MOLECULAR PLANT-MICROBE INTERACTIONS
Vol. 4, No. 6, pp. 619-622, 1991

Research Notes

The *glnA* Gene of *Rhizobium leguminosarum* bv. *phaseoli* and Its Role In Symbiosis

Soledad Moreno[1], Roberto Meza[1], Josefina Guzman[1], Alfonso Carabez[2], and Guadalupe Espin[1]

[1] Centro de Investigacion sobre Ingenieria Genetica Biotecnologia and [2]Instituto de Fisiologia Celular Universidad Nacional Autonoma de Mexico, Apartado Postal 510-3, Cuernavaca, Mor. 62271 Mexico.
Received April 30 1991. Accepted 9 July 1991.

We have determined glutamine synthetase (GS) activity in bacteroids isolated from nodules of *Phaseolus vulgaris* induced by *Rhizobium leguminosarum* biovar *phaseoli* CE3 wild type strain. GSI but not GSII activity was detected. No GS activity was detected in bacteroids of strain SM58, which carries a *glnA*::Km insertion mutation. Acetylene reduction activity of nodules induced by strain SM58 was about 50% of that found in nodules induced by the wild type. Microscopic analysis of nodules induced by strain SM58 showed that the number of bacteroids within the infected cells was reduced as compared to the number of bacteroids present in the wild type-induced nodules. In addition, SM58 bacteroids contained high amounts of poly-β-hydroxybutyrate granules.

Additional keywords: glutamine synthetase in bacteriods, *Rhizobium gln* genes, *Rhizobium leguminosarum* bv. *phaseoli-Phaseolus vulgaris* symbiosis.

Rhizobium species assimilate ammonia for growth in the free-living state through the action of the GSI, GSII, and glutamate synthase (GOGAT) enzymes. In nodules, the nitrogen-fixing bacteroids export the ammonia produced by the nitrogenase to the plant cells (Bergersen and Turner 1967; O'Gara and Shanmugam 1976), where it is assimilated by the plant glutamine synthetase (GS). The rhizobial GSI and GSII are products of the *glnA* and *glnII* genes, respectively (Somerville and Kahn 1983; Carlson *et al.* 1985; Carlson and Chelm 1986; Colonna-Romano *et al.* 1987; Espin *et al.* 1988; de Bruijn *et al.* 1989). *glnT*, a locus first identified in *Agrobacterium tumefaciens* (Rossbach *et al.* 1988) and *Rhizobium meliloti* (de Bruijn *et al.* 1989) that complements *Escherichia coli* glutamine auxotrophs and is neither *glnA* nor *glnII*, has been cloned from *Rhizobium leguminosarum* bv. *phaseoli* Jordan and shown to code for a glutamine synthetase activity (GSIII) (Espin *et al.* 1990). However, the physiological conditions for GSIII expression or activity in *Rhizobium* have not been found yet.

Rhizobial GSI is similar to the GS of enteric bacteria: it is a dodecameric protein, and its activity is regulated by an adenylylation-deadenylylation mechanism (Darrow 1980; Ludwig 1980; Rossi *et al.* 1989). GSII is an octameric enzyme, is heat sensitive, and is similar to eukaryotic GSs (Darrow 1980; Carlson and Chelm 1986). Transcription of *glnA* is not significantly regulated by the nitrogen source in the medium (Carlson *et al.* 1987; Szeto *et al.* 1987; Rossi *et al.* 1989; Chiurazzi and Iaccarino 1990). In contrast, the structural gene for GSII, *glnII*, is regulated in response to the nitrogen and oxygen availability (Adams and Chelm 1988; Martin *et al.* 1988; Rossi *et al.* 1989; Shatters *et al.* 1989; de Bruijn *et al.* 1989). The presence of GS activity in bacteroids of several *Rhizobium* species was demonstrated earlier by Brown and Dilworth (1975). However, at that time, the presence of two GS isoenzymes in these bacteria was unknown. In that study, the bacteroid GS activity was found to be significantly lower as compared with that of the plant GS (Brown and Dilworth 1975). In a recent study, no GSII protein was detected in crude extracts of alfalfa, pea, or soybean nodules using an anti-GSII-specific antiserum (Shatters *et al.* 1989). Taken together these data suggest that GSI but not GSII might be present in bacteroids of *Rhizobium* and *Bradyrhizobium* species. In regard to the role of the *R. l.* bv. *phaseoli* GSI and GSII in symbiosis, we have previously reported a mutant lacking GSII activity that has a Nod^+ Fix^+ phenotype (Morett *et al.* 1985). To investigate the role of *R. l.* bv. *phaseoli* GSs in nitrogen metabolism and in symbiosis, we have cloned the *glnA* gene and constructed by site-directed mutagenesis a strain carrying a *glnA*::Km mutation.

