutant strains have been characterized as to the nature of their genetic defect, and most were selected in a manner that eliminated auxotrophs and thus would preclude the isolation of heme biosynthetic mutants. Leong *et al.* (11) reported that alfalfa plants inoculated with an ALA synthase-deficient strain of *R. meliloti* grew no better than uninoculated controls and formed only small, white (and therefore presumably leghemoglobin-free) nodules which had no nitrogenase activity. Because of the growing number of differences that have been shown to exist between the *Rhizobium* and *Bradyrhizobium* microsymbionts (12, 13), we have initiated a study of the genetics and regulation of the heme biosynthetic pathway of *B. japonicum*, the soybean symbiont, in order to clarify its role in the development of nitrogen-fixing root nodules. In this paper we describe the isolation and characterization of a gene, *hemA*, encoding ALA synthase. We also present evidence that ALA synthase activity by *B. japonicum* is not essential for the formation of effective nodules containing leghemoglobin. Evidently the soybean plant is able to rescue the ALA synthase-deficient *B. japonicum* strain. Thus, the regulation

Abbreviations: Lb-heme, the heme moiety of leghemoglobin; ALA, δ -aminolevulinic acid; bp, base pair(s).

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of the synthesis of the heme moiety in soybean nodules might be more complex than previously believed.

MATERIALS AND METHODS

Chemicals. Ethyl 3-ketobutanoate, ALA, coenzyme A, glycine, hemin, kanamycin sulfate, 2-mercaptoethanol, protoporphyrin IX, pyridine, pyridoxal phosphate, rifampicin, and sodium succinate were obtained from Sigma; ATP, from P-L Biochemicals; and tetracycline hydrochloride and Hepes, from United States Biochemical (Cleveland, OH).

Bacteria, Plasmids, and Cosmids. All experiments were carried out using a small-colony derivative of *B. japonicum* strain USDA 311b110 (for a description of the stable derivatives of this strain, see refs. 14 and 15), designated here as USDA 110. *B. japonicum* strain MLG 1 is a Δ hemA derivative of USDA 110 as described in this paper. The *Escherichia coli* strains ED8654 (*galK*, *galT*, *trpR*, *metB*, *hsdR*, *supE*, *supF*, *lacY*) (16) and HB101 [*hsdS* (r_B^- , m_B^-), *recA*, *ara*, *proA*, *lacY*, *galK*, *rpsL*, *xyl*, *mtl*, *supE*] (17) were used for routine plasmid construction and maintenance. SASX41B (Hfr PO2A/*hemA*, *metB*, *spoT*, *relA*), an ALA synthase-deficient strain, was used for complementation tests. The cosmid library of *B. japonicum* DNA used was that described by Adams *et al.* (18). pRJcos15-89 is a cosmid, identified from this library, that carries the *B. japonicum* *hemA* gene. pRK2013 is a helper plasmid that carries the conjugal-transfer genes of RK2 (19). pDS4101 is a derivative of ColK that can mobilize derivatives of pBR322 (20). pKC7 carries the *nptII* gene encoding kanamycin resistance (21). pSL1 carries the *R. meliloti* *hemA* gene (11). pBJ110 and pBJ131 are derivatives of pBR322 whose construction is described in this paper.

Bacterial Media and Growth Conditions. *B. japonicum* was routinely cultured in broth or on agar plates of YEX medium (18). To check the ALA auxotrophy of strain MLG 1, a mineral salts base medium adapted from O'Gara and Shanmugam (22) was used, which had the following composition per liter: (NH₄)₂SO₄, 0.33 g; KH₂PO₄, 0.3 g; Na₂HPO₄, 0.3 g; MgSO₄·7H₂O, 0.12 g; CaCl₂, 0.05 g. Trace elements were added (22) and the pH was adjusted to 6.8. After autoclaving, 1 ml of a filter-sterilized vitamin solution (composition per 100 ml: riboflavin, 20 mg; biotin, 12 mg; thiamine-HCl, 80 mg; inositol, 48 mg; *p*-aminobenzoic acid, 8 mg; nicotinic acid, 50 mg; calcium pantothenate, 80 mg; cyanocobalamin, 0.1 mg) and 7.5 ml of filter-sterilized 20% (wt/vol) xylose were added per liter. Strain MLG 1 was routinely grown by supplementing the medium with ALA at 50 μ g/ml. For plating exconjugates from the matings, YEGG agar with the following composition (per liter) was used: gluconic acid, 5 g; sodium glutamate, 1 g; yeast extract, 1 g; K₂HPO₄, 0.5 g; MgSO₄·7H₂O, 0.2 g; NaCl, 0.1 g. *E. coli* strains were routinely grown on LB medium (23). To test the complementation of the ALA auxotrophy of SASX41B, cells were plated onto M9 medium (23) containing 0.2% maltose with or without ALA (50 μ g/ml).

