MOLECULAR PLANT-MICROBE INTERACTIONS
Vol. 4, No. 3, pp. 254-261, 1991

Bradyrhizobium japonicum ntrBC/glnA and *nifA/glnA* Mutants: Further Evidence that Separate Regulatory Pathways Govern *glnII* Expression in Free-Living and Symbiotic Cells

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Received 26 September 1990. Accepted 18 January 1991.

Bradyrhizobium japonicum **produces two glutamine synthetases that are encoded by the genes *glnA* and *glnII*. We have previously hypothesized that two separate regulatory networks involving either *ntrC* or *nifA* control *glnII* expression in *B. japonicum* (G. B. Martin, K. A. Chapman, and B. K. Chelm, Journal of Bacteriology 170:5452-5459, 1988). Here we test this model by constructing strains of *B. japonicum* that carry a deletion in *glnA* and in either the *ntrBC* operon or *nifA*. These double mutants were compared with strains carrying single mutations in *glnA*, *ntrBC*, or *nifA* for the ability to utilize various nitrogen sources, nodulate soybeans, and fix atmospheric nitrogen. The *ntrBC/glnA* mutant was unable to grow on nitrate or ammonia as the sole nitrogen source when grown in aerobic cultures. In addition, it produced fewer nodules per plant than did the wild-type strain or the single mutants and was altered in its ability to fix nitrogen. The *nifA/glnA* mutant had no nitrogen utilization defects in aerobic, free-living culture, but it produced many fewer nodules on soybeans than did the wild-type strain or a *nifA* mutant. These observations are interpreted in the context of a model of separate regulatory networks controlling *glnII* expression in *B. japonicum*.**

Additional keywords: nitrogen regulation.

During free-living growth, *Bradyrhizobium japonicum* (Buchanan) Jordan, the endosymbiont of soybean, assimilates ammonia primarily by the coordinate activities of glutamine synthetase (GS) and glutamate synthase (Brown and Dilworth 1975; Vairinhos *et al.* 1983). In *B. japonicum* bacteroids, the symbiotic form of these bacteria, GS activity decreases in concert with the derepression of nitrogenase activity, thereby allowing fixed ammonia to be exported to the plant cytoplasm (Brown and Dilworth 1975). The differential expression of GS during free-living growth, during incipient nodule formation, and in mature bacteroids is therefore an integral part of the developmental process in *B. japonicum*.

B. japonicum contains two GS enzymes, GSI and GSII, encoded by *glnA* and *glnII*, respectively (Carlson *et al.* 1987; Darrow and Knotts 1977; Darrow *et al.* 1981). The *B. japonicum glnA* gene has sequence similarity to the *Escherichia coli glnA* gene and is not appreciably nitrogen-regulated (Carlson *et al.* 1985). In contrast, the GS encoded by *glnII* has sequence similarity with eucaryotic GSs and is transcriptionally regulated in response to nitrogen availability (Carlson and Chelm 1986; Carlson *et al.* 1987).

In *Klebsiella pneumoniae* (Schroeter) Trevisan, the best studied nitrogen-fixing organism, genes involved in nitrogen metabolism are subject to two levels of positive regulation in response to ammonia and oxygen (Ausubel 1984). The first level, which is specific to nitrogen fixation (*nif*) genes, is mediated by the transcriptional activator encoded by *nifA* (NifA; Ausubel 1984). The second level is mediated by the central nitrogen regulation (Ntr) system, which controls the expression of a variety of nitrogen assimilation genes in the enteric bacteria (Magasanik 1982). In the Ntr system, the gene product of *ntrC* (NtrC) functions as a transcriptional activator of many genes, including *nifA* (Gussin *et al.* 1986). Both *nifA* and *ntrC* homologous genes are present in members of the Rhizobiaceae, and these genes have been isolated and characterized in several species (Fischer *et al.* 1986; Martin *et al.* 1988; Pawlowski *et al.* 1987; Rossbach *et al.* 1987; Szeto *et al.* 1984, 1987). These studies have established that the rhizobial *nifA* gene is expressed independently of the Ntr system and is not dependent on NtrC for expression. *Azorhizobium caulinodans* Dreyfus *et al.*, where NtrC activation of *nifA* has been reported, appears to be the exception (Pawlowski *et al.* 1987).

We have shown previously that in *B. japonicum* NtrC is required for the transcriptional regulation of *glnII* under conditions of nitrogen-starved aerobic growth (Martin *et al.* 1988). Under microaerobic conditions and during symbiotic growth, however, *glnII* is expressed but does not require NtrC (Martin *et al.* 1988). Szeto *et al.* (1987) have shown in *Rhizobium meliloti* Dangeard that, under symbiotic conditions, NifA activates several genes (i.e. *nifHDK* and *fixABC*) which are regulated by NtrC during aerobic growth. We have suggested that the NtrC-independent expression of *glnII* during microaerobic and symbiotic

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growth also involves NifA control (Martin *et al.* 1988). Thus in both *B. japonicum* and *R. meliloti*, several genes involved in nitrogen metabolism appear to be regulated by separate regulatory networks in free-living and symbiotic cells.

To examine the physiological effects of specific regulatory proteins on the expression of *glnII*, we constructed double mutants that carry a *glnA* deletion along with a deletion in either *ntrBC* or *nifA*. By eliminating the GS activity encoded by *glnA*, we were able to assess the role of NtrC and NifA in regulating *glnII* and the subsequent effects of this control on soybean nodulation. The results reported here support a model of separate regulatory networks in *B. japonicum* responding to nitrogen deprivation or a symbiotic signal that probably involves oxygen limitation.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, growth conditions, and GS assays. BJ110d is a small-colony derivative of *B. japonicum* 3I1b110 (Guerinot and Chelm 1986). All other bacterial strains and recombinant plasmids that were used in this study are listed in Table 1.