A cosmid gene library of *R. l.*bv. *phaseoli* CE3 strain (Cevallos *et al.* 1989) was transferred by conjugation into the *glnA* mutant of *Klebsiella pneumoniae* (Schroeter) Trevisan UNF1811 (Espin *et al.* 1981) A transconjugant UNF1811(pSM240) able to grow in the absence of glutamine was identified and shown to contain a plasmid (pSM240). Two *Eco*RI fragments of 2.8 and 1.2 kb of pSM240 were subcloned into plasmid pSUP202 (Simon *et al.* 1983). The resultant plasmid, pSM245, complemented the deficiency in GS activity of UNF1811. The two *Eco*RI fragments of pSM245 were subcloned separately in both orientations into pSUP202 vector, neither fragment alone complemented strain UNF1811, suggesting that the complementing sequence lies within these two *Eco*RI sites. This suggestion was confirmed by constructing a derivative of pSM245 that had the *kan* gene from plasmid pGV97 (Leemans *et al.* 1982) inserted into the *Eco*RI site that separates the two inserts. The resulting kanamycin resistant plasmid, pSM58, failed to complement strain UNF1811.

Please address correspondence to Guadalupe Espin.

To confirm that the complementing plasmid pSM245 encoded for a GS gene, and to determine which *R. l.* bv. *phaseoli* gene had been cloned, we compared the electrophoretic mobility of the GS activity of strain UNF1811 (pSM245) with those of GSI and GSII of *R. l.* bv. *phaseoli* and with the *K. pneumoniae* GS. Strain UNF1811 (pSM245) showed a heat stable activity that comigrated in polyacrylamide gels with GSI (Fig 1).

The *glnA*::Km mutation created in the construction of pSM58 was used to replace a genomic *glnA*$^+$ allele in CE3. Plasmid pSM58, which cannot replicate in *Rhizobium*, was transferred by conjugation into strain CE3 for gene replacement. Strain SM58, a CE3 kanamycin resistant derivative, was isolated and confirmed by Southern blot hybridization experiments to carry the *glnA*::Km insertion mutation (data not shown).

We determined the growth and the GS activities of the CE3 and SM58 strains. When CE3 is grown on minimal medium with glutamate as the sole nitrogen source GSII activity is about 20-fold higher than when this strain is grown on ammonia. The level of GSI activity is about twofold higher in glutamate (Table 1). No GSI activity was detected in strain SM58 (Table 1; Fig. 1.), and GSII activity was found higher than in the wild type (Table 1). However, although different overall GS levels can be measured, no effect on the growth rate of mutant and wild type strains can be observed (Table 1).

