This is to certify that the

dissertation entitled

Characterization of a nitrogen regulatory (Ntr) system in Bradyrhizobium japonicum.

presented by

Gregory B. Martin

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Genetics

Major professor

Date 4/14/89

CHARACTERIZATION OF A NITROGEN REGULATORY (Ntr) SYSTEM IN *BRADYRHIZOBIUM JAPONICUM*

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Genetics Program

1989

ABSTRACT

CHARACTERIZATION OF A NITROGEN REGULATORY (Ntr) SYSTEM IN *BRADYRHIZOBIUM JAPONICUM*

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Bradyrhizobium japonicum reduces dinitrogen to ammonia in symbiotic association with the soybean, *Glycine max*. In contrast to free-living cells, symbiotic cells of this bacterium repress glutamine synthetase so that fixed nitrogen can be exported to the host plant. This dissertation describes the characterization of five *B. japonicum* loci that are involved in glutamine biosythesis or its regulation. Analysis of mutants lacking one or both of the isoforms of glutamine synthetase, GSI and GSII, revealed that either isoform alone is sufficient to provide glutamine prototrophy and to enable the effective nodulation of soybean. A double mutation, *glnA/glnII*, produces glutamine auxotrophy and prevents soybean nodulation. A regulatory gene, *ntrC*, was isolated and its gene product was found to activate transcription of *glnII* in aerobically grown cells. The activation of *glnII* in microaerobically-grown cells and in nodule bacteria was NtrC-independent. In related work two double mutant strains, *glnA/ntrBC* and *glnA/nifA*, were constructed to look at the physiological effects of NtrB, NtrC and NifA on the regulation of *glnII* during free-living growth and nodulation. Both strains were poor symbionts but only the *glnA/ntrBC* strain was a glutamine auxotroph in free-living cultures. Another putative nitrogen regulatory gene, *glnB*, was sequenced and its expression was analyzed.

The *B. japonicum glnB* gene was highly homologous to the enteric *glnB* genes, but in contrast to them it was located directly upstream of *glnA*. The *glnB* gene was expressed from tandem promoters which were differentially regulated in response to the nitrogen status, but not oxygen status, of the medium. The expression of the *glnB* downstream promoter required the *B. japonicum ntrC* gene product. In contrast to *glnII* regulation, expression from one of the *glnB* promoters was NtrC dependent in nodule bacteria. These results are considered with respect to possible models of global gene regulation in free-living and symbiotic cells in response to nitrogen and oxygen status.

to Susan, Kelly, and Michael

ACKNOWLEDGMENTS

I express my appreciation to Barry Chelm for initiating me into Bradyrhizobium lore and for providing a dynamic, competitive laboratory environment for this work. Barry's perceptive feel for important questions initiated this research and inspired me to follow it through. I express my gratitude to Mike Thomashow and Fred Ausubel for serving as advisors to me for the latter part of my thesis work. Their suggestions regarding research goals, and their critical listening, questioning and reading made this research challenging and enjoyable. I thank the members of my graduate committee, Jim Tiedje, Andrew Hanson, Dennis Fulbright, for their guidance and advice. I also thank Lisa Albright, Jeff Elhai, and Larry Snyder for critical readings of various parts of this thesis and for many helpful discussions. I am grateful to members of the Chelm lab, Tom Adams, Todd Carlson, John Scott-Craig and John Somerville, Bill Holben, Ken Chapman, Elizabeth Verkamp, and Todd Cotter for sharing advice, experience, plasmids and strains. I express my gratitude to Boris Magasanik and Larry Reitzer for the NtrC antiserum, Tracy Nixon for plasmids, and Stuart Pankratz for expert electron microscopy. I acknowledge the DOE Plant Research Laboratory and the USDA National Needs in Biotechnology Program for financial support of my graduate studies. Finally, special thanks go to Susan, and our children, Kelly and Michael, for creating a warm and loving home.

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INTRODUCTION

Bacterial global gene regulation.

The cosmopolitan lifestyles of many bacteria demand coordinated and rapid changes in gene expression. The response to an environmental stress must be coordinated since the production of unnecessary gene products wastes metabolic energy. In addition, coping with some stresses requires the concurrent activation of many interrelated cell functions. The response must be rapid because bacterial cell growth is rapid and bacterial metabolism is affected quickly by its environment. Global gene regulation is one system whereby bacteria are equipped to respond to stress quickly and coordinately.

Global regulatory networks are composed of a hierarchy of interrelated and interdependent protein-encoding genes (17). In skeletal form this hierarchy consists of a sensor, usually a pair of regulators - one a transcriptional activator protein and one a regulator of this positive factor - and finally the regulon itself. The regulon consists of all of the genes under the regulation of a particular transcriptional activator. Genes or gene products fulfilling each of these roles have been characterized in several global regulatory networks (17, 20, 25, 32, 37, 47).

To postulate a sensor implies the presence of a stimulus. This stimulus may be oxygen limitation, heat shock, phosphate limitation or

nitrogen limitation depending on the regulatory network (20, 25, 41, 47). In many cases the precise stimulus is unknown - the glutamine:2 ketoglutarate ratio may be what is actually sensed in the nitrogen regulated response (42). Similarly, cessation of phosphate transport rather than intracellular phosphate concentration per se may be the stimulus in the phosphate limiting response (52). Molecular aspects of stimulus reception are usually unknown (but see below). If the sensor is a specific gene product as is thought for the nitrogen limited response (GlnD; 25,42), the response to a stimulus may be simply increased transcription of the gene encoding this product. Exactly how this transcription is induced, however, is also unknown in most bacterial systems.