To check growth on various nitrogen sources, 50-ml cultures of a mineral salts base medium adapted from O'Gara and Shanmugam (1976) were used and had the following composition per liter: KH_2PO_4, 0.3 g; Na_2HPO_4, 0.3 g; and $MgSO_4 \cdot 7H_2O$, 0.12 g. Trace elements were added (O'Gara and Shanmugam 1976), and the pH was adjusted to 6.8. After autoclaving, 1 ml of a filter-sterilized vitamin solution (Guerinot and Chelm 1986), 0.05 g of $CaCl_2$, and 7.5 ml of filter-sterilized 20% (w/v) D-xylose were added per liter. The various nitrogen sources were added at 1.0 mM from filter-sterilized stock solutions.

GS activity was assayed by the whole cell γ-glutamyl transferase method of Bender *et al.* (1977) with the following modifications. A 1-ml sample of saturated culture was inoculated into 25 ml of prewarmed (30° C) xylose-glutamate minimal medium, and the cultures were shaken at 250 rpm in 125-ml flasks. At $OD_{600\ nm} = 0.8$, a 1.5-ml sample was taken. The cells were centrifuged and washed once with 1% KCl (4° C), resuspended in 1% KCl with 1 mg/ml of DNase, and held on ice. The cells were subjected to three cycles of freeze-thawing using a isopropanol dry ice bath and a room temperature water bath. Fifty microliters of this sample was incubated at 50° C for 60 min to test for the presence of GSII, which is unstable under these conditions. Samples (50-μl) of heat-treated and untreated cells were assayed for 20 min at 37° C. The protein content of a 50-μl sample was determined as described by Smith *et al.* (1985).

DNA biochemistry. Southern hybridizations and isolation of plasmid DNA were conducted as described previously (Adams *et al.* 1984). Total genomic DNA from *B. japonicum* was purified by phenol extraction (Maniatis *et al.* 1982). The isolation of *glnA* and the *ntrBC* operon has been described previously (Carlson *et al.* 1985; Martin *et al.* 1988). The *nifA* locus was isolated from a *B. japonicum* cosmid library using a 950-base pair (bp) *Pst*I-*Eco*RV fragment from the *K. pneumoniae nifA* gene as a probe (Adams 1986).

Gene-directed mutagenesis. Recombinant plasmids were manipulated *in vitro* and in *E. coli* to construct mutant alleles of the *glnA* and *ntrBC* loci (pBJ284 and pBJ326, respectively; Table 1, Fig. 1). The mutant *ntrBC* region was constructed by deleting the 1.8-kilobase (kb) *Sal*I fragment from the center of the *ntrBC* operon and replacing it with a 1.35-kb kanamycin resistance cassette, which has been described previously (Martin *et al.* 1988). This cassette is homologous to the kanamycin resistance gene present

Table 1. Bacterial strains and plasmids used in this study

Designation	Description[a]	Source or reference
BJ110d	Wild type	Guerinot and Chelm 1986
BJ2841	*glnA*::*spc*; *glnA* mutant, Sp^r	Carlson *et al.* 1987
BJ3263	*ntrBC*::*nptII*; *ntrBC* mutant, Km^r	This study
BJ2101	*nifA*::*nptII*; *nifA* mutant, Km^r	Adams 1986
GM10-1	*glnA*::*spc*, *nitrBC*::*nptII*; *glnA*/*ntrBC* mutant, Sp^r, Km^r	This study
GM4-33	*glnA*::*spc*, *nifA*::*nptII*; *glnA*/*nifA* mutant, Sp^r, Km^r	This study
pBJ284	Sp^r; *glnA* deletion allele cloned into pKC7	Carlson *et al.* 1987
pBJ326	Km^r, Ap^r; *nitrBC* deletion allele cloned into pBR322	This study
pHC45Ω	Sp^r; vector	Prentki and Krisch 1984
pKC7	Km^r, Ap^r; vector	Rao and Rogers 1979
pBR322	Ap^r, Tc^r; vector	Bolivar *et al.* 1977

[a] Sp, spectinomycin; Km, kanamycin; Ap, ampicillin; Tc, tetracycline; and ^{r}, resistant.

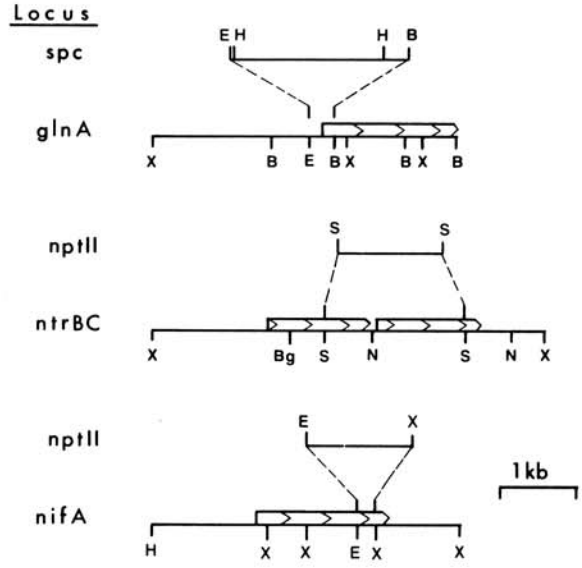

Fig. 1. Physical maps of the *Bradyrhizobium japonicum glnA*, *ntrBC*, and *nifA* wild-type and mutant alleles. The coding regions of the genes are indicated by the boxed regions containing arrowheads, and transcription is from left to right as drawn. The insertion locations are indicated for the spectinomycin resistance gene (*spc*) and the kanamycin resistance gene (*nptII*). (See the text for details of constructions.) Abbreviations for restriction endonuclease sites are as follows: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; N, *Nru*I; and X, *Xho*I.

on pKC7. The mutant *glnA* allele was constructed by deleting the 300-bp *Eco*RI-*Bam*HI fragment from *glnA* and replacing it with a *Hin*dIII fragment containing a spectinomycin resistance gene (Table 1, Fig. 1). The *Hin*dIII fragment was a derivative of plasmid pHC45Ω (Prentki and Krisch 1984), to which *Eco*RI-*Hin*dIII and *Bam*HI-*Hin*dIII adapters obtained from plasmid pKC7 (Rao and Rogers 1979) had been added (Fig. 1).

