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The Rhizobial *hemA* Gene Is Required for Symbiosis in Species with Deficient δ-Aminolevulinic Acid Uptake Activity[1]

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Most rhizobial *hemA* mutants induce root nodules on their respective legume hosts that lack nitrogen fixation activity and leghemoglobin expression. However, a *Bradyrhizobium japonicum hemA* mutant elicits effective nodules on soybean, and we proposed previously that synthesis and uptake of the heme precursor δ-aminolevulinic acid (ALA) by the plant and bacterial symbiont, respectively, allow mutant rescue (I. Sangwan, M.R. O'Brian [1991] Science 251: 1220–1222). In the present work, the *B. japonicum hemA* mutant MLG1 elicited normal nodules on three hosts, including cowpea, a plant that is not effectively nodulated by a *hemA* mutant of *Rhizobium* sp. These data indicate that *B. japonicum* rather than soybean possesses the unique trait that allows normal nodule development by a *hemA* mutant. Cowpea expressed glutamate-dependent ALA formation activity in nodules induced by *B. japonicum* strains I110 or MLG1 and by *Rhizobium* sp. ANU240. Exogenous ALA was taken up by *B. japonicum* bacteroids isolated from soybean or cowpea nodules, and the kinetics of uptake were biphasic. By comparison, *Rhizobium* sp. ANU240 had very low ALA uptake activity. In addition, ALA uptake was observed in cultured cells of *B. japonicum* but not in cultured cells of three other rhizobial species tested. We suggest that the differential success of legume-rhizobial *hemA* symbioses is due to an ALA uptake activity in *B. japonicum* that is deficient in other rhizobia, thereby further validating the ALA rescue hypothesis.

Rhizobia establish a symbiotic relationship with certain legumes whereby the plant assimilates nitrogen fixed by the bacterium and the endosymbiont utilizes carbon fixed by the host plant. The symbiosis is manifest as highly specialized root nodules comprised of differentiated plant and bacterial cells. An increase in both plant and bacterial hemes, which is necessary to accommodate the high energy demand of nitrogen fixation (Appleby, 1984; Sangwan and O'Brian, 1992), is one of many changes that occurs in each cell type during nodule development. Plant hemoglobins (leghemoglobin) facilitate oxygen diffusion to the respiring bacteroids and buffer the free oxygen concentration in nodules at a low tension (Appleby, 1984), whereas bacterial Cyts are necessary for oxidative phosphorylation, which drives nitrogen fixation.

Studies with rhizobial heme synthesis mutants show that formation of bacterial heme is a complex process. A *Bradyrhizobium japonicum* mutant defective in the gene encoding ALA synthase (*hemA*), the first committed step in heme synthesis, is rescued symbiotically with respect to bacterial heme formation; thus, it can establish an effective symbiosis (Sangwan and O'Brian, 1991). However, defects in ALA dehydratase or ferrochelatase, enzymes that catalyze the second and final steps in heme synthesis, respectively, elicit soybean nodules that contain few bacteria, do not fix nitrogen, and lack leghemoglobin (Frustaci and O'Brian, 1992; Chauhan and O'Brian, 1993).

An inducible soybean nodule ALA synthesis activity and a *B. japonicum* ALA uptake activity have been demonstrated (Sangwan and O'Brian, 1991, 1992), and we proposed that these traits allow a *B. japonicum hemA* mutant to be rescued by provision of plant ALA to the endosymbiont. Induction of glutamate-dependent ALA synthesis by soybean in nodules is due, at least in part, to activation of *Gsa1* (Sangwan and O'Brian, 1993; Frustaci et al., 1995), a gene encoding the C_5 pathway enzyme glutamate 1-semialdehyde aminotransferase (reviewed by Beale and Weinstein, 1991). *Gsa1* is also expressed in soybean leaves for Chl synthesis; thus, its activation in nodules likely results from an altered spatial expression of a gene not normally expressed strongly in root-derived cells (Frustaci et al., 1995).