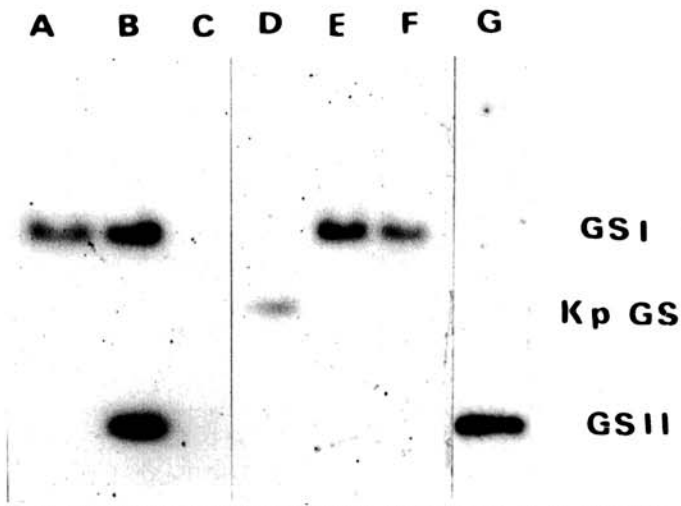

Fig. 1. Electropherograms of GSI and GSII stained in situ for the transferase activity. Strains were grown on minimal medium (Noel *et al.* 1984) to an $OD_{540\ nm}$ of 0.3. Cell extracts were prepared by sonication. Extracts were heated for 1 h at 50° C to inactivate GSII. Heated (e,f) and nonheated (a,b,c,d,g) portions of the extracts containing 150 μg of protein were loaded onto polyacrylamide gels for electrophoresis. GS assays were conducted for 3 h at 30° C. Strain CE3, lanes b and e; UNF1811(pSM245), lanes a and f; UNF1811, lane c; UNF5022, lane d; SM58, lane g.

GS activity was also determined in bacteroids of strains CE3 and SM58. *Phaseolus vulgaris* L. seedlings were inoculated with strains CE3 or SM58. Inoculated plants were grown in a Conviron plant growth chamber at 25 C with 75% relative humidity and 12-hr light-dark periods. After 20 days bacteroids were isolated as described by Fortin *et al.* (1985). The heat resistant GSI, but not the heat labile GSII activity, was present in bacteroids of strain CE3 (Table 1). No GS activity was detected in bacteroids of strain SM58.

Acetylene reduction activity of 20-day-old nodules produced by strains CE3 and SM58 was determined. This activity was reduced in nodules induced by SM58 (Table 1). We therefore explored the histological organization and subcellular ultrastructure of nodules induced by the SM58 mutant. As Figure 2 shows, 20 days after inoculation, infected cells filled with bacteroids were observed in nodules induced by strain CE3, whereas fewer bacteroids were observed in nodules induced by strain SM58. In contrast to the wild type, bacteroids of the SM58 contained pronounced deposits of poly-β-hydroxybutyrate (PHB)

In this study, we determined GS activity of nitrogen-fixing *R. l.* bv. *phaseoli* bacteroids of the wild type CE3. We found, under the conditions tested, that GSI is the unique GS activity that is present. This was confirmed by the fact that no GS activity was detected in bacteroids of the GSI mutant. We also found that nodules induced by this strain have a reduced capacity to fix nitrogen. In contrast to the situation in *R. l.* bv. *phaseoli*, *Bradyrhizobium japonicum glnA* strains induced higher numbers of nodules per plant and higher acetylene reduction activity than did the wild type (Carlson *et al.* 1987). A similar symbiotic phenotype was reported for *B. japonicum glnII* mutants (Carlson *et al.* 1987). Nodulation and nitrogen fixation in *glnA*, and *glnII* mutants of *R. meliloti* were reported to be normal (Somerville and Kahn 1983; Somerville *et al.* 1989; de Bruijn *et al.* 1989), suggesting

Table 1. Growth, glutamine synthetase, and acetylene reduction activities of CE3 and SM58 strains of *Rhizobium leguminosarum* bv. *phaseoli*[a]

Strain	Medium	DT[b] (min)	Transferase[a]		Biosynthetic[a]		Acetylene reduction[c]
			GSI	GSII	GSI	GSII	
CE3	MM + glutamate	150	1.38	2.29	0.07	0.08	...
SM58	MM + glutamate	150	<0.01	2.56	<0.01	0.09	...
CE3	MM + NH^{4-}	140	0.63	0.12	0.03	<0.01	...
SM58	MM + NH^{4-}	160	<0.01	0.22	<0.01	<0.01	...
CE3	Bacteroids	...	0.35	<0.01	0.02	<0.01	706 (57)[d]
SM58	Bacteroids	...	<0.01	<0.01	<0.01	<0.01	370 (26)