The mutant *nifA* allele (pBJ210) was constructed by deleting the 300-bp *Eco*RI-*Xho*I fragment internal to *nifA* (Fischer *et al.* 1986) and replacing it with a 1.6-kb *Eco*RI-*Xho*I kanamycin resistance cassette isolated from pKC7 (Table 1, Fig. 1; Adams 1986).

The plasmids carrying the mutant alleles were conjugally transferred from *E. coli* to *B. japonicum* by methods described previously (Guerinot and Chelm 1986). Stable *B. japonicum* recombinants were selected by antibiotic resistance conferred by the constructed allele, and those in which the mutant allele had replaced the wild-type allele were screened by hybridization as described previously (Guerinot and Chelm 1986). All of the constructed genotypes were confirmed by Southern hybridization analyses of genomic DNA. In all of the steps of strain construction, the growth medium was supplemented with 10 mM glutamine.

Plant inoculation, growth conditions, and acetylene reduction assays. Soybean (*Glycine max* (L.) Merr. cv. Amsoy 71) seeds were sterilized as described previously (Guerinot and Chelm 1986) and then soaked for 1 hr in a late log phase culture of the appropriate strain of *B. japonicum*. Seeds were planted in modified Leonard jars as described by Vincent (1978) using the nitrogen-free medium of Johnson *et al.* (1966). The plants were grown with a 16-hr daily light period, day and night temperatures of 28° and 25° C, respectively, a relative humidity of 60%, and a photon flux rate of 300 $\mu mol \cdot m^{-2} \cdot sec^{-1}$. All plants were harvested at 28 days, and the nodules were removed, rinsed with H_2O, counted, weighed, and then used in acetylene reduction assays (Hardy *et al.* 1968). Bacteria were isolated from nodules and subjected to DNA hybridization analyses to confirm the presence of the mutant strains.

Electron microscopy. Nodules were harvested from 4-wk-old root systems, rinsed in deionized water, and cut with a razor in slices approximately 1 mm thick. The slices were then immediately fixed in 4% glutaraldehyde in 0.15 M sodium cacodylate, pH 7.2, for 2 hr under vacuum and then at 4° C overnight. After fixation, the tissue was rinsed three times over a period of 3 hr at room temperature with buffer A (Maupin and Pollard 1983) containing 0.2% (w/v) tannic acid and then for 15 min in buffer A alone. Postfixation was in 1% (w/v) osmium tetroxide in buffer A for 1 hr at room temperature. The tissue was then dehydrated through a graded ethanol series, cleaned in propylene oxide, and embedded in VCD/HSXA ultralow viscosity medium (Ladd Research Industries, Burlington, VT). Thin sections were cut on an LKB Ultrotome III (Pharmacia-LKB, Piscataway, NJ) with a Du Pont diamond knife. Sections were subsequently stained with uranyl acetate and lead citrate and examined in a Philips EM300 electron microscope.

RESULTS AND DISCUSSION

Construction of *B. japonicum ntrBC/glnA* and *nifA/glnA* mutants. To investigate the transcriptional control of *glnII*, we have previously relied on S1 protection assays (Martin *et al.* 1988). However, because nodules incited by *B. japonicum nifA* mutants contain severely degraded bacteroids, we have been unable to isolate high-quality RNA and thereby test transcriptional control directly. Therefore, we monitored *glnII* transcription indirectly by studying the physiological and symbiotic consequences of *nifA/glnA* and *ntrBC/glnA* mutations in *B. japonicum*.

Recombinant plasmids containing the *ntrBC* mutant allele (pBJ326) and the *glnA* mutant allele (pBJ284) were conjugated into the wild-type strain of *B. japonicum* (BJ110d) and recombined into the BJ110d genome to obtain the *ntrBC* mutant (BJ3263) and the *glnA* mutant (BJ2841, see Materials and Methods). The *B. japonicum nifA* mutant, BJ2101, was constructed in an analogous manner (Adams 1986).

The *ntrBC/glnA* and *nifA/glnA* double mutants were obtained by processing the *ntrBC* and *nifA* deletion mutants (BJ3263 and BJ2101, respectively) through a second cycle of mutagenesis using the *glnA* mutant allele plasmid pBJ284 (Table 1).

The presence of pKC7-homologous sequences in all of the mutant allele constructions allowed us to use the pKC7 plasmid as a probe to verify the presence of the disrupted alleles in all strains (Fig. 2); the *glnA*, *ntrBC*, and *nifA*

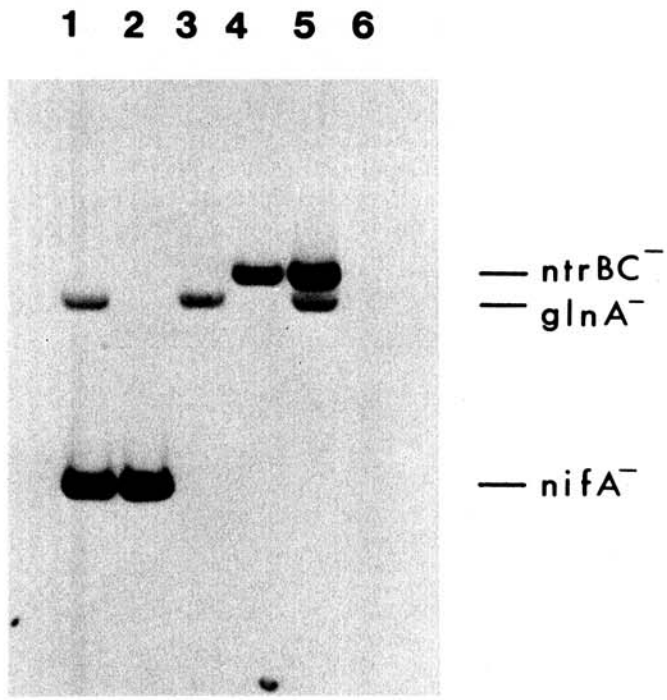

Fig. 2. Hybridization analysis of pKC7 to *Bradyrhizobium japonicum* genomic DNA from wild-type and mutant strains. *Xho*I digests of total cellular DNA were transferred to cellulose nitrate and hybridized to radiolabeled pKC7. Lane 1 contains GM4-33 (*nifA/glnA* mutant); lane 2, BJ2101 (*nifA* mutant); lane 3, BJ2841 (*glnA* mutant); lane 4, BJ3263 (*ntrBC* mutant); lane 5, GM10-1 (*ntrBC/glnA* mutant); and lane 6, BJ110d (wild type).

mutant alleles were carried on 4.4-, 4.7-, and 2.3-kb *Xho*I fragments, respectively. This, and additional experiments using probes containing the *glnA*, *ntrBC*, and *nifA* genes (data not shown), showed that the mutations were recombined into the expected regions of the *B. japonicum* chromosome by a double-crossover event, thus replacing the wild-type allele with the mutant allele.