Plant Tests. Soybean seeds [*Glycine max* (L.) Merr. cv. Amsoy 71] were surface-sterilized by soaking for 5 min in 1% sodium hypochlorite followed by 30 min in 3% hydrogen peroxide. Seeds were subsequently given four 15-min rinses in sterile distilled water and then soaked for 1 hr either in a late log phase culture of the appropriate strain of *B. japonicum* or in sterile YEX broth in the case of uninoculated control seeds. Seeds were planted in modified Leonard jars as described by Vincent (24), using the nitrogen-free medium of Johnson *et al.* (25). The plants were grown with a 13-hr daily light period, day/night temperatures of 28/25°C, a relative humidity of 60%, and a photon fluence rate of 300 μ mol·m⁻²·sec⁻¹. Twenty-three to twenty-six days after planting the shoots were separated from the roots and dried at 100°C in a forced-air oven prior to weighing. The acetylene-

reduction assay (26) was used to estimate nitrogenase activity of the excised root systems. After the assay, the nodules were excised, rinsed, counted, weighed, and then immediately frozen in liquid nitrogen and stored at -70°C for later use. Freezing and storage of the nodules had no effect on the measurement of heme concentrations or ALA synthase activity.

Strain Verification of Bacteria from Nodules. Nodules were chosen at random, immersed in 95% ethanol for 30 sec and then in 3% hydrogen peroxide for 4 min, and then washed six times with sterile water. These surface-sterilized nodules were crushed aseptically in 1 ml of sterile 0.01% Tween 80. Serial dilutions were plated onto nonselective medium and colonies were subsequently checked for genetic markers.

DNA Methods. All Southern hybridizations, colony hybridizations, and isolation of plasmid and total genomic DNA were carried out as described (18).

Gene-Directed Mutagenesis. A 2.1-kilobase-pair (kbp) region delimited by *EcoRI* and *Xho I* restriction sites was deleted from pBJ110 and replaced with a 1.7-kbp *EcoRI*-*Xho I* restriction fragment containing *nptII* from pKC7, by use of standard DNA manipulation procedures (Fig. 1b). The resultant plasmid, pBJ131 (Fig. 1a), was transformed into a HB101 strain carrying pDS4101 for use as the donor strain in conjugal mating with *B. japonicum*. The efficiency of conjugal transfer was found to increase $\approx$ 10-fold when the mobilizer plasmid pDS4101 was present in the donor cell along with pBJ131. For the mating, cells (ml of culture $\times$ OD₆₀₀) were mixed at a ratio of 1.8 *B. japonicum* recipients to 1 HB101/pBJ131/pDS4101 donor to 1 HB101/pRK2013 helper. They were then pelleted, washed once in 5% LB medium, resuspended in 5% LB medium, and spread-plated onto YEX agar. After incubation for 4 days at 30°C, the cells were removed from the mating plate by suspension in 0.01% Tween 80 and plated onto YEGG agar containing kanamycin (100 μ g/ml), to select for *B. japonicum* exconjugates that had stably integrated the *nptII* gene-containing region, and rifampicin (50 μ g/ml) for counterselection against the *E. coli* donor and helper cells. Colonies from these selective plates were screened by colony hybridization for the absence of all vector sequences, the absence of the deleted *hemA* gene region, and the presence of the *nptII* gene. The DNA structure of these deletion mutants was verified by hybridization analysis of restriction digests of total genomic DNA.