Unlike the *B. japonicum*-soybean symbiosis, *hemA* mutants of *Rhizobium meliloti*, *Rhizobium* sp., and *Azorhizobium caulinodans* induce ineffective (non-nitrogen fixing), hemoglobin-defective nodules on alfalfa, cowpea, and *Sesbania rostrata*, respectively (Leong et al., 1982; Stanley et al., 1988; Pawlowski et al., 1993). Those studies are often discussed in the context of the hypothesis that legume hemoglobin heme is synthesized by the bacterial symbiont; however, alfalfa nodules elicited by the *R. meliloti hemA* mutant are arrested in early development (Dickstein et al., 1991), and hence the late nodule protein leghemoglobin is unlikely to be expressed in that organ irrespective of the source of the hemoglobin heme. Therefore, those studies address differences in bacterial heme synthesis rather than formation of plant hemes. Nevertheless, leghemoglobin expression and nitrogen fixation serve as conspicuous markers of plant and bacterial differentiation in nodules, respectively;

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Abbreviation: ALA, δ-aminolevulinic acid.

hence, they are meaningful parameters in analyzing the *hemA* mutants. In the current work, we show that the ALA uptake activity found in *B. japonicum* is deficient in other rhizobia, and we propose that this trait accounts for the differential success of *hemA* species in symbiosis.

MATERIALS AND METHODS

Chemicals and Reagents

Chemicals were obtained from Sigma, J.T. Baker, or Boehringer Mannheim. ALA was obtained from Porphyrin Products (Logan, UT). L-[3,4-^{3}H]Glu (40–60 Ci/mmol) and [4-^{14}C]ALA (50 mCi/mmol) were obtained from Dupont-NEN. Ecoscint scintillation fluor was purchased from National Diagnostics (Manville, NJ).

Plant and Bacterial Material

The *Bradyrhizobium japonicum hemA* mutant MLG1 and parent strain I110 were described previously (Guerinot and Chelm, 1986). The *B. japonicum hemB* mutant KP32 was described previously (Chauhan and O'Brian, 1993) and was grown in media containing 10 μM hemin. *Rhizobium* sp. ANU240 is a streptomycin-resistant derivative of *Rhizobium* sp. NGR234 (Morrison et al., 1983). The Sm^r derivative was used in the present work to verify its identity by its antibiotic resistance in cultures and from nodules. *Rhizobium meliloti* 102F34 and *Azorhizobium caulinodans* ORS571 were cultured as described previously (Leong et al., 1982; Pawlowski et al., 1993). Soybeans (*Glycine max* cv Essex), cowpeas (*Vigna unguiculata* cv California black eye 5), and mung bean (*Vigna radiata*) were grown in a Conviron environmental growth chamber on a 16-/8-h light/dark cycle. The light temperature and RH were 25°C and 80%, respectively, and the dark temperature and RH were 22°C and 70%, respectively. Surface-sterilized seeds were germinated on H_2O-agar plates for 2 d and then planted in vermiculite, at which time approximately 10^8 cells of *B. japonicum* strain I110 or MLG1 or *Rhizobium* sp. ANU240 were added. Plants were harvested 30 to 40 d after planting and root nodules were picked. Plant cytosol and bacteroids were prepared from fresh nodules as previously described (Sangwan and O'Brian, 1992). These fractions were frozen at −70°C for heme determinations and ALA synthesis activity assays. Bacteroids were isolated from fresh nodules and assayed immediately for ALA uptake assays.

ALA Synthesis Activity Assays

ALA synthase activity in bacterial extracts was measured as ALA formed from Gly and succinyl-CoA as previously described (Sangwan and O'Brian, 1992). The ALA was purified from the reaction mixture using a Dowex-50W (Na^+) column and quantified colorimetrically as the ALA pyrrole with modified Ehrlich reagent as described by Sangwan and O'Brian (1992). ALA formation from glutamate by plant nodule cytosol was measured as incorporation of radiolabel from 10 μCi of 3,4-[^{3}H]glutamate into ALA as previously described (Sangwan and O'Brian, 1992). The data are presented as the averages of triplicate trials. Both plant and bacterial extracts could be stored at −70°C without loss of activity.