[a] Glutamine synthetase activity was determined to whole cells as previously described (Morett *et al.* 1985). Specific activity is expressed as micromoles of product formed per minute per milligram of protein.
[b] Duplication time.
[c] Acetylene reduction was determined as described previously (Morett *et al.* 1988). Specific activity is given as arbitrary units of ethylene produced per hour, per weight (g) of nodules. Values are the mean of 45 determinations.
[d] Number in parenthesis are standard deviations. The Student *t* test showed that the difference between media was highly significant ($P < 0.01$).

that neither GSI or GSII are essential for the symbiosis. However, glutamine synthesis seems to be important at several stages in the symbiosis, because a *B. japonicum glnA,glnII* double mutant is a glutamine auxotroph and is defective in nodulation (Carlson *et al.* 1987), whereas in *R. meliloti* a *glnA,glnII* mutant, which under certain conditions is not a glutamine auxotroph, has a Nod^+ Fix^+ phenotype (Somerville *et al.* 1989). It is generally assumed that bacteroids do not assimilate ammonia, still it is likely that in *R. l.* bv. *phaseoli* bacteroids some glutamine is synthesized, because the utilization of its amide moiety by the glutamine amido transferase leads to the synthesis of the pyridine nucleotide coenzymes (Meister 1980) that are essential for the nitrogenase activity. Glutamine synthesis might also be important at some state in the symbiosis before nitrogen fixation is started, such as bacteroid proliferation or differentiation. Reduction of nitrogen fixation in nodules induced by strain SM58 is likely to be related to the reduction in the number of bacteroids seen in the infected cells. Accumulation of PHB in bacteroids of strain SM58 is a phenotype similar to that of *nifH* mutants of *B. japonicum* (Hahn *et al.* 1984). Thus it might be related to the reduction in nitrogen fixation. PHB accumulation could be the result of an increase in its synthesis or an inhibition of its degradation or both. Catabolism of PHB proceeds through the action of a PHB depolymerase and of a D(−)-3-hydroxybutyrate dehydrogenase. These enzymes have been identified in *Rhizobium* bacteroids (Wong and Evans 1971; Dawes and Senior 1973; Karr *et al.* 1984); the later enzyme is inhibited by 2-oxoglutarate in *Azotobacter vinelandii* (Dawes and Senior 1973). Glutamine and 2-oxoglutarate are the initial substrates in the GOGAT/GS pathway; therefore there is a possibility that in the SM58 bacteroids, 2-oxoglutarate accumulates and thus inhibits PHB degradation.

The experiments presented here indicate that in *R. l.* bv. *phaseoli* GSI is the unique isoenzyme present in nitrogen-fixing bacteroids; when it is removed by mutation, the effectiveness of the nodule is impaired.