Free-living and symbiotic phenotypes of the *B. japonicum glnA*, *ntrBC*, and *ntrBC/glnA* mutants. The *ntrBC*, *glnA*, and *ntrBC/glnA* mutants were screened for defects in nitrogen utilization by observing growth on media containing various nitrogen sources and by performing GS assays (Table 2). It has been reported for several rhizobial species, including *B. japonicum*, that NtrC is required for aerobic growth on nitrate as a sole nitrogen source (Martin *et al.* 1988; Rossbach *et al.* 1987; Szeto *et al.* 1987). Thus, as expected, we found that the *ntrBC* region was required for growth on nitrate (Table 2). The *ntrBC/glnA* mutant, GM10-1, was also unable to utilize nitrate, but in addition, it could not utilize ammonia and grew poorly on glutamate and glutamine as sole nitrogen sources (Table 2). We reported previously that *B. japonicum* strains with single *glnA* and *glnII* mutations showed no nitrogen utilization defects, but that a *glnA/glnII* double mutant is a glutamine auxotroph (Carlson *et al.* 1987). The fact that the *ntrBC/glnA* mutant was also unable to utilize ammonia as a sole nitrogen source indicated an absolute requirement of the *ntrBC* region for *glnII* expression during aerobic, free-living growth. GS assays confirmed that no GSII activity was present in BJ3263 (Table 2).

Based on the observation that a *glnII/glnA* mutant is Nod^- (Carlson *et al.* 1987), we expected that the *ntrBC/glnA* mutant would be altered in its ability to form an effective symbiosis with soybean. A significant difference, however, between the *ntrBC/glnA* and the *glnII/glnA* mutants is that *glnII* might be activated by NifA in the *ntrBC/glnA* mutant if this mutant were able to invade root cells.

The effects of the *ntrBC*, *glnA*, and *ntrBC/glnA* mutations on soybean nodulation and nitrogen fixation were determined by growing cultures in minimal medium supplemented with 1 mM glutamine, washing the cultures in unsupplemented medium, and inoculating soybean seeds in modified Leonard jars. We found that the *ntrBC* mutant, BJ3263, formed similar numbers of nodules on soybean as did the wild-type strain (BJ110d; Table 3). In addition, the average plant dry weight and acetylene reduction rates with this mutant were similar to the wild-type strain (Table 3). In contrast, the *ntrBC/glnA* mutant, GM10-1, produced many fewer nodules than did the wild-type or *ntrBC* strain, and these nodules were of two distinct types (Table 3, Fig. 3C). Nodules of the less common type were apparently normal, leghemoglobin-containing nodules of a similar size as wild-type nodules. Electron microscopy of these normal-appearing nodules revealed numerous mature bacteroids surrounded by well-formed peribacteroid membranes; in all respects, the ultrastructure of these nodules appeared to be identical to the wild-type (BJ110d) nodule ultrastructure (Fig. 4A, D). The nodules of the second and more common type were smaller than wild-type nodules and were greenish white inside. Electron microscopy of these nodules revealed very few bacteroids (Fig. 4B).

These observations indicate that, in the absence of *glnA*, the activation of *glnII* by NtrC is necessary for prolific nodulation of soybean roots. The presence of a few wild-type nodules, however, suggests that when *ntrBC/glnA* mutant cells do occasionally invade soybean root hairs, *glnII* is expressed and a normal symbiosis is possible. The low rate of acetylene reduction detected from the GM10-1 nodules is presumably attributable to these wild-type nodules (Table 3).

The inability of GM10-1 to prolifically nodulate soybean is probably due simply to the nitrogen utilization defects of this strain. An *A. caulinodans* glutamine auxotroph also forms a defective symbiosis (De Bruijn *et al.* 1988). It appears, however, that the inability of *B. japonicum* and *A. caulinodans* glutamine auxotrophs to form effective symbioses is not a general phenomenon in rhizobial-plant associations. An *R. meliloti glnA/glnII* mutant is a glutamine auxotroph but is Nod^+ Fix^+ on *Medicago sativa* L. (De Bruijn *et al.* 1989). A possible explanation for these contrasting results is that the plant partners in these associations may differ in their ability to provide glutamine to their bacterial symbiont.

Free-living and symbiotic phenotype of the *B. japonicum nifA/glnA* mutant. No nitrogen utilization deficiencies have been reported for aerobically grown, free-living rhizobial *nifA* mutants (Fischer *et al.* 1986; Szeto *et al.* 1984), including a *B. japonicum nifA* mutant strain (BJ2101) constructed

Table 2. Aerobic growth properties and glutamine synthetase activities of *Bradyrhizobium japonicum* strains

Strain	Genotype	Nitrogen source[a]				Activities[b]	
		Nitrate	Ammonia	Glutamate	Glutamine	GSI	GSII
BJ110d	Wild type	++	++	++	++	215	735
BJ2841	*glnA*	++	++	++	++	0	575
BJ3263	*nitrBC*	−	++	++	++	280	0
BJ2101	*nifA*	++	++	++	++	115	640
GM10-1	*glnA/ntrBC*	−	−	+	+	0	0
GM4-33	*glnA/nifA*	++	++	++	++	0	610

[a] Growth was determined in liquid minimal medium and is indicated as follows: ++, good; +, poor; and −, none. Nitrogen sources were tested at 1.0 mM concentration. All nitrogen sources were added from freshly prepared, filter-sterilized stock solutions.