Nitrogen Fixation Activity

Nitrogen fixation activity by nodules attached to roots was discerned as the reduction of acetylene to ethylene as previously described (O'Brian et al., 1987). The nodules were removed from the roots and weighed after the assay was completed. The data are expressed as the averages of four or five individual measurements for each nodule type.

Heme Determinations

Nodule cytosol heme (leghemoglobin) was quantified by measuring the absorption difference between 556 and 540 nm of the reduced pyridine hemochromagen using an E_{mM} of 24.5 (Smith, 1975), and the data are expressed as the averages of quadruplicate trials. Bacterial hemes were analyzed by dithionite-reduced minus ferricyanide-oxidized absorption spectra using an SLM-Aminco DW-2000 spectrophotometer in the dual-beam mode (Frustaci et al., 1991).

ALA Uptake Activity

Bacteroids were isolated from freshly harvested nodules, washed, and suspended in 50 mM $NaPO_4$, pH 7.4, and kept on ice until use. Cells were diluted to 3.6 mg protein mL^{-1} with $NaPO_4$ and incubated at 37°C with shaking for 5 min. Then unlabeled and [4-^{14}C]ALA were added to the desired concentration and specific activity, and shaking was continued at 37°C. Fifty-microliter aliquots were removed at the desired times, filtered through a Gelman (Ann Arbor, MI) Metricel membrane (0.45-μm pore size), and then washed twice with 1 mL of $NaPO_4$ buffer containing 1 mM unlabeled ALA. Further washings did not decrease the radiolabel found on the filter. The filters were then immediately added to scintillation fluor and counted in a scintillation spectrometer. In some experiments, cells were washed by centrifugation rather than applied to a filter, but this was not useful for short (1–2 min) times. For determination of initial velocities, ALA uptake was measured for 2 min, which was within the linear range for uptake. The data were analyzed by Eadie-Scatchard and Lineweaver-Burk plots (Segel, 1975), and the K_m and V_{max} values reported were derived from Lineweaver-Burk analysis. For inhibitor studies, cells were incubated with 1 mM sodium cyanide, 10 mM sodium azide, or 1 mM dinitrophenol. The ALA uptake data are the averages of triplicate trials.

RESULTS

Phenotype of Legume Nodules Elicited by a *B. japonicum hemA* Mutant

Rhizobial *hemA* mutants are defective in ALA synthase, the first committed step in heme biosynthesis; hence, they cannot make ALA from Gly and succinyl-CoA. A *B. japonicum hemA* mutant elicits effective nodules on soybean (Guerinot and Chelm, 1986), but other rhizobial *hemA* mutants induce non-nitrogen fixing, hemoglobin-deficient

nodules on their respective hosts (Leong et al., 1982; Stanley et al., 1988; Pawlowski et al., 1993). Those observations indicate that a trait unique to either *B. japonicum* or soybean allows a successful symbiosis despite the bacterial *hemA* mutation. To address this further, we took advantage of the ability of *B. japonicum* to elicit nodules on cowpea and mung bean, in addition to soybean. We reasoned that, if cowpea and mung bean nodules elicited by the *B. japonicum hemA* mutant MLG1 were normal, then it is likely that the trait of interest resides with the bacterial symbiont; however, abnormal nodules formed from those symbioses would imply that soybean possesses some trait that is missing in the other legumes. We found that the *B. japonicum hemA* mutant strain MLG1 elicited nodules on both cowpea and mung bean that contained leghemoglobin (Table I) as has been found for soybean (Table I; Guerinot and Chelm, 1986). Although the mung bean nodules contained leghemoglobin and appeared normal, few nodules were elicited by either *B. japonicum* strain, and thus experiments with that plant were not pursued further. In addition to hemoglobin expression, cowpea nodules elicited by *B. japonicum* mutant strain MLG1 had nitrogenase activity comparable to those induced by parent strain I110 (Table I), and thus both the plant and bacterial cells in nodules were apparently differentiated to a mature symbiotic state. By contrast, a *hemA* mutant of *Rhizobium* sp. NGR234 induces ineffective, hemoglobin-defective nodules on cowpea (Stanley et al., 1988); thus, *B. japonicum* must possess a trait lacking in *Rhizobium* sp. NGR234, which allows a *hemA* mutant to form normal nodules on that host.