All global regulatory models have a central positive regulatory factor (37). It is this factor, usually found to be a DNA-binding protein (9, 29, 37, 50) that activates transcription from the various genes and/or operons making up the regulon (17). A regulon may consist of obviously related functions such as the phosphate permease and the phosphate binding protein in the Pho regulon of *E. coli* (17, 47). However, regulons often consist of what appear at first sight to be unrelated gene functions, as for example nitrate and nitrite reductase and the tripeptide permease in the aerobic/anaerobic regulon of *S. typhimurium* (20, 44).

Although analogous roles for specific positive regulatory factors in different global systems are suggested by models, only recently has the structural homology of these proteins been recognized (32, 53). Striking homologies have been found between the N-terminal amino acids

of several positive regulatory proteins including the gene products of *ntrC*, *phoB*, *ompR*, and *virG*. Similarly, there are C-terminal homologies between the residues of gene products thought to modify or regulate these activators: *ntrB*, *phoR*, *envZ*, and *virA*. A model has been proposed that tandem regulatory genes, perhaps arising from an ancestral pair, exist in a relationship in which one gene product acts as a transducer of an environmental stimulus while the other, modified by this transducer, activates transcription of genes needed for coping with a specific environmental stress. Strong evidence supporting this model has come from work in *E. coli*. Ninfa et al. showed that the protein kinases that regulate chemotaxis and transcription of nitrogen-regulated genes, CheA and NtrB, respectively, have cross-specificities. CheA can phosphorylate the Ntr transcription factor, NtrC and NtrB can phosphorylate CheY, the modifier of CheA (31).

Global nitrogen regulation

A global regulatory system responding to limitation of ammonia or other fixed nitrogen was characterized first in *E. coli* (25, 49). Since then analogous networks but with slightly different characteristics have been identified in other enterics including *S. typhimurium* (7, 23), *Klebsiella. aerogenes* (16, 25), *K. pneumoniae* (18), and in some nonenterics: *Rhodopseudomonas capsulata* (21), *Azotobacter vinelandii* (48), *Rhizobium meliloti* (46) and *Bradyrhizobium japonicum* (1, 2, 10, 11). I will summarize the similarities and differences of the various Ntr systems with emphasis on the role of the positive regulator, NtrC, in these networks.

Nitrogen regulation in enteric non-nitrogen-fixers

The nitrogen regulatory (Ntr) model for the enteric bacteria *Escherichia coli* and *Klebsiella pneumoniae* will be presented here in detail because it provides the rationale for several of the experimental approaches that were explored in this work (Figure 1). A list of enteric nitrogen regulatory and nitrogen-regulated genes, their gene products and functions is presented in Table 1.

The enteric non-nitrogen-fixers, *E. coli* and *S. typhimurium* possess an operon, *glnA-ntrBC*, that has three distinct promoters (6, 27, 51). The promoters *glnA*p1, distal to the structural gene for glutamine synthetase (GS), and *ntrB*p upstream of *ntrBC*, are both relatively weak and function to provide the cell with low levels of GS, NtrB, and NtrC (19, 30, 38). The promoter *glnA*p2, downstream of *glnA*p1, is stronger and is activated by NtrC (and a unique sigma factor NtrA) during nitrogen limitation (30, 39). This activation provides the cell with high levels of GS under limited nitrogen conditions (38). In addition to this positive regulation, NtrC also represses transcription from *glnA*p1 and *ntrB*p under limiting nitrogen conditions. This basic regulatory scheme is further modified by the gene products of *glnB*, *glnD* and *glnE* (25). Glutamine synthetase undergoes post-translational modification via an adenlylylation reaction (43). The biosynthetic activity of GS is progressively decreased by the specific covalent attachment of adenylyl groups to each of its 12 subunits (43). This reversible modification occurs in response to nitrogen limitation and is mediated by the gene product of *glnE* (18, 25), an adenylyltransferase