[b] Glutamine synthetase (GS) activities. GSI is the activity of heat-treated cells. GSII is the difference between the activity of untreated cells and that of heat-treated cells. Units are in nanomoles of γ-glutamyl hydroxamate formed per minute per milligram of protein. 0, activity was below the limit of detection of this assay.

Table 3. Symbiotic properties of *Bradyrhizobium japonicum* strains[a]

Strain	Genotype	Number of nodules	Acetylene reduction (μmoles per plant)	Plant dry weight (g)
BJ110d	Wild type	63.7 ± 17.6	19.8 ± 4.64	4.33 ± 0.28
BJ2841	*glnA*	82.3 ± 7.5	27.3 ± 0.6	3.20 ± 0.35
BJ3263	*ntrBC*	58.8 ± 9.6	21.3 ± 7.4	3.00 ± 0.38
BJ2101	*nifA*	151.5 ± 15.2	0	0.94 ± 0.07
GM10-1	*glnA/ntrBC*	13.1 ± 5.3	0.2 ± 0.1	0.86 ± 0.16
GM4-33	*glnA/nifA*	29.5 ± 13.3	0	0.93 ± 0.15

[a] The numbers represent the means and standard errors of the means from three nodulation tests per strain. Plants were inoculated, grown, and harvested as described in the text.

in our laboratory (Table 2). We predicted that a *nifA/glnA* mutant (GM4-33) would not exhibit nitrogen utilization defects in aerobic cultures, because *glnII* expression is activated by NtrC under these conditions (Martin *et al.* 1988). This was the case, and further experiments confirmed the presence of GSII activity in GM4-33, indicating that

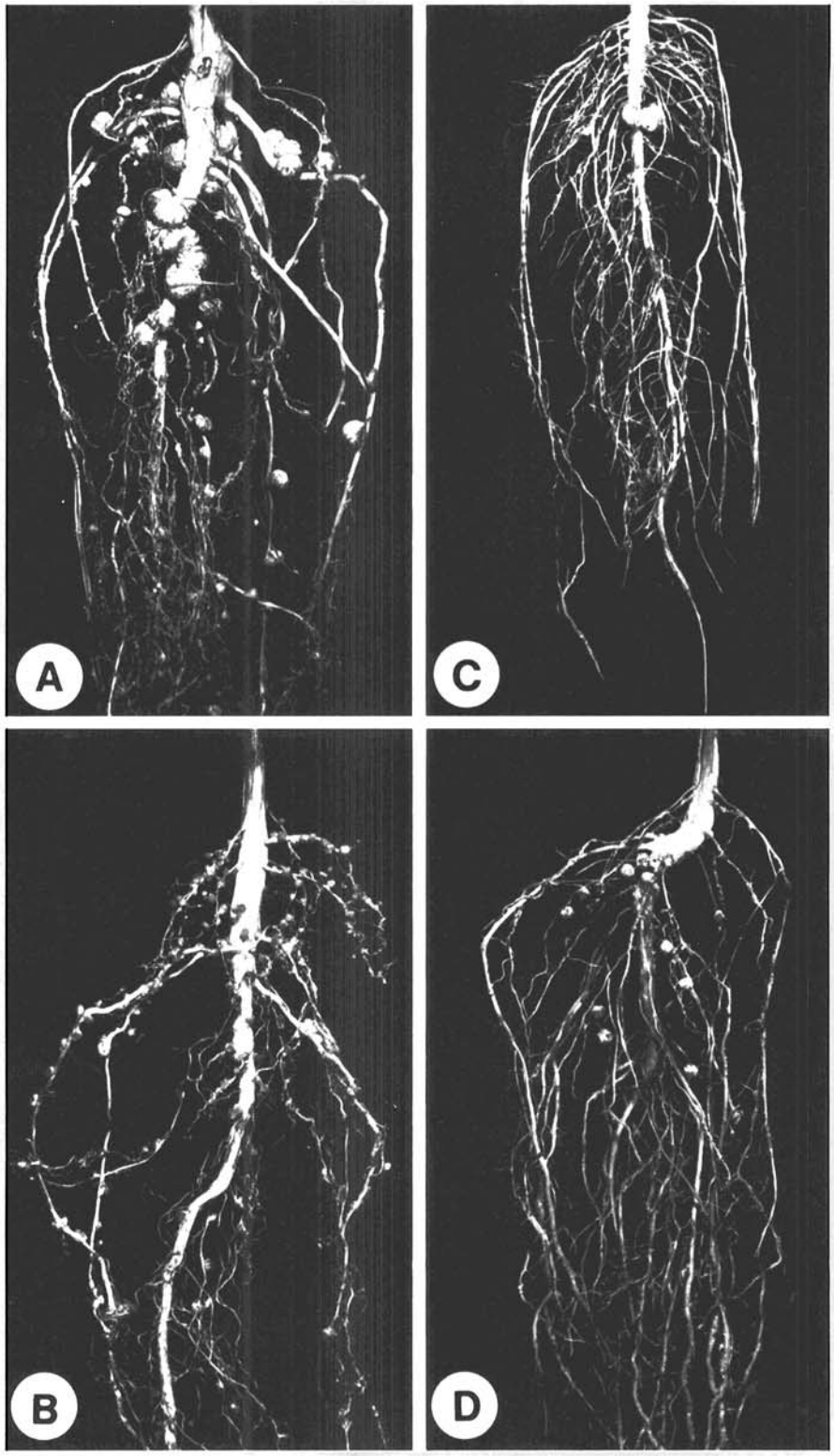

Fig. 3. Comparison of symbiotic phenotypes of *Bradyrhizobium japonicum* BJ110d (wild type) and mutant strains BJ2101 (*nifA*), GM10-1 (*ntrBC/glnA*), and GM4-33 (*nifA/glnA*). Sterilized soybean seeds were inoculated with the appropriate *B. japonicum* strain and grown in modified Leonard jars (see the text). Plants were harvested 4 wk after sowing. **A**, BJ110d (wild type); **B**, BJ2101; **C**, GM10-1; and **D**, GM4-33.

nifA, therefore, does not play an essential role in activating *glnII* expression during aerobic, free-living growth (Table 2). In contrast, under microaerobic conditions GM4-33 was unable to grow on KNO_3, NH_4Cl, or glutamate; this suggests a requirement of *nifA* for *glnII* expression under oxygen-limiting conditions.