Bacterial Heme Expression in the *B. japonicum hemA* Mutant in Nodules

Cyt hemes were analyzed in *B. japonicum* I110 and MLG1 isolated from soybean or cowpea nodules and in cultured cells (Fig. 1). The heme-defective phenotype of cultured cells of the *hemA* mutant was rescued in cowpea bacteroids (Fig. 1), as was observed in those from soybean (Fig 1; Sangwan and O'Brian, 1991). The *hemA* mutant expressed almost wild-type levels of heme in cowpea nodules; both the increase in total Cyt hemes and the disappearance of Cyt aa_3 at 603 nm (Fig. 1) are typical of bacteria that have differentiated into bacteroids, and it is consistent with the nitrogen fixation activity found in nodules. The symbiotic rescue of the heme defect in the *hemA* mutant was not due to reversion of the ALA synthase-defective phenotype, since the activity was absent in bacteroids from soybean and cowpea nodules (Table II). The rescue of heme in nodules is sufficient to explain the effective symbiosis between the *hemA* mutant and the legumes examined (Table I).

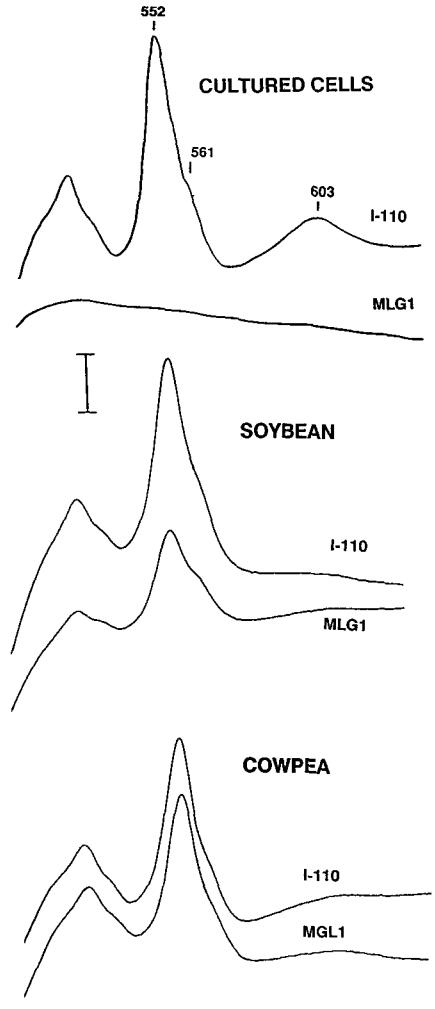

Figure 1. Cyt spectra of *B. japonicum* I110 or *hemA* strain MLG1 from cultured cells, soybean nodules, or cowpea nodules. The protein concentrations in the samples are 3 mg/mL for bacteroids and 10 mg/mL for cultured cells. The vertical bar represents a ΔA of 0.007.

Table I. *Acetylene reduction activities and hemoglobin content in legume nodules elicited by B. japonicum strains I110 or MLG1*

N.D., Not determined. The data are expressed as the averages ± SD of four samples.

Nodule Source (plant [bacterium])	Leghemoglobin Heme	Acetylene Reduction
	nmol g^{-1} nodule	*μmol C_2H_4 formed h^{-1} g^{-1} nodule*
Soybean (I110)	136 ± 2	7.0 ± 0.4
Soybean (MLG1)	79 ± 3	6.8 ± 0.9
Cowpea (I110)	138 ± 5	6.5 ± 0.7
Cowpea (MLG1)	123 ± 7	7.2 ± 0.7
Mungbean (I110)	157 ± 2	N.D.[a]
Mungbean (MLG1)	119 ± 2	N.D.