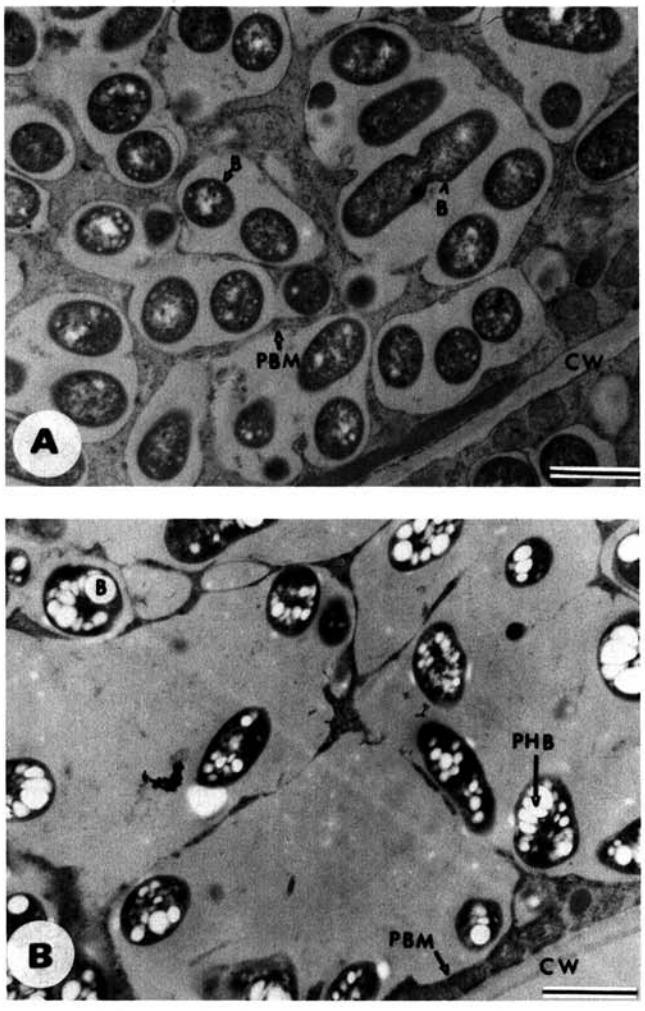

Fig. 2. Electron micrographs of thin sections of nodules from *Phaseolus vulgaris* beans 20 days after inoculation with **A,** CE3 and **B,** SM58 strains. Structural features are abbreviated as follows: B, bacteroids; PM, peribacteroid membrane; PHB, poly-β-hydroxybutyrate granules; CW, plant cell wall. The magnification is 12,000-fold; a 1-μ scale is included. Microscopic examination was carried out as described by Soberon *et al.* 1990.

ACKNOWLEDGMENTS

This study was supported by EEC collaborative grant CI1-0410-ME/CI1-0408-I. We thank M. Iaccarino for discussions and A. Gonzalez and E. Merino for reviewing the manuscript.

LITERATURE CITED

Adams, T. H., and Chelm, B. K. 1988. Effects of oxygen levels on the transcription of *nif* and *gln* genes in *Bradyrhizobium janonicum*. J. Gen. Microbiol. 134:611-618.

Bergersen, F. J., and Turner G. L. 1967. Nitrogen fixation by bacteroids fraction of soybean root nodules. Biochim. Biophys. Acta. 141:507-515.

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

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

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.

Cevallos, M. A., Vazquez, M., Davalos, A., Espin, G., Sepulveda, J., and Quinto, C. 1989. Characterization of *Rhizobium phaseoli* Sym plasmid regions involved in nodule morphogenesis and host range specificity. Mol. Microbiol. 3:879-889.

Chiurazzi, M., and Iaccarino, M. 1990. Transcriptional analysis of the *glnB-glnA* region of *Rhizobium leguminosarum* biovar *viciae*. Mol. Microbiol. 4:1727-1735.

Colonna-Romano, S., Riccio, A., Guida, M., Defez, R., Lamberti, A., Iaccarino, M., Arnold, W., Priefer, U., Puhler, A. 1987. Tight linkage of and a putative regulatory gene in *Rhizobium leguminosarum*. Nucleic Acids Res. 15:1951-1964.

Darrow, R. A. 1980. Role of glutamine synthetase in nitrogen fixation. Pages 139-166 in Glutamine Synthetase: Metabolism, Enzymology and Regulation. J. Mora and R. Palacios, eds. Academic Press, New York.

Dawes, E. A., and Senior, J. P. 1973. The role and regulation of energy reserve polymers in micro-organisms. Pages 135-266 in: Advances in Microbial Physiology. Vol. 10. A. H. Rose and D. W. Tempest, eds. Academic Press, London.

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.

Espin, G., Alvarez-Morales, A., and Merrick. M. J. 1981. Complementation analysis of *glnA* linked mutations which affect nitrogen fixation in *Klebsiella pneumoniae*. Mol. Gen. Genet. 184:213-217.

Espin, G., Moreno, S., Wild, M., and Meza, R. 1988. *Rhizobium phaseoli* glutamine synthetases. Page 517 in: Nitrogen Fixation: Hundred years After. H. Bothe, F. J. de Bruijn, and W. E. Newton, eds. Gustav Fisher, New York.

Espin, G., Moreno, S., Wild, M., Meza, R., and Iaccarino, M. 1990. A previously unrecognized glutamine synthetase expressed in *Klebsiella pneumoniae* from the *glnT* locus of *Rhizobium leguminosarum*. Mol. Gen. Genet. 223:513-516.