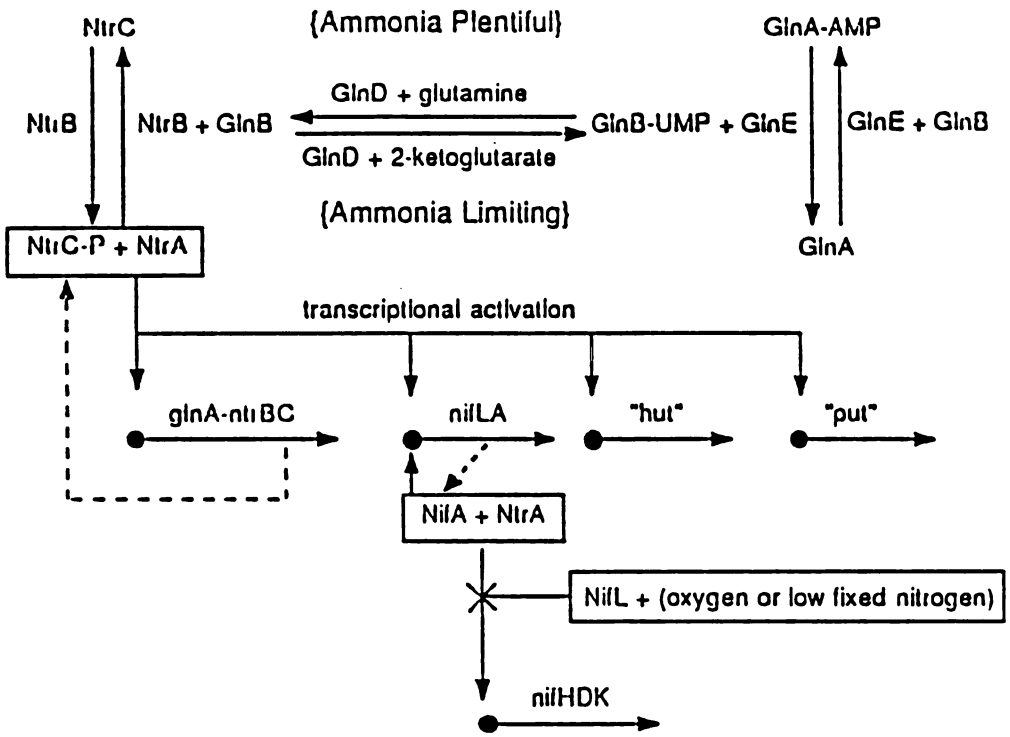

Figure 1: Nitrogen regulatory network (Ntr) in the enteric bacteria (18).

Table 1. Components of nitrogen regulatory (Ntr) systems in enteric bacteria

Gene	(synonym)	Gene product	Function
Regulatory			
ntrA	rpoN, glnF	NtrA	RNA polymerase binding of Ntr/Nif promoters
ntrB	glnL	NtrB, NR_{II}	NtrC phosphorylation
ntrC	glnG	NtrC, NR_{I}	Activator/Repressor of of Ntr promoters
glnB		GlnB, P_{II}	Stimulates GS adenylation and NtrC phosphorylation
glnD		GlnD, UTase	Uridylylates GlnB
glnE		GlnE, ATase	Adenylylates GS
nifA		NifA	Activator of Nif promoters
nifL		NifL	Regulates NifA
Regulon			
glnA	Glutamine synthetase		Nitrogen assimilation
hut	Histidine utilization enzymes		Nitrogen assimilation
glnHPQ	Glutamine permease		Glutamine transport
nifHDK	Nitrogenase		Nitrogen fixation

(ATase). Adenylylation is stimulated by the gene product encoded by *glnB* - a tetramer with small, 12,000 kd subunits (GlnB; 16). Deadenylylation is catalyzed by the adenylyltransferase (GlnE) and is stimulated by the uridylylated form of GlnB (GlnB-UMP 7, 25, 40). Thus the uridylylation of GlnB is a critical step in the activation/deactivation of GS. A uridylyltransferase (UTase), encoded by *glnD*, is responsible for this reaction (8, 25). GlnD may sense the nitrogen status of the cell via the glutamine:2-ketoglutarate ratio (42, 43). The activity of the positive transcriptional regulator, NtrC, is also regulated by GlnB, GlnD as well as NtrB (8, 16, 19, 25, 30). NtrB is a kinase/phosphatase that post-translationally modifies the NtrC protein (12, 54). This modification results in its activation (phosphorylation) or deactivation (dephosphorylation). As mentioned earlier GlnB is deuridylylated by GlnD and is then involved in adenylylating GS (8, 16). Coordinate with this reaction the deuridylylated GlnB also stimulates the dephosphorylation of NtrC by NtrB (16, 30).

The function of NtrC in activating transcription has recently been clarified. It had been known that NtrC binds to specific sequence elements adjacent to Ntr-regulated promoters (39). Using a purified in vitro transcription system Popham et al. (36) found that after binding at these sites, NtrC catalyzes the isomerization of closed recognition complexes to open complexes in which DNA in the region of transcription start site is locally denatured. Whether NtrC makes physical contact with the NtrA:RNA polymerase complex in this reaction is not yet known (36).

The specific interactions involved in enteric nitrogen global regulation are presented in model form in Figure 1 (18). In summary:

Under nitrogen limitation:

1. *glnD* or its gene product senses a low glutamine: 2-ketoglutarate ratio and GlnD uridylylates GlnB (GlnB-UMP), (8, 42).
2. Uridylylated GlnB is no longer active in stimulating the dephosphorylation of NtrC, (16).
3. NtrB phosphorylates NtrC (NtrC-P) which then becomes active (coordinate with NtrA, a sigma factor) in inducing transcription from the *glnA*p2 promoter (30); GS activity increases.
4. Adenylylated GS already present in the cell is deadenylylated by ATase stimulated by GlnB-UMP, (40).
5. Newly transcribed/translated GS becomes available and is not adenylylated due to the presence of GlnB-UMP (16).

Under nitrogen excess:

1. *glnD* or its gene product senses a high glutamine: 2-ketoglutarate ratio and deuridylylates GlnB (8, 42).
2. GlnB stimulates the phosphatase activity of NtrB and NtrC-P is dephosphorylated and thus made inactive, (30).
3. The decreased NtrC level causes transcription to revert to the weaker *glnA*p1 promoter thus lowering GS levels (38).
4. Concurrently, the deuridylylated GlnB stimulates the adenylyltransferase activity of GlnE and GS is inactivated, (16, 40).