Plant ALA Formation Activity in Nodules

The data indicate that *B. japonicum* possesses a trait lacking in *Rhizobium* sp. NGR234 and perhaps other rhizobia, which allows a *hemA* mutant of the former to establish a symbiosis on numerous hosts. Two phenomena were previously described for soybean nodules induced by *B. japonicum* that are proposed to allow bacterial heme expression in a *hemA* mutant: (a) an inducible glutamate-dependent ALA formation activity by soybean in nodules and (b) an ALA uptake activity by *B. japonicum* bacteroids

Table II. *Glutamate-dependent ALA formation activities in soybean and cowpea in nodules elicited by various rhizobial strains and bacterial ALA synthase activities in bacteroids and cultured cells*

The data are expressed as the averages of triplicate trials. The SD values were less than 10%.

Source of Nodules	Plant ALA Formation Activity	Bacterial ALA Synthase Activity
	cpm h^{-1} mg^{-1} protein	*nmol h^{-1} mg^{-1} protein*
B. japonicum I110		
Culture		5.9
Soybean	6996	5.2
Cowpea	4734	4.0
B. japonicum MLG1		
Culture		0
Soybean	6952	0
Cowpea	3954	0
Rhizobium sp. ANU240		
Cowpea	7238	3.1

(Sangwan and O'Brian, 1991). If these traits are relevant to the current work, we would predict that *B. japonicum* is unique either in its ability to induce plant ALA synthesis in nodules or in its ALA uptake activity. We measured glutamate-dependent ALA synthesis in the plant fraction of soybean and cowpea nodules induced either by *B. japonicum* strain I110 or MLG1 or with *Rhizobium* sp. ANU240, a streptomycin-resistant derivative of NGR234 (Table II).

ALA synthesis activity was found in the plant fraction of all cowpea nodules tested, whether induced with wild-type strains or with the *hemA* mutant MLG1. Therefore, plant ALA synthesis activity in nodules is not restricted to soybean, which further supports the idea that the unique trait of interest resides with *B. japonicum* rather than with the host. In addition, the data rule out *B. japonicum*-specific induction of plant ALA synthesis activity since cowpea nodules elicited by *Rhizobium* sp. ANU240 express that activity as well (Table II).

ALA Uptake by *B. japonicum*

B. japonicum bacteroids possess ALA uptake activity (Sangwan and O'Brian, 1991), and it was present in bacteroids isolated from soybean or from cowpea (Fig. 2). However, *Rhizobium* sp. ANU240 bacteroids had very poor uptake activity (Fig. 2), and little ALA was found in those cells even after 40 min. Thus, the ALA uptake phenotype of *B. japonicum* is not dependent on the host, and the deficient activity in *Rhizobium* sp. (Fig. 2) may explain the failure of a *hemA* mutant of that species to establish successful symbioses (Stanley et al., 1988), since a mutant that cannot make ALA requires an exogenous source.

ALA uptake by *B. japonicum* strain I110 bacteroids isolated from soybean nodules was analyzed by measuring initial uptake rates as a function of ALA concentration, and the data are shown as an Eadie-Scatchard plot (Fig. 3). The kinetics appeared to be biphasic, indicative of two mechanisms of ALA uptake. In three separate experiments, the high-affinity mechanism had an average apparent K_m of 74

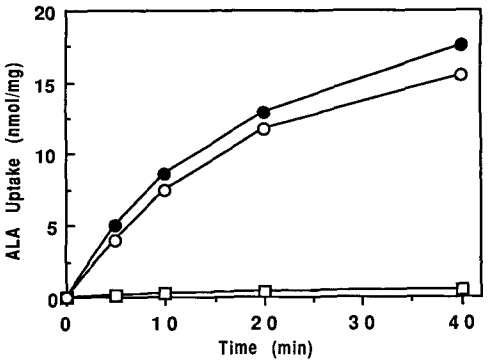

Figure 2. ALA uptake by *B. japonicum* I110 bacteroids from soybean (○) or from cowpea (●) and in *Rhizobium* sp. ANU240 bacteroids from cowpea (□). ALA (1 mM) was used as substrate, and each point is the average of duplicate trials.