Other Biochemical Methods. Nodule extracts were prepared from frozen nodules as follows, with all steps carried out at 4°C. Nodules (5 g) were allowed to thaw in 15 ml of 25 mM phosphate buffer (pH 7.0), washed twice, and then macerated in 15 ml of the same buffer with a mortar and pestle. The nodule brei was squeezed through four layers of Miracloth (Chicopee Mills, Milford, NJ), and the filtrate was centrifuged three times at 300 $\times$ g for 10 min and then centrifuged at 16,300 $\times$ g for 20 min to pellet the bacteria. The supernatant from this last centrifugation was used for determination of heme, and the pelleted bacteria were used to prepare cell-free extracts for ALA synthase determinations.

The procedure for measuring ALA synthase activity in cell-free extracts was adapted from that described by Roessler and Nadler (27) as follows. Cells collected from nodules as described above or from free-living culture were washed at 4°C in 3 ml of 200 mM Hepes, pH 8.0/33 mM MgCl₂/1 mM 2-mercaptoethanol, centrifuged at 16,300 $\times$ g for 10 min, and resuspended in 3 ml of the same buffer. The cells were then ruptured by passage through a French pressure cell at 844 kg/cm² at 4°C. The extracts were centrifuged at 23,300 $\times$ g for 20 min at 4°C, and the supernatant was used for measurement of ALA synthase activity. Reaction mixtures (1 ml) containing 100 mM Hepes (pH 8.0), 16.5 mM MgCl₂, 100 mM sodium succinate, 100 mM glycine, 7 mM ATP, 50 μ M coenzyme A, 0.3 mM pyridoxal

A

B

FIG. 1. (A) Restriction endonuclease map of the *B. japonicum* *hemA* region. Shown are the *Pst* I inserts of pBJ110 and pBJ131, which carry the wild-type and deleted *hemA* regions, respectively, within pBR322. Restriction sites are *Bgl* II (B), *Eco*RI (R), *Pst* I (P), *Sal* I (S), and *Xho* I (X). The arrows indicate the positions and directions of transcription of the *hemA* and *npII* genes. (B) Protocol for the construction of the deleted *hemA* gene region. The *Eco*RI-*Xho* I region of pBJ110 was excised and replaced by the *npII*-containing *Eco*RI-*Xho* I fragment from pKC7 (thick line), to form pBJ132. The 3' flanking region of the *hemA* gene was then replaced by the addition of the *Eco*RI fragment from pBJ110 (thin line) into the single *Eco*RI site of pBJ132, to form pBJ131. pBJ131 was checked by restriction analysis for the correct orientation of the added *Eco*RI fragment.

phosphate, 0.5 mM 2-mercaptoethanol, and 0.5–1.0 mg of protein were incubated for 1 hr at 30°C. The reaction was terminated by the addition of 65 μ l of 100% (wt/vol) trichloroacetic acid. After 15 min on ice, the protein was pelleted by centrifugation. One milliliter of the supernatant was combined with 0.25 ml of 0.65 M Na_2PO_4 and 0.1 ml of ethyl 3-ketobutanoate. The tubes were capped and incubated at 100°C for 15 min. After cooling, 1.35 ml of modified Ehrlich reagent (28) was added and the resulting colored complex was analyzed at 553 nm after 15 min. The molar extinction coefficient was determined to be 6.1×10^4 , in good agreement with other reported values (27). The absorption spectra produced by the incubation product and by authentic ALA standards were recorded and compared in order to confirm the identity of the product.

Heme concentrations in the nodule cytosol were determined by the pyridine hemochrome method as described by Bisseling *et al.* (29), with the following modification: 0.5 ml of pyridine and 0.25 ml of 1 M NaOH were added to 2.1 ml of sample. Protein was determined by a modification of the Lowry procedure (30) using egg white lysozyme as a standard.

RESULTS

Isolation of the *B. japonicum* *hemA* Gene. A genomic library of *B. japonicum* USDA 110 DNA, constructed in the broad-host-range cosmid pLAFR1 (18), was screened for recombinant cosmids carrying the gene encoding ALA synthase (*hemA*), with a 1.4-kbp *Sal* I-*Hind*III restriction endonuclease fragment of the *R. meliloti* *hemA* gene isolated from pSL1 (11) as hybridization probe. Several cosmids were identified that had homology to the *R. meliloti* probe. Further screening of the cosmids identified a 4.2-kbp *Pst* I fragment of *B. japonicum* DNA that contained the entire region of homology. The restriction endonuclease map of this region is illustrated in Fig. 1A.