It is important to note that GS is maintained at a low level, unadenylylated, even under nitrogen excess in order to provide the cell with some nitrogen assimilatory capacity. In addition to GS, there are several other proteins under nitrogen control which can be considered part of the Ntr regulon. It has recently been reported in *E. coli* that the glutamine transport operon (*glnHPQ*) has two transcriptional start sites, one having homology to other Ntr promoters (33, 34). In *S. typhimurium* several amino acid transport functions are known to be under Ntr control including: the histidase (*hisY*), glutamine (*glnH*), and lysine-arginine-ornithine (*argT*) permeases (23). In the non-nitrogen-

fixing strain of *K. aerogenes* studied by Magasanik and co-workers (25) it has been found that urease and the tryptophan permease are under nitrogen control, as well as enzymes involved in degrading histidine (Hut system) and proline (Put system).

Nitrogen regulation in the enteric, nitrogen-fixers.

Enteric free-living nitrogen-fixers such as *K. pneumoniae* share much of the regulatory circuitry modeled in the *E. coli* system (3, 9, 15, 18, 54). They however have additional genes in the Ntr regulon as result of their nitrogen-fixing capability. For example, it has been shown that NtrC is involved indirectly in the activation of *nif* structural genes via the activation of the *nifLA* operon (18, 45, 54). This operon encodes the negative regulatory factor NifL that under anaerobiosis or in the presence of sufficient nitrogen inhibits *nifA* (18, 28). NifA is the direct positive regulatory factor responsible for activating, along with NtrA, the transcription of the *nifHDK* operon and other *nif* genes which encode the components necessary for nitrogen-fixation (5, 15, 29, 35).

Nitrogen regulation in nonenteric, free-living nitrogen-fixers.

Nonenteric, free-living nitrogen-fixers with demonstrated Ntr systems include *Azotobacter vinelandii* (48) and *Rhodopseudomonas capsulata* (21). In *R. capsulata* regulatory genes were isolated that are unable to activate a *nifH::lacZ* fusion (21). Of four such characterized genes one, *nifR1*, was found to be partially homologous to the *E. coli ntrC* gene based on Southern hybridizations. A *ntrC::Tn*5 mutant is Nif- but differs from the typical *ntrC* phenotype in several respects. The *R.*

capsulata ntrC strain is able to use proline, arginine, glutamine, or NH_4 as sole nitrogen sources. In addition, in contrast to *E. coli* where *ntrC* transcript is 10-12 times more abundant in nitrogen-starved than nitrogen-excess cultures, *R. capsulata ntrC* transcript increased only two-fold in derepression conditions (21).

The *A. vinelandii ntrC* gene was isolated by identifying cosmids that complemented *E. coli ntrC* strains (48). A mutant with a *Tn*5 insertion in the putative *ntrC* gene was unable to grow on NO_3^-; nitrate reductase activity was 3-11% of wild type. However, this strain was able to fix nitrogen and was unaltered in glutamine synthetase activity. *A. vinelandii* expresses genes for an alternative nitrogenase when grown without molybdenum. This alternative nitrogenase was also found to be unaffected in the *ntrC* strain (48).

Nitrogen regulation in symbiotic nitrogen-fixers

The *ntrC* gene has been isolated and characterized in the symbiotic nitrogen-fixer *Rhizobium meliloti* (46). This species contains among others, two operons, *nifHDK* and *fixABC*, that require the *nifA* regulatory gene product for transcription under symbiotic conditions (15, 35). In addition, like most members of the *Rhizobiaceae* this organism contains two glutamine synthetases, I and II, encoded by different genes (13). *R. meliloti ntrC*::*Tn*5 insertion mutants were observed for their ability to grow on various amino acids, NH_4SO_4, NO_3^-, for transcription of *glnA*, *nifA*, *nifHD*, *nifB*, and *fixABC* under free-living conditions and for symbiotic phenotype. Unlike *K pneumoniae* the *R. meliloti ntrC* was not required for utilization of histidine or proline. The *ntrC* strain was

also able to grow on urea, glutamine, and NH_4SO_4. However, it was unable to grow on 0.5 mM NO_3^- indicating a similarity to *A. vinelandii* where nitrate reductase activity is decreased in an *ntrC* mutant (46, 48). Unlike the *E. coli* Ntr system (25), *glnA* transcript levels were found to be unaffected in the *ntrC* strain (46), thus providing an explanation for the ability to grow on NH_4SO_4. The *glnII* transcript level was not studied, but GSII activity has been shown to be nitrogen-controlled in *Bradyrhizobia* (1, 2, 10, 11). Different results were obtained regarding *nif* expression depending on if free-living or symbiotic growth conditions were studied. Although nitrogen-deprived, free-living *R. meliloti* have not been shown to fix nitrogen, the *ntrC* gene was found to be required under these conditions for transcription of *nifA*, *nifHDK*, and *fixABC*, (21). The *ntrC* strains were able to nodulate alfalfa plants and showed wildtype levels of nitrogenase activity. Thus, in contrast to *K. pneumoniae* (18), the *R. meliloti ntrC* does not appear to activate *nifA* - at least not during symbiotic nitrogen fixation. Rather, it has been proposed that a symbiosis-specific signal is responsible for *nifA* activation in *Rhizobia* (46). Alternatively, *nifA* transcription may be constitutive or regulated in an *ntrA*-independent manner.

Previous to the work presented here no *ntrC* mutants had been reported in any *Bradyrhizobia*. Nitrogen-regulated control of *glnII* expression under aerobic conditions had been demonstrated (10, 11), however, and GSI had been shown to exist in adenylylated and unadenylylated forms in response to nitrogen conditions (14, 24). These observations suggested that global nitrogen regulatory circuitry existed in the *Bradyrhizobia*.