± 10 μM and a V_{max} of 1.4 ± 0.18 nmol min^{-1} mg^{-1} protein. Saturation kinetics of uptake infers that the observed mechanism is protein mediated. ALA uptake by *B. japonicum* with 10 μM substrate, a concentration at which the high-affinity mechanism should predominate, was inhibited by about 40% with cyanide or azide and 80% by dinitrophenol, indicating that this phase required energy. The average apparent K_m of the low-affinity ALA uptake mechanism was 9 ± 1.6 mM and the V_{max} was 59 ± 9 nmol min^{-1} mg^{-1} protein. Although kinetics analysis yields a K_m value for the low-affinity phase of ALA uptake, we do not distinguish this high value from an unsaturable mechanism. ALA uptake in the presence of 1 mM ALA was only slightly (10–15%) inhibited by cyanide or azide, but it was inhibited 80% by the proton ionophore dinitrophenol; thus, the low-affinity ALA uptake may depend on a proton gradient across the membrane.

Having established two kinetics phases for ALA uptake by *B. japonicum* strain I110 bacteroids, we measured initial uptake rates by *B. japonicum* strains I110 and MLG1 isolated from soybean or cowpea nodules, and rates from *Rhizobium* sp. ANU240 from cowpea were measured at

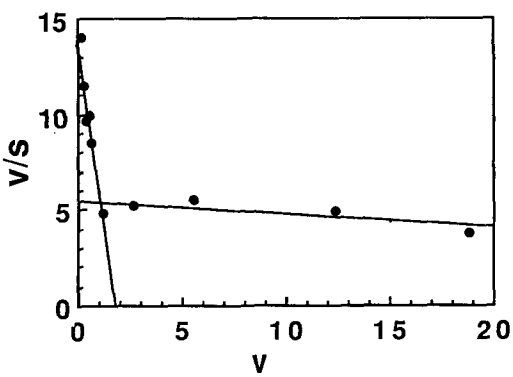

Figure 3. Eadie-Scatchard plot of the initial velocity of ALA uptake as a function of ALA concentration by *B. japonicum* I110 bacteroids from soybean nodules. Exogenous ALA was taken up by bacteroids for 2 min at ALA concentrations ranging from 0.01 to 5 mM. Each point is the average of triplicate trials. V, Velocity in nmol ALA taken up min^{-1} mg^{-1} protein. V/S, Velocity/substrate.

Table III. *ALA uptake by B. japonicum or Rhizobium sp. ANU240 bacteroids at ALA concentrations of 0.01 or 1 mM*

The data are expressed as the averages of ± SD of triplicate trials.

Bacteroids (nodule source)	ALA Uptake	
	0.01 mM ALA	1 mM ALA
	nmol min^{-1} mg^{-1} protein	
B. japonicum strain I110		
Soybean	0.22 ± 0.02	7.6 ± 0.3
Cowpea	0.22 ± 0.01	7.6 ± 0.6
B. japonicum strain MLG1		
Soybean	0.24 ± 0.02	4.1 ± 0.2
Cowpea	0.26 ± 0.002	4.4 ± 0.4
Rhizobium sp. ANU240		
Cowpea	0.005 ± 0.002	0.2 ± 0.02

10 μM and 1 mM ALA, where the high- and low-affinity systems should predominate, respectively (Table III). Similar ALA uptake rates were observed in *B. japonicum* wild type and in *hemA* backgrounds at each substrate concentration, and the observed rates did not depend on whether those bacteroids were obtained from soybean or cowpea nodules (Table III). However, initial velocities of ALA uptake by *Rhizobium* sp. ANU240 were very low at either substrate concentration relative to that observed in *B. japonicum*; the rate at 10 μM was more than 40-fold less than was observed by *B. japonicum* and was 20- to 35-fold less at 1 mM ALA. Therefore, *Rhizobium* sp. ANU240 bacteroids were deficient in both the high- and low-affinity ALA uptake systems.