Characterization of the *B. japonicum* *hemA* Gene. To verify that the *Pst* I fragment identified on the basis of homology was a contiguous, nonrearranged region of the bacterial genome, the fragment was transferred from one of the cosmids (pRJcos15-89) into the *Pst* I site of pBR322 (designated pBJ110) and used to probe cellulose nitrate transfers of various restriction digests of *B. japonicum* USDA 110 genomic DNA. All hybridization was in agreement with the restriction map presented in Fig. 1A, indicating that no rearrangement had occurred during the DNA manipulations

and that only one copy of the *hemA* gene exists in the *B. japonicum* genome (Fig. 2). To localize the region of pBJ110 that is homologous to the *R. meliloti* *hemA* gene, two different restriction endonuclease fragments were used as hybridization probes. These fragments represent the 5' half and the 3' half of the *R. meliloti* gene. Each probe hybridized to a different region of the *B. japonicum* DNA, indicating the approximate location as well as the direction of transcription of the *hemA* gene (Fig. 1). This localization has been confirmed by nucleotide sequence determination (unpublished data).

To confirm that this region encoded an ALA synthase, a *B. japonicum* mutant strain was constructed by way of gene-directed mutagenesis. The technique utilized takes advantage of the fact that pBR322 derivatives can be conjugally mobilized with the aid of a helper plasmid into *Bradyrhizobium* but

FIG. 2. Hybridization analyses of *B. japonicum* genomic DNA. *Bgl* II digests of total cellular DNA from strain 110 (lanes 1, 3, and 5) or strain MLG 1 (lanes 2, 4, and 6) were transferred to cellulose nitrate and hybridized to the following radiolabeled DNA probes: the 1.4-kbp *Xho* I-*Eco*RI fragment from pBJ110 (lanes 1 and 2), pKC7 (lanes 3 and 4), and pBJ110 (lanes 5 and 6).

are unable to replicate in the recipient because of their narrow host range (31). The pBR322 derivative pBJ131, shown in Fig. 1, was used to effect the conjugal transfer of a mutated version of the *hemA* gene region into wild-type *B. japonicum*. In this plasmid, a major portion of *hemA* has been deleted and replaced, for purposes of manipulation, with a fragment containing the *ntpII* gene which encodes kanamycin resistance. By antibiotic selection, strains of *B. japonicum* were identified in which the mutated version of the *hemA* gene region carried on pBJ131 had become stably maintained in the recipient by apparent homologous recombination into the chromosome. This and all further manipulations were carried out in the presence of exogenous ALA. The frequency of antibiotic resistance due to chromosomal integration was found to be at least an order of magnitude greater than that of resistance resulting from spontaneous mutation. Single and double recombinants were initially distinguished among the kanamycin-resistant exconjugates by the use of colony hybridizations. Strains with no homology to vector sequences presumably arose from double-crossover events and were considered further. Whereas the formation of cointegrates by a single crossover was the predominant recombinational event, strains in which the double crossover had occurred comprised about 10% of the colonies screened. In order to verify the deletion of the *hemA* region and the correct gene replacement, total genomic DNA was extracted from several different presumptive mutant strains, and cellulose nitrate transfers of restriction endonuclease digests were hybridized to various DNA probes (Fig. 2). As expected, a 3.8-kbp *Bgl* II fragment that hybridizes to the 1.4-kbp *Xho* I-*Eco*RI fragment is present in wild type but absent in the mutant (lanes 1 and 2). The *ntpII*-containing plasmid pKC7 hybridizes to *Bgl* II fragments of 2.4 and 2.2 kbp that are present in the mutant but absent in the wild type (lanes 3 and 4), and the 3.8- and 1.1-kbp *Bgl* II fragments in wild type that hybridize to the plasmid pBJ110 are shifted to 2.4 and 2.2 kbp in the mutant (lanes 5 and 6). DNA sequence data (not shown) indicate that all but the first 282 bp of the *hemA* coding region have been deleted in addition to ≈ 900 bp of downstream sequence.