In this dissertation I present experiments that utilized various *B. japonicum* mutants constructed by gene-directed mutagenesis. These experiments were designed to characterize the regulation of specific *B. japonicum* genes in response to shifts in nitrogen status and oxygen status. Chapter 1 reports the construction of *glnA*, *glnII* and *glnA/glnII* mutants and presents evidence indicating that neither *glnA* or *glnII* plays an essential role in the symbiotic association with soybean. Chapters 2 and 3 report the isolation of a regulatory gene, *ntrC*, and the construction of various mutants designed to elucidate the role of *ntrC* and another regulatory gene, *nifA*, in the control of gene expression in response to nitrogen and oxygen stress. The results of these studies suggest that separate regulatory networks exist in *B. japonicum* free-living and symbiotic cells. These networks are shown to be governed either by nitrogen or oxygen availability. Finally, Chapter 4 presents the isolation and regulation of the *B. japonicum glnB* gene. This gene was found to be regulated by the *ntrC* gene product at tandem promoters in response to nitrogen status, but not oxygen status. The work reported in chapters 1 and 2 has been presented elsewhere (11, 26). Chapters 3 and 4 will be submitted for publication to Molecular and General Genetics and Journal of Bacteriology, respectively.

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Chapter 1

Physiological roles of the two isoforms of glutamine synthetase in ammonia assimilation and symbiotic nitrogen fixation in *Bradyrhizobium japonicum*

INTRODUCTION

During free-living growth *Bradyrhizobium japonicum*, the endosymbiont of soybean, assimilates ammonia primarily by the coordinate activity of glutamine synthetase (GS) and glutamate synthase (3, 23). However, in bacteroids, the symbiotic form of these bacteria, GS activity decreases in concert with the derepression of nitrogenase activity (3). The physiological role of GS during free-living growth, incipient nodule formation and in mature bacteroids is therefore an integral part of the developmental process in *B. japonicum*.

Bacteria of the *Rhizobiaceae* family, including *B. japonicum*, are unusual in that most members contain two GS enzymes GSI and GSII encoded by *glnA* and *glnII*, respectively (6, 12, 8, 9). The *B. japonicum glnA* gene has sequence similarity to the *E. coli glnA* gene and is not appreciably nitrogen-regulated (4). The activity of GSI is post-translationally regulated by reversible adenylylation (8). The GS encoded by *glnII* has sequence similarity with eucaryotic GS's and it has been suggested that GSII was acquired from plants (5). GSII is not known to be post-translationally modified, but is instead transcriptionally regulated in response to nitrogen and carbon source (6, 9, 16) and oxygen concentration (20).

Because most members of the *Rhizobiaceae* can associate with plants it has been speculated that there may be a unique symbiotic role for one or both of the two glutamine synthetases found in rhizobial species (7, 9). Previously the lack of isolated genes has made it difficult to make well-defined mutations at these two loci and thereby rigorously test this hypothesis. I have used gene-directed mutagenesis to develop single *glnA* and *glnII* mutant strains and a double *glnA/glnII* mutant strain. I show here that *glnA* or *glnII* alone is sufficient to provide glutamine prototrophy while the double mutant *glnA/glnII* is a glutamine auxotroph. Both single mutants form effective symbioses with soybean. The double mutant, however, is unable to form nodules on soybean.

MATERIALS AND METHODS

Bacterial strains, plasmids, media and growth conditions. BJ110d is a small-colony derivative of *B. japonicum* 3I1b110 (13). All other bacterial strains and recombinant plasmids 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 (18) was used which had the following composition per liter: KH_2PO_4, 0.3 g; Na_2HPO_4, 0.3 g; $MgSO_4.7H_2O$, 0.12 g. Trace elements were added (18) and the pH was adjusted to 6.8. After autoclaving, 1 ml of a filter-sterilized vitamin solution (13), 0.05 g $CaCl_2$ and 7.5 ml of filter-sterilized 20% (wt/vol) D-xylose were added per liter. The various nitrogen sources were added from filter-sterilized stock solutions at the concentrations indicated in Table 2.

DNA biochemistry. Southern hybridizations and isolation of plasmid DNA were carried out as described previously (1). Total genomic DNA from *B. japonicum* was purified by phenol extraction (17). The isolation of *glnA* and *glnII* has been described (4, 5).

Gene-directed mutagenesis. Recombinant plasmids were manipulated in vitro and in *E. coli* to construct mutant alleles of the *glnA* and *glnII* loci (Table 1, Figure 1). The mutant allele of the *glnA* gene was constructed by deletion of the *Eco*RI to *Bam*HI region indicated in Figure 1 and replacement of this region with the *Hind*III Omega fragment from the plasmid pHC45 (19) to which *Eco*RI-*Hind*III and *Hind*III-*Bam*HI adaptors (from pKC7; [21]) had been added. This fragment confers spectinomycin resistance upon *B. japonicum*. The mutant allele of the *glnII* gene was constructed by deletion of the *Xho*I fragment indicated in Figure 1 and replacement of this region with a *Sal*I fragment containing the *nptII* gene from plasmid pRL161 (25). This fragment confers kanamycin resistance upon *B. japonicum*. The plasmids carrying the mutant alleles were conjugally transferred from *E. coli* to *B. japonicum* by methods previously described (13). 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 (13). 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. In the case of the double mutations, the confirmed *glnII* mutant strain, BJ2513, was

Table 1. Bacteria strains and plasmids.