ALA Uptake in Cultured Cells

ALA uptake activity was observed in cultured cells of *B. japonicum* strain I110 (Fig. 4A; Table IV), and thus it appears to be a constitutive activity. Eadie-Scatchard analysis showed that the kinetics of ALA uptake were biphasic (Fig. 4A), as was observed for bacteroids. The K_m and V_m values for the high-affinity phase were 32 μM and 1.9 nmol min^{-1} mg^{-1} protein, respectively, and thus were similar to the corresponding phase in bacteroids. The low-affinity uptake by cultured cells had K_m and V_m values of 2.3 mM and 9 nmol h^{-1} mg^{-1} protein; hence, these parameters were somewhat lower than those observed for bacteroids. We measured initial ALA rates by cultured cells of *B. japonicum* I110, *Rhizobium* sp. ANU240, *R. meliloti*, and *A. caulinodans* at 10 μM and 1 mM ALA (Table IV). As compared to *B. japonicum*, *R. meliloti*, *A. caulinodans*, and *Rhizobium* sp. ANU240 had very low uptake activities. Thus, the bacterial *hemA* gene is required for symbiosis in the three rhizobial species that are deficient in ALA uptake, but it is not essential in *B. japonicum*, which expresses ALA uptake activity.

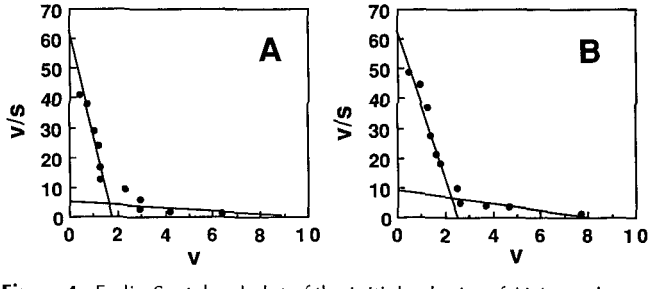

Figure 4. Eadie-Scatchard plot of the initial velocity of ALA uptake as a function of ALA concentration by cultured cells of *B. japonicum* I110 (A) and the *hemB* mutant strain KP32 (B). Conditions are as described in Figure 3.

Expression of ALA uptake in cultured cells allowed us to address whether the relatively high activity was due to a high ALA metabolism subsequent to uptake by using a mutant that cannot metabolize ALA (Fig. 4). The *hemB* mutant KP32 is defective in ALA dehydratase (Chauhan and O'Brian, 1993); thus, it cannot convert ALA to porphobilinogen in the heme pathway and is a hemin (heme hydrochloride) auxotroph. The kinetics of ALA uptake in strain KP32 were very similar to that found in cultured cells of the parent strain I110 (Fig. 4), and therefore intercellular ALA metabolism did not appear to contribute significantly to the observed ALA uptake activities.

DISCUSSION

The nonessentiality of the *B. japonicum hemA* gene for symbiosis with soybean is remarkable in three respects: (a) The *hemA* gene product ALA synthase is the only known ALA-forming enzyme in *B. japonicum*, and yet bacteroids of the mutant are viable, fix nitrogen (Guerinot and Chelm, 1986), and express heme (Sangwan and O'Brian, 1991). (b) Unlike *hemA*, the *B. japonicum* heme synthesis genes *hemB* and *hemH* are required for symbiosis with soybean (Frustaci and O'Brian, 1992; Chauhan and O'Brian, 1993). (c) Bacterial *hemA* is essential for other rhizobia-legume symbioses (Leong et al., 1982; Stanley et al., 1988; Pawlowski et al., 1993). The first two issues have been addressed previously in proposing that *B. japonicum* heme can be synthesized from soybean-derived ALA but not from any other plant heme precursor (Sangwan and O'Brian, 1991; Chauhan and O'Brian, 1993), and the current work suggests that the third set of observations can be explained by this scheme as well.