B. japonicum nifA mutants fail to activate many genes involved in an effective symbiosis; these include *nifH*, *nifDK*, *fixA*, and *fixBCX* (Fischer *et al.* 1986; Hennecke *et al.* 1988). In addition, Fischer *et al.* (1986) reported that *B. japonicum nifA* mutants show an altered nodulation phenotype inducing numerous, small, widely distributed soybean nodules in which the bacteroids are subject to severe degradation. It was suggested that this hypernodulation is due to a circumvention of a plant phenomenon termed "autoregulation" in which effective nodulation events inhibit further nodulation on other parts of the roots (Fischer *et al.* 1986; Pierce and Bauer 1983). Here, we used an independently constructed *B. japonicum nifA* mutant (BJ2101) as a control and confirmed that more than double

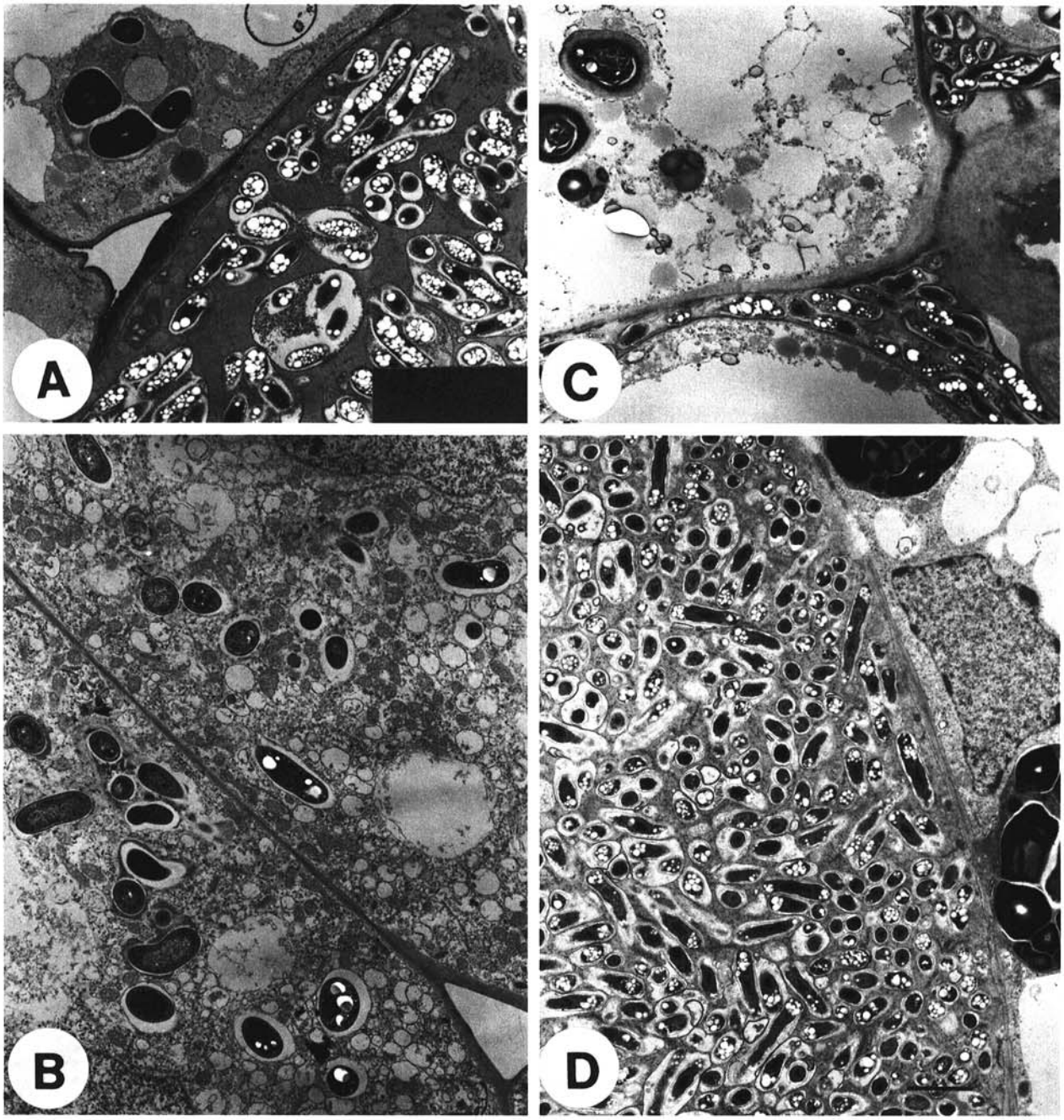

Fig. 4. Transmission electron micrographs of nodule tissue infected by *Bradyrhizobium japonicum* BJ110d (wild type) and mutant strains GM10-1 (*ntrBC*/*glnA*) and GM4-33 (*nifA*/*glnA*). The tissue was harvested 4 wk after seed inoculation and sowing. All micrographs were taken at the same magnification (×5,000) with the bar in **D** representing 1 μm. **A**, BJ110d; **B**, GM10-1 (greenish white nodule); **C**, GM4-33; and **D**, GM10-1 (normal-appearing nodule).

the number of nodules are observed on a root system inoculated with the *nifA* mutant than are seen in a symbiosis induced by the wild-type strain (Table 3, Fig. 3B). In contrast, we found that the *B. japonicum nifA/glnA* mutant, GM4-33, formed significantly fewer nodules than did the *nifA* mutant or the wild-type strain (Table 3, Fig. 3D). The nodules that were formed were larger than nodules induced by the *nifA* mutant but, like those nodules, they were Fix$^-$ (Table 3, Fig. 3D). The fact that some nodules were formed by strain GM4-33 suggested that *glnII* may be expressed in this mutant in response to a symbiotic signal, albeit at a low level.