Strain or plasmid	Description	Source or reference
BJ110d	Wild type	(13)
BJ2841	*glnA*::spc; *glnA* mutant, Spc^r	This work
BJ2513	*glnII*::*nptII*; *glnII* mutant, Kan^r	This work
GM3-21	*glnA*::spc, *glnII*::*nptII*; *glnA/glnII* mutant, Spc^r, Kan^r	This work
Plasmids		
pBJ196	Ap^r; *glnII* locus cloned into pBR322	(5)
pBJ53A	Ap^r; *glnA* locus cloned into pUC8	(4)
pBJ251	Kan^r; *glnII* deletion allele cloned into pBR322	This work
pBJ284	Spc^r; *glnA* deletion allele cloned into pKC7	This work
pDS4101	Ap^r; derivative of ColK	(25)
pHC45	Spc^r, source of Spc cassette	(21)
pRL161	Kan^r, Source of *nptII* cassette	P. Wolk and J. Elhai (unpublished)
pBR322	Ap^r, Tc^r; vector	(2)
pKC7	Km^r, Ap^r; vector	(21)

processed through a second mating round using an *E. coli* strain carrying the plasmids pBJ284 and pDS4101 (see Table 1) to mutate the *glnA* gene.

Plant inoculation and growth conditions. Soybean seeds (*Glycine max* [L.] Merr. cv. Amsoy 71) were sterilized as described previously (13) 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 (24) using the nitrogen-free medium of Johnson et al. (15). The plants were grown with a 16 hr daily light period, day/night temperatures of 28/25^{o}C, a relative humidity of 60%, and a photon fluence rate of 300 umol$\cdot$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 (13, 14).

RESULTS AND DISCUSSION

Construction of *B. japonicum glnA*, *glnII* and *glnA/glnII* mutants. Since most members of the family *Rhizobiaceae* contain two forms of glutamine synthetase the function of these two enzymes has been a matter of speculation (e.g. 7, 8, 9, 16). In order to determine if both enzymes are capable of fulfilling the requirement for the biosynthesis of glutamine, we used the isolated *glnA* and *glnII* genes to construct mutant *B. japonicum* strains that were missing either one, or both of the enzymes GSI and GSII (see Materials and Methods; Figure 1). The resulting three derivatives of the wildtype strain BJ110 were BJ2841, *glnA*; BJ2513, *glnII*; and GM3-21, *glnA/glnII* (Table 1).

Figure 1. Physical maps of the *glnA* and *glnII* wildtype and mutant alleles. The restriction maps of the *glnA* and *glnII* gene regions are shown with endonuclease sites indicated as follows: *Bam*HI (B), *Sal*I (S), *Eco*RI (E), *Hin*dIII (H), and *Xho*I (X). The coding regions of the genes are indicated by the boxed regions containing arrowheads with transcription for both genes from left to right as drawn. The promoters for both genes are indicated (P).

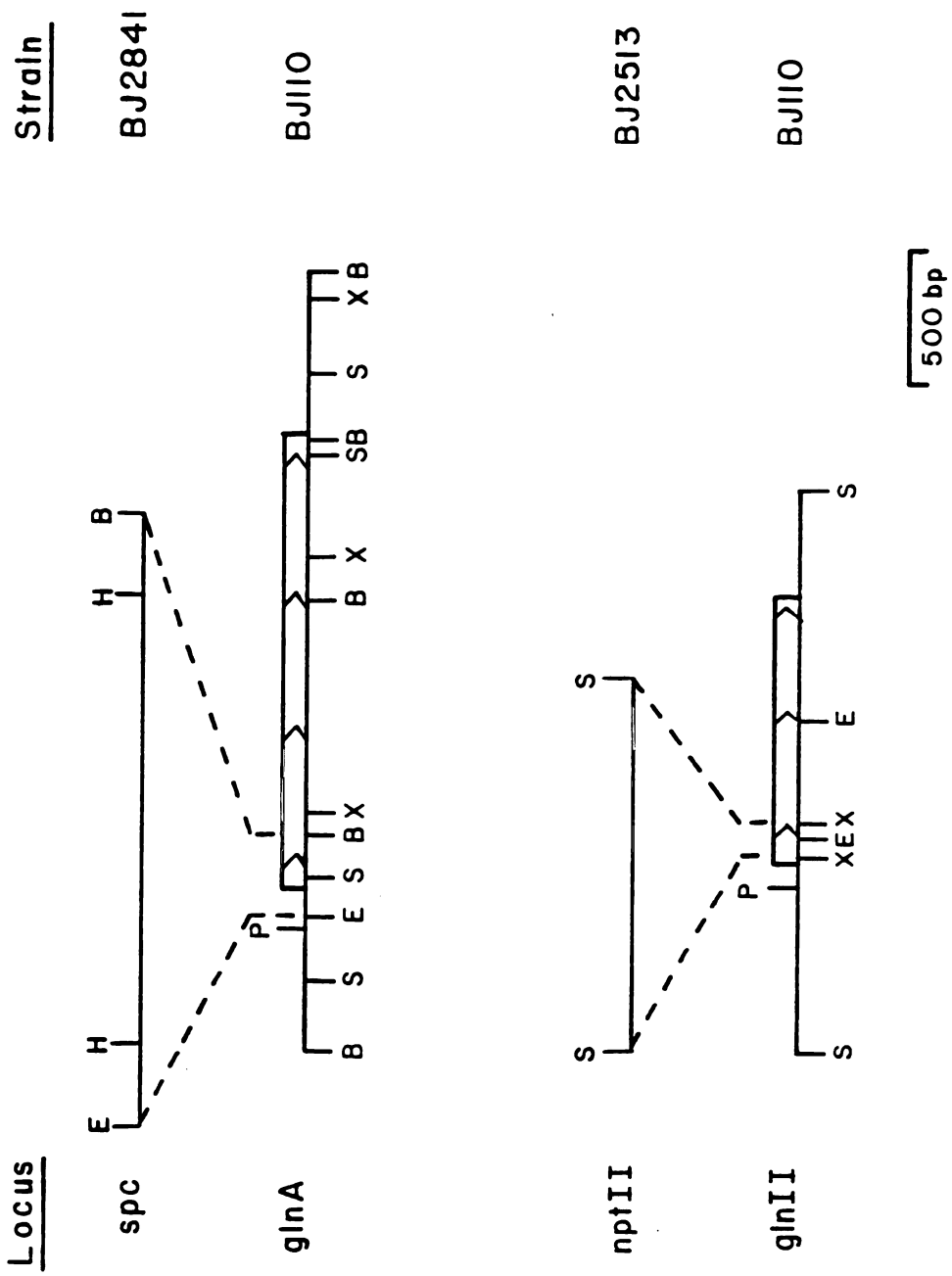
Strain
BJ2841
BJ110
BJ2513
BJ110
Locus
spc
glnA
nptII
glnII
E H H B
P
B S E S B X
B X
S B
S X B
S S
P
S X E X E
S S
500 bp