The constructed strain, MLG 1, was unable to grow on minimal medium without exogenous ALA and no ALA synthase activity was detected in extracts prepared from cells of the mutant strain. ALA synthase activity was determined in the wild-type parental strain and was similar to that reported by other workers (27). ALA synthase activity in USDA 110 cell-free extracts was absolutely dependent on the addition of succinate and glycine, and the absorption spectrum of the assay product was identical to that obtained when pure ALA was treated in an identical fashion.

One of the cosmids originally identified as carrying the *hemA* gene, pRJcos15-89, was conjugally transferred (19) into strain MLG 1. There was a 1:1 correspondence between presence of the cosmid and the ability of MLG 1 to grow in the absence of exogenous ALA, further verifying that the mutant phenotype of MLG 1 results directly from the deletion

of the DNA region carried on the cosmid. The cosmid did not complement the ALA auxotrophy of an *E. coli hemA* mutant, SASX41B.

Symbiotic Phenotype of *B. japonicum* MLG 1. To test the symbiotic effectiveness of strain MLG 1, it was necessary to grow the inoculum for plant trials in the presence of ALA, as MLG 1 was auxotrophic for this amino acid. Cells of MLG 1 were then starved of ALA for 48 hr before they were used to inoculate surface-sterilized soybean seeds. The healthy, green appearance of MLG 1-inoculated plants at the time of harvest, as compared to the chlorotic uninoculated control plants, was indicative of this mutant strain's ability to form effective nodules on soybeans. The nodules appeared normal, contained heme, and were capable of high levels of acetylene reduction (Table 1). To ensure that these nodules had been formed by MLG 1, bacteria were isolated from randomly chosen nodules and tested for genetic markers. All of the 100 colonies tested were both kanamycin-resistant and auxotrophic for ALA, indicating that all the bacteria present in the nodules were mutant and that no phenotypic reversion was occurring during nodulation. Further, bacterial extracts prepared from nodules incited by MLG 1 had no detectable ALA synthase activity (Table 1). Keeping in mind the small number of plants analyzed to date, there appear to be significant differences between plants inoculated with the wild-type parental strain USDA 110 and those inoculated with the *hemA* strain MLG 1 with respect to nodule and shoot weights, heme content, and acetylene-reducing activity (Table 1). Plants inoculated with MLG 1 have about 70% of the nodule mass and shoot dry weight as plants inoculated with USDA 110 and have about 60% as much heme. MLG 1-inoculated plants, however, reduce acetylene at twice the rate of those inoculated with USDA 110, on a per-gram-of-nodule basis. These results indicate that the physiological interaction between the plant and bacteria has been modified by the *hemA* deletion.

DISCUSSION

In the bacteria studied to date, ALA, the first committed intermediate in the biosynthesis of heme, is formed from the condensation of succinyl-CoA and glycine in a reaction catalyzed by ALA synthase (32). Using gene-directed mutagenesis, we have shown that a *B. japonicum* strain (MLG 1) that can no longer form ALA by this pathway still nodulates soybeans effectively, even though this strain is auxotrophic for ALA. Although it is not unusual for mutant strains of rhizobia with various nutritional requirements to induce effective nodules, bacterial ALA synthase activity had been implicated in the formation of the prosthetic group of leghemoglobin (5) and has therefore been considered essential for symbiotic nitrogen fixation. Although the data presented here do not rule out the possibility that the bacterial partner normally produces the bulk of the ALA that is needed to form Lb-heme, they do exclude bacterial

Table 1. Comparison of symbiotic effectiveness of *B. japonicum* USDA 110 and MLG 1

Inoculum	No. of plants	No. of nodules per plant	Nodule fresh weight (g) per plant	Shoot dry weight (g) per plant	Nitrogenase activity*	ALA synthase activity†	Heme content‡
USDA 110	18	37 $\pm$ 3	0.70 $\pm$ 0.05	1.30 $\pm$ 0.10	2.15 $\pm$ 0.24§	8.7	91.4
MLG 1	18	33 $\pm$ 2	0.47 $\pm$ 0.03	0.91 $\pm$ 0.04	4.48 $\pm$ 0.51	0	57.1
None	5	0	0	0.63 $\pm$ 0.09	0	ND	ND

Data are expressed as mean $\pm$ SEM. Due to intertrial variability, data from two separate trials have been pooled. ND, not done.