The severe degradation of bacteroids in nodules incited by *B. japonicum* strains carrying a mutant *nifA* allele precluded the isolation of high-quality RNA. We were therefore unable to study *glnII* expression at the transcriptional level in nodules incited by *nifA* mutants. Nevertheless, the observation that many fewer nodules were formed with the *nifA/glnA* mutant than with the *nifA* mutant is consistent with the notion that NifA activates *glnII* expression and, in addition, indicated that the GS activity encoded by *glnA* is required for the formation of the numerous, small nodules seen in the symbiosis incited by *nifA* mutants.

Electron microscopy of the nodules incited by the *nifA/glnA* mutant revealed bacteroid degradation and the presence of numerous intercellular bacteria (Fig. 4C). It is not clear why the nodules induced by the *nifA/glnA* mutant were larger than the nodules induced by the *nifA* mutant. It is possible that the plant becomes limited in some way in the resources it allocates to an ineffective symbiosis involving a strain with a mutant *nifA* locus and that more plant resources are available to the few individual nodules formed with the *nifA/glnA* mutant than with the numerous nodules induced by the *nifA* mutant.

The *glnA* mutant, BJ2841, was also included in these experiments as a control, and as reported earlier (Carlson *et al.* 1987), it formed slightly more nodules and had a higher acetylene reduction rate per plant than did the wild-type strain. A *B. japonicum glnII* mutant exhibits a similar phenotype (Carlson *et al.* 1987). The significance of these observations remains unclear.

The results presented here are consistent with a model for *glnII* regulation that involves separate regulatory networks existing in free-living and symbiotic cells. This dual regulation would allow *B. japonicum* to continually transcribe *glnII* as the bacterium makes the transition from free-living growth in the soil to the symbiotic state as a bacteroid. The *ntrBC/glnA* mutant (GM10-1), according to the model, was defective in nodule formation, because it lacked NtrC needed for the activation of expression during aerobic growth and also lacked the ancillary GS activity encoded by *glnA*. A *B. japonicum glnII/glnA* mutant, a glutamine auxotroph, is similarly ineffective in establishing a symbiosis with soybean (Carlson *et al.* 1987). The defect in nodulation observed in GM10-1 is presumably the result of an inability to maintain cell growth long enough to efficiently invade root hairs and thereby reach the microaerobic environment of the vesicle bound by the peribacteroid membrane where symbiotic activation of *glnII* expression could occur.

The symbiosis induced by the *nifA/glnA* mutant suffered from numerous defects caused by the absence of NifA, and conclusions regarding the loss of *glnII* regulation by NifA are less clear. We can conclude, however, that the inability of the *nifA/glnA* mutant (GM4-33) to hypernodulate soybean indicates that the GS activity encoded by *glnA* allows the interaction induced by the *nifA* mutant, BJ2101, to proceed to the stage where numerous infections and subsequent hypernodulation occur. In addition, the inability of GM4-33 to hypernodulate soybean indicates that sufficient ancillary GS activity is not supplied by *glnII* in this mutant. This suggests, but does not prove, that NifA is required for the activation of *glnII* in symbiotic cells.

ACKNOWLEDGMENTS

We thank Mike Thomashow, Fred Ausubel, and Mary Lou Guerinot for comments that helped us to improve this manuscript, Tom Adams for the *B. japonicum nifA* mutant, and Stuart Pankratz for the expert electron microscopy.

This research was supported by the U.S. Department of Energy, Division of Biological Energy Research, under contract DE-AC02-76ER01-1338 and by the U.S. Department of Agriculture (USDA) grant 85-CRCR-1-1739. G. B. Martin was supported, in part, by a USDA National Needs in Biotechnology fellowship. This article is journal series 13060 of the Michigan Agricultural Experiment Station.

LITERATURE CITED

Adams, T. H. 1986. Transcriptional regulation of nitrogen fixation and nitrogen assimilation genes in *Bradyrhizobium japonicum*. Ph.D. thesis, Michigan State University, East Lansing. 176 pp.

Adams, T. H., and Chelm, B. K. 1984. The *nifH* and *nifDK* promoter regions from *Rhizobium japonicum* share structural homologies with each other and with nitrogen-regulated promoters from other organisms. J. Mol. Appl. Genet. 2:392-405.

Ausubel, F. M. 1984. Regulation of nitrogen fixation genes. Cell 37:5-6.

Bender, R. A., Janssen, K. A., Resnick, A. D., Blumenberg, M., Foor, F., and Magasanik, B. 1977. Biochemical parameters of glutamine synthetase from *Klebsiella aerogenes*. J. Bacteriol. 129:1001-1009.

Bolivar, F., Rodrigues, R. L., Greene, P. J., Betlach, H., Heynecker, H. L., Boyer, H. W., Crossa, J. H., and Falkow, S. 1977. Construction and characterization of new cloning vehicles. A multiple cloning system. Gene 2:95-100.

Brown, C. M., and Dilworth, M. J. 1975. Ammonia assimilation by *Rhizobium* cultures and bacteroids. J. Gen. Microbiol. 86:39-48.

Carlson, T. A., and Chelm, B. K. 1986. Apparent eucaryotic origin of glutamine synthetase II from the bacterium *Bradyrhizobium japonicum*. Nature (London) 322:568-570.

Carlson, T. A., Guerinot, M. L., and Chelm, B. K. 1985. Characterization of the gene encoding glutamine synthetase I (*glnA*) from *Bradyrhizobium japonicum*. J. Bacteriol. 162:698-703.

Carlson, T. A., Martin, G. B., and Chelm, B. K. 1987. Differential transcription of the two glutamine synthetase genes of *Bradyrhizobium japonicum*. J. Bacteriol. 169:5861-5866.

Darrow, R. A., and Knotts, R. R. 1977. Two forms of glutamine synthetase in free-living root-nodule bacteria. Biochem. Biophys. Res. Commun. 78:554-559.