Figure 1.

Growth of *B. japonicum glnA, glnII* and *glnA/glnII* mutant strains on various nitrogen sources. The growth properties of the glutamine synthetase deficient strains are summarized in Table 2. Both the *glnA* (BJ2841) and *glnII* (BJ2513) single mutant strains were glutamine prototrophs and were able to utilize nitrate, and ammonia as sole nitrogen sources. The *glnA/glnII* double mutant strain (GM3-21) was unable to grow on ammonium or nitrate as sole nitrogen sources and could be rescued by exogenous glutamine indicating glutamine auxotrophy (Table 2). The growth of GM3-21 in the presence of exogenous glutamine was not as good as for a glutamine prototroph indicating that there might be an additional defect, possibly in the transport of glutamine. This decreased growth was not investigated further.

Symbiotic properties of *B. japonicum glnA, glnII* and *glnA/glnII* mutant strains. Both the *glnA* (BJ2841) and *glnII* (BJ2513) strains were able to effectively nodulate soybeans but surprisingly they induced greater numbers of nodules per plant than the wildtype (BJ110d) parental strain (Table 3). The reason for this hypernodulation is unknown. Higher levels of nitrogen fixation ability as assayed by acetylene reduction activity were also found for the plants inoculated with these two mutant strains. The *glnA glnII* double mutant (GM3-21) was unable to nodulate soybeans (Table 3). We found that the addition of glutamine to the potting medium resulted in the partial rescue of nodulation by the double mutant, yielding small white Fix$^-$ nodules. Unless there are polar effects of the constructed alleles on genes which are as yet uncharacterized, these results taken together indicate that GSI and GSII

Table 2. Growth properties of *B. japonicum gln* mutants.[a]

Strain	Nitrogen Source[b]		
	Glutamine	Ammonium	Nitrate
BJ110d (wt)	++	++	++
BJ2841 (*glnA*)	++	++	++
BJ2513 (*glnII*)	++	++	++
GM3-21 (*glnA/glnII*)	+	-	-

[a]Growth was determined in minimal media (18), both in liquid and on plates, and is indicated as follows: ++, very good; +, good; -, none.

[b]Both glutamine and ammonium chloride were tested at 10 mM. Potassium nitrate was tested at a variety of concentrations within the range of 0.05 to 2 mM; the results were the same at all concentrations.

Table 3. Symbiotic phenotypes *B. japonicum gln* mutants.[a]

Strain	Number of nodules[a] (Total wet weight/ plant, g.)	Acetylene reduction[b] (umol of C_2H_4/hour per plant root system)
BJ110 (wt)	74.0 ± 7.8 (1.61 ± 0.28)	24.0 ± 3.1
BJ2841 (*glnA*)	90.3 ± 14.2 (1.51 ± 0.24)	40.6 ± 1.5
BJ2513 (*glnII*)	116.0 ± 11.4 (1.82 ± 0.3)	36.8 ± 2.1
GM3-21 (*glnA/glnII*)	0	0

[a]The number of nodules per plant and, in parentheses, the total wet weight of nodules per plant in grams, respectively, are indicated. The numbers represent the mean +/- SEM of 3 nodulation tests each.

[b]Nitrogen fixation activity was measured by the acetylene reduction assay as described previously (13, 14). Activity is reported as umoles of ethylene produced per hour per plant root system. The numbers represent the mean +/- SEM of the analyses of three plants each.

are the only two glutamine synthetases in *B. japonicum* which are active in glutamine biosynthesis under standard laboratory conditions and in symbiotic cells.

Recently there have been reports of glutamine auxotrophs in other rhizobial species: *Agrobacterium tumefaciens* (22), *Azorhizobium caulinodans* (10), and *Rhizobium meliloti* (11). In each of these cases the isolated genes were used, as in this work, to make defined mutations in the gene(s) for glutamine synthetase. The *A. tumefaciens* and *A. caulinodans* glutamine auxotrophs were both found to be defective in association with their host plants (10, 22). A *R. meliloti glnA/glnII* mutant, however, was a glutamine auxotroph but still formed wild-type nodules on alfalfa plants (11). A possible explanation for these contrasting results is that the plant partners in the associations may differ in their ability to provide glutamine to their bacterial symbiont.