* μ mol of C_2H_4 formed per g of nodule per hr.

†nmol of ALA formed per mg of protein per hr.

‡nmol of heme per g of nodule (mean of duplicate determinations of pooled nodules from 9 plants).

§n = 12 for C_2H_2 -reduction assays on plants inoculated with USDA 110.

production of ALA via ALA synthase as the sole source of ALA for heme formation in soybean nodules.

It is possible that MLG 1 has an alternative pathway of ALA formation that can be utilized as a backup in the presence but not in the absence of the host plant. However, the simplest explanation for the symbiotic phenotype of this mutant is that the plant provides the ALA needed to form the various bacterial tetrapyrroles, including the one needed for formation of Lb-heme. Whether plant-synthesized ALA normally contributes to Lb-heme formation remains to be determined. The host plant has the ability to make ALA (for reviews, see refs. 33 and 34). There is mounting evidence that in both plants and algae, two pathways of ALA formation exist. In *Euglena gracilis*, an ALA synthase pathway like the one found in bacteria, fungi, and animal cells has been proposed to lead to the formation of nonchloroplast cytochrome hemes, and a 5-carbon pathway (so called because it incorporates the 5-carbon skeleton of glutamate directly into ALA) leads to the formation of chloroplast tetrapyrroles, including chlorophyll and chloroplast cytochromes (35). By analogy with animal mitochondria, the plant ALA synthase should be localized in the mitochondria; the ALA formed by this enzyme would be transported into the cytoplasm where the next step in heme biosynthesis is carried out. As all nodule cells contain mitochondria, mitochondrial ALA synthase could provide a source of ALA for the intracellular bacteria. However, the number of mitochondria per cell is small compared to the number of bacteria per infected cell (7). In our preparations of bacterial fractions from nodules, mitochondria would probably be pelleted with the bacterial cells. We did not detect any ALA synthase activity in this fraction prepared from nodules formed by MLG 1, but until a careful effort is made to measure mitochondrial ALA synthase activity, mitochondria remain the most probable source of ALA. Of course, the green tissues of the host plant may be forming ALA and transporting it to the nodule.

Leong *et al.* (11) showed that *R. meliloti hema* mutants formed ineffective nodules on alfalfa plants and that these nodules apparently lacked Lb-heme, as deduced from their white color. This difference in phenotypes between *R. meliloti* ALA synthase mutants and a *B. japonicum* ALA synthase mutant may reflect either inherent differences between the two genera of rhizobia or differences between the host plants that these strains infect. For example, it is possible that rescue of MLG 1 is by an intermediate other than ALA, and that the difference between MLG 1 and the *R. meliloti* mutants is that *B. japonicum* is permeable to that compound whereas *R. meliloti* is not. Permeability to heme precursors appears to vary among organisms (32).

It will be of interest to look at the time-course of leghemoglobin formation as well as that of other nodule-specific proteins in nodules formed by MLG 1 as compared to nodules formed by the wild-type parental strain. Preliminary observations indicate that there might be a delay in the onset of nitrogen fixation, which may explain the differences observed in nodule and shoot weights. It would also suggest that although bacterial ALA synthase is nonessential for an effective symbiosis, it is necessary for optimal interaction between the two organisms.

We thank Gary Ditta and Sally Leong for generously providing plasmids and information. We also acknowledge Ken Poff and Ken Nadler for the use of equipment, Peter Wolk and Jeff Elhai for plasmids and advice on bacterial conjugation, Nick Jacobs and Ken Nadler for helpful discussion, and all the members of our lab for criticism. This work was supported by the U.S. Department of

Energy Division of Biological Energy Research under Contract DE-AC02-76ER01-1338 and by U.S. Department of Agriculture Grant 83-CRCR-1-1307. This is article no. 11860 from the Michigan Agricultural Experiment Station.

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