Darrow, R. A., Crist, D., Evans, W. R., Jones, B. L., Keister, D., and Knotts, R. R. 1981. Biochemical and physiological studies on the two glutamine synthetases of *Rhizobium*. Pages 182-185 in: Current Perspectives in Nitrogen Fixation. A. H. Gibson and W. E. Newton, eds. Australian Academy of Science, Canberra.

de Bruijn, F. J., Pawlowski, K., Ratet, P., Hilgert, U., Wang, C. H., Schneider, M., Meyer, H., and Schell, J. 1988. Molecular genetics of nitrogen fixation by *Azorhizobium caulinodans* ORS571, the diazotrophic stem-nodulating symbiont of *Sesbania rostrata*. Pages 351-355 in: Nitrogen Fixation: Hundred Years After. H. Bothe, F. J. de Bruijn, and W. E. Newton, eds. Gustav Fischer, Stuttgart.

de Bruijn, F. J., Rossbach, S., Schneider, M., Ratet, P., Messmer, S., Szeto, W. W., Ausubel, F. M., and Schell, J. 1989. *Rhizobium meliloti*

1021 has three differentially regulated loci involved in glutamine biosynthesis, none of which is essential for symbiotic nitrogen fixation. J. Bacteriol. 171:1673-1682.

Fischer, H.-F., Alvarez-Morales, A., and Hennecke, H. 1986. The pleiotropic nature of symbiotic regulatory mutants: *Bradyrhizobium japonicum nifA* gene is involved in control of *nif* gene expression and formation of determinate symbiosis. EMBO J. 5:1165-1173.

Guerinot, M. L., and Chelm, B. K. 1986. Bacterial α-aminolevulinic acid synthase activity is not essential for leghemoglobin formation in the soybean/*Bradyrhizobium japonicum* symbiosis. Proc. Natl. Acad. Sci. USA 83:1837-1841.

Gussin, G. N., Ronson, C. W., and Ausubel, F. M. 1986. Regulation of nitrogen fixation genes. Annu. Rev. Genet. 20:567-592.

Hardy, R. W. F., Holsten, R. D., Jackson, E. K., and Burns, R. C. 1968. The acetylene-ethylene assay for N_2 fixation: Laboratory and field evaluation. Plant Physiol. 43:1185-1207.

Hennecke, H., Fischer, H.-M., Gubler, M., Thony, B., Anthamatten, D., Kullik, I., Ebeling, S., Fritsche, S., and Zurcher, T. 1988. Regulation of *nif* and *fix* genes in *Bradyrhizobium japonicum* occurs by a cascade of two consecutive gene activation steps of which the second one is oxygen sensitive. Pages 339-344 in: Nitrogen Fixation: Hundred Years After. H. Bothe, F. J. de Bruijn, and W. E. Newton, eds. Gustav Fischer, Stuttgart.

Johnson, G. V., Evans, H. J., and Ching, T. M. 1966. Enzymes of the glyoxylate cycle in rhizobia and nodules of legumes. Plant Physiol. 41:1330-1336.

Magasanik, B. 1982. Genetic control of nitrogen assimilation in bacteria. Annu. Rev. Genet. 16:135-168.

Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 545 pp.

Martin, G. B., Chapman, K. A., and Chelm, B. K. 1988. Role of the *Bradyrhizobium japonicum ntrC* gene product in differential regulation of the glutamine synthetase II gene (*glnII*). J. Bacteriol. 170:5452-5459.

Maupin, P., and Pollard, T. D. 1983. Improved preservation and staining of HeLa cell actin filaments, clathrin-coated membranes, and other cytoplasmic structures by tannic acid-glutaraldehyde-saponin fixation. J. Cell Biol. 96:51-62.

O'Gara, F., and Shanmugam, K. T. 1976. Regulation of nitrogen fixation by rhizobia: Export of fixed N_2 as NH_4. Biochim. Biophys. Acta 437:313-321.

Pawlowski, K., Ratet, P., Schell, J., and de Bruijn, F. J. 1987. Cloning and characterization of *nifA* and *ntrC* genes of the stem nodulating bacterium ORS571, the nitrogen fixing symbiont of *Sesbania rostrata*: Regulation of the nitrogen fixation (*nif*) genes in the free living versus symbiotic state. Mol. Gen. Genet. 206:207-219.

Pierce, M., and Bauer, W. D. 1983. A rapid regulatory response governing nodulation in soybean. Plant Physiol. 73:286-290.

Prentki, P., and Krisch, H. M. 1984. In vitro insertional mutagenesis with a selectable DNA fragment. Gene 29:303-313.

Rao, R. N., and Rogers, S. G. 1979. Plasmid pKC7: A vector containing ten restriction endonuclease sites suitable for cloning DNA segments. Gene 7:79-82.

Rossbach, S., Schell, J., and de Bruijn, F. J. 1987. The *ntrC* gene of *Agrobacterium tumefaciens* C58 controls glutamine synthetase (GSII) activity, growth on nitrate and chromosomal but not Ti-encoded arginine catabolism pathways. Mol. Gen. Genet. 209:419-426.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. 1985. Measurement of pritein using bicinchoninic acid. Anal. Biochem. 150:76-85.

Szeto, W. W., Zimmerman, J. L., Sundaresan, V., and Ausubel, F. M. 1984. A *Rhizobium meliloti* symbiotic regulatory gene. Cell 36:535-543.

Szeto, W. W., Nixon, B. T., Ronson, C. W., and Ausubel, F. M. 1987. Identification and characterization of the *Rhizobium meliloti ntrC* gene: *R. meliloti* has separate regulatory pathways for activation of nitrogen fixation genes in free-living and symbiotic cells. J. Bacteriol. 169:1423-1432.

Vairinhos, F., Bhandari, B., and Nicholas, D. J. D. 1983. Glutamine synthetase, glutamate synthase and glutamate dehydrogenase in *Rhizobium japonicum* strains grown in cultures and in bacteroids from root nodules of *Glycine max*. Planta 159:207-215.

Vincent, J. M. 1978. Factors controlling the legume-*Rhizobium* symbiosis. Pages 103-129 in: Nitrogen Fixation, Vol. II. W. E. Newton and W. H. Orme-Johnson, eds. University Park Press, Baltimore, MD.