According to the model, ALA synthesis by the legume host and subsequent uptake by the endosymbiont are necessary to rescue a bacterial *hemA* mutant; hence, it predicts that a deficiency in either activity renders the *hemA* gene essential for symbiosis. Herein we demonstrated that the *B. japonicum hemA* mutant MLG1 formed effective symbioses with numerous hosts (Table I), including cowpea, a legume that cannot be effectively nodulated by a *hemA* mutant of *Rhizobium* sp. (Stanley et al., 1988). Also, the heme defect of cultured cells of the *B. japonicum* mutant was rescued in symbiosis with either soybean or cowpea, and the Cyt heme pattern was typical of differentiated bacteroids (Fig. 1). These observations strongly indicate that *B. japonicum* expresses a trait missing in *Rhizobium* sp. and that hosts other than soybean can rescue a *hemA* mutant. These conclusions are consistent with the observed ALA synthesis activity in cowpea nodules elicited by either *B. japonicum* or *Rhizobium* sp. (Table II) and by the defective ALA uptake activity in *Rhizobium* sp. (Fig. 2; Table III). In addition, *B. japonicum* ALA uptake by bacteroids was not dependent on

Table IV. *ALA uptake by several rhizobial strains grown in culture at ALA concentrations of 0.01 and 1 mM*

Rhizobial Species	ALA Uptake	
	0.01 mM ALA	1 mM ALA
	nmol min^{-1} mg^{-1} protein	
B. japonicum strain I110	0.40	18.6
Rhizobium sp. ANU240	0.008	0.8
R. meliloti 102F34	0.003	0.3
A. caulinodans ORS571	0.003	1.9

the host plant (Fig. 2; Table III). We propose that both soybean and cowpea have the synthetic capacity to rescue a *hemA* mutant, but only rhizobial cells with ALA uptake activity can utilize the exogenous precursor for bacterial heme synthesis. Although we have not examined ALA uptake by *R. meliloti* or *A. caulinodans* bacteroids, cultured cells are defective in ALA uptake, as was observed for *Rhizobium* sp. ANU240 (Table IV), which may explain the failure of *hemA* mutants of those species to form effective symbioses with their respective hosts. Finally, the ability to explain the apparent contradictory observations involving rhizobial heme synthesis mutants enumerated above in terms of an interorganismic heme pathway (Sangwan and O'Brian, 1991) further argues that the proposed scheme is tenable.

ALA uptake by *B. japonicum* bacteroids was found to be biphasic, indicating two kinetics mechanisms with differing affinities for ALA. The K_m values obtained for each phase were approximately 70 μM and 9 mM hence, the low-affinity mechanism may be unsaturable. The aqueous phase of soybean nodules contained approximately 6 μM ALA as discerned by purification of ALA from that fraction followed by quantitation of the ALA pyrrole (see "Materials and Methods"). Thus, although we do not know the ALA concentration of the bacteroid milieu in nodules, this low amount of ALA makes it likely that uptake in situ would be carried out predominantly by the high-affinity mechanism. *B. japonicum* ALA uptake appeared to be constitutive, because activity was observed in cultured cells of that species but was defective in *Rhizobium* sp. ANU240, *R. meliloti*, and *A. caulinodans*. We note that these rhizobia are ALA auxotrophs in culture, as are ALA synthesis mutants in numerous Gram-positive and Gram-negative bacteria; thus, ALA must be able to enter those cells at concentrations used to culture them (approximately 300 μM). However, this high amount of ALA likely compensates for the deficient ALA uptake. The *B. japonicum hemA* strain MLG1 grew in the presence of 1 μM ALA in minimal media, but *R. meliloti hemA* strain A102 required 20 μM ALA. Also, recent data show that ALA can be taken up by *Escherichia coli* and *Salmonella typhimurium* via a dipeptide permease (dpp) system (Elliot, 1993; Verkamp et al., 1993); dpp *hemA* double mutants are ALA auxotrophs but require 100-fold more ALA for growth than do dpp$^+$ auxotrophs. A genetic characterization of *B. japonicum* ALA uptake is currently in progress.

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