Fortin, M. G., Zelechowska, M., and Verma, D. S. P. 1985. Specific targeting of membrane nodulins to the bacteroid-enclosing compartment in soybean nodules. EMB0 J. 4:3041-3046.

Hahn, M., Meyer, L., Studer, D., Regensburger, B., and Hennecke, H. 1984. Insertion and deletion mutations within the *nif* region of *Rhizobium japonicum*. Plant Mol. Biol. 3:159-168.

Karr, D. B., Waters, J. K., Susuki, F., and Emerich, D. W. 1984. Enzymes of the poly-β-hydroxybutyrate and citric acids cycles of *Rhizobium japonicum* bacteroids. Plant Physiol. 75:1158-1162.

Leemans, J., Dehlaere, R., Willmitzer, L. De Greve, H., Hernalsteens, J. P., Van Montagu, M., and Schell. J. 1982. Genetic identification of functions of TL-DNA transcripts in octopine crown galls. EMBO J. 1:147-152.

Ludwig, R. A. 1980. Physiological roles of glutamine synthetases I and II in ammonium assimilation in *Rhizobium* sp. 32H1. J. Bacteriol. 141:1209-1216.

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

Meister, A. 1980. Catalytic mechanism of glutamine synthetase: Overview of glutamine metabolism. Pages 1-40 in: Glutamine: Metabolism, Enzymology, and Regulation. J. Mora and R. Palacios, eds. Academic Press, New York.

Morett, E., Moreno, S., and Espin, G. 1985. Impaired nitrogen fixation and glutamine synthesis in methionine sulfoximine sensitive (MS^S) mutants of *Rhizobium phaseoli*. Mol. Gen. Genet. 200:229-234.

Morett, E., Moreno, S., and Espin, G. 1988. Transcription analysis of the three *nifH* genes of *Rhizobium phaseoli* with gene fusions. Mol. Gen. Genet. 213:499-504.

Noel, K. D., Sanchez, A., Fernandez, L., Leemans, J., Cevallos, M. A. 1984. *Rhizobium phaseoli* symbiotic mutants with transposon Tn*5* insertions. J. Bacteriol. 158:148-155.

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

Rossi, M., Defez, R., Chiurazzi, M., Lamberti, A., Fuggi, A., and Iaccarino, M. 1989. Regulation of glutamine synthetases isoenzymes in *Rhizobium leguminosarum* biovar *viciae*. J. Gen. Microbiol. 135:629-637.

Rossbach, S., Schell, J., and de Bruijn, F. J. 1988. Cloning and analysis of *Agrobacterium tumefaciens* C58 loci involved in glutamine biosynthesis: Neither the *glnA* (GSI) nor the *glnII* (GSII) gene plays a special role in virulence. Mol. Gen. Genet. 212:38-47.

Shatters, R. G., Somerville, J. E., Kahn, M. L. 1989. Regulation of glutamine synthetase II activity in *Rhizobium meliloti* 104A14. J. Bacteriol. 171:5087-5094.

Simon, R., Priefer, U., and Puhler, A. 1983. A broad host range mobilization system for in vivo genetic engineering: Transposon mutagenesis in Gram-negative bacteria. Bio/Technol. 1:784-791.

Soberon, M., Membrillo-Hernandez, J., Aguilar, G., and Sanchez, F. 1990. Isolation of *Rhizobium phaseoli* Tn*5*-induced mutants with altered expression of cytochrome terminal oxydases O and aa_3. J. Bacteriol. 172:1676-1680.

Somerville, J. E., and Kahn, M. L. 1983. Cloning of the glutamine synthetase I gene from *Rhizobium meliloti*. J. Bacteriol. 156:167-176.

Somerville, J. E., Shatters, R. G., and Kahn, M. L. 1989. Isolation, characterization and complementation of *Rhizobium meliloti* 104A14 mutants that lack glutamine synthetase II activity. J. Bacteriol. 171:5079-5086.

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

Wong, P. P., and Evans, H. J. 1971. Poly-β-hydroxybutyrate utilization by soybean (*Glycine max* Merr.) nodules and assessment of its role in maintenance of nitrogenase activity. Plant Physiol. 47:750-755.