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Chapter 2

Role of the *Bradyrhizobium japonicum ntrC* Gene Product in Differential Regulation of the Glutamine Synthetase II gene, *glnII*.

SUMMARY

The *ntrC* gene was isolated from *Bradyrhizobium japonicum*, the endosymbiont of soybean (*Glycine max*), and its role in regulating nitrogen assimilation was examined. Two independent *ntrC* mutants were constructed by gene replacement techniques. One mutant was unable to produce NtrC protein, while the other constitutively produced a stable, truncated NtrC protein that lacked a postulated DNA-binding region. Both *ntrC* mutants were unable to utilize potassium nitrate as a sole nitrogen source. In contrast to wild-type *B. japonicum*, the NtrC null mutant lacked *glnII* transcript in aerobic, nitrogen-starved cultures. However, the truncated-NtrC mutant expressed *glnII* under both nitrogen-starved and nitrogen-excess cultures. Both mutants expressed *glnII* under oxygen-limited culture conditions and in symbiotic cells. These results suggest that nitrogen assimilation in *B. japonicum* is regulated in response to both nitrogen limitation and oxygen limitation and that separate regulatory networks exist in free-living and symbiotic cells.

INTRODUCTION

Free-living *Bradyrhizobium japonicum* cells assimilate ammonia primarily by the coordinate activity of glutamine synthetase (GS) and glutamate synthase (6, 51). However, in bacteroids, the symbiotic form

of these bacteria, GS activity decreases in concert with the derepression of nitrogenase activity (6, 50). The regulation of nitrogen assimilation pathways is therefore an integral part of the bacterial developmental process and the symbiotic interaction.

In the *Enterobacteriaceae*, genes involved in nitrogen metabolism are coordinately regulated by the Ntr system (22, 30, references below). Genes regulated by the Ntr system have a unique promoter that bears no homology to the canonical *Escherichia coli* promoter (15, 37). These genes also have protein-binding sequences that display dyad symmetry (4). Transcriptional activation of Ntr promoters requires RNA polymerase core, an alternative sigma factor (54) which is the product of the *ntrA* gene (NtrA), and the regulatory protein NtrC encoded by the *ntrC* (*glnG*) gene (24, 25, 33). NtrC has sequence-specific DNA binding activity (4, 42, 49) that has been implicated in transcriptional activation (42), repression (23, 49) and antitermination (4). NtrC can be phosphorylated by the *ntrB* gene product which converts NtrC to its promoter activating form (34). In the *Enterobacteriaceae*, *glnA*, the only gene encoding glutamine synthetase, is expressed from two promoters (16, 41). Expression from the upstream promoter, *glnA*p1 (an *E. coli* consensus-type promoter) occurs under conditions of carbon deficiency and nitrogen excess and is repressed by NtrC (16, 41). Expression from the downstream promoter, *glnA*p2, requires NtrC and occurs under conditions of nitrogen limitation (16, 41). Bacteria of the *Rhizobiaceae*, including *B. japonicum*, are distinct from the *Enterobactericeae* in that most members contain two glutamine synthetase enzymes, GSI and GSII, encoded by *glnA* and *glnII* respectively (7, 8, 12,

13). Interestingly, the dual promoter control of *glnA* seen in the *Enterobactericeae* is replaced in *B. japonicum* with two genes coding for GS which are differentially regulated (9). The *B. japonicum glnA* gene is homologous to the *E. coli glnA* gene and is not appreciably nitrogen regulated (8, 9). In contrast, the GS encoded by *glnII* shares homology with eucaryotic glutamine synthetases, responds to nitrogen availability, has an Ntr-type promoter, and has sequence elements upstream that are homologous to the *Enterobacteriaceae* NtrC binding sequence (7, 9, T.A. Carlson, Ph.D. dissertation, Michigan State University, 1986).

Several lines of evidence suggest the presence of an Ntr system in the *Rhizobiaceae* (35, 43, 45). The isolation of *ntrC* genes has been reported in *Rhizobium meliloti* (45), *Bradyrhizobium parasponiae* (35), *Agrobacterium tumefaciens* (43), and *Azorhizobium caulinodans* (38). *A. caulinodans ntrC* mutants are affected in histidine and arginine utilization, in free-living nitrogen fixation and in their ability to form Fix^+ nodules (38). *A. tumefaciens ntrC* mutants are unable to grow on nitrate, lack GSII activity, and are unable to use arginine unless the T_i-encoded arginine catabolism pathway is induced (43). In *R. meliloti*, *ntrC* is required for growth on nitrate and for the *ex planta*, but not symbiotic, transcription of several nif genes and the *fixABC* operon (45). However, neither *R. meliloti* or *A. tumefaciens ntrC* mutants are affected in their ability to interact normally with their plant hosts (43, 45). Because of the differential regulation of *nif* genes *ex planta* and symbiotically, Szeto et al. (45) postulated a dual regulatory system governing nitrogen utilization in the *Rhizobiaceae*.

This model posits that during aerobic nitrogen-limited growth, NtrC activates the transcription of genes required for nitrogen utilization but that during symbiotic growth another activator, the *nifA* gene product, regulates the expression of these genes.

In this chapter I show that the *B. japonicum ntrC* gene product is required for the transcriptional regulation of *glnII* under conditions of nitrogen starved aerobic growth. Under microaerobic conditions and during symbiosis, however, *glnII* is expressed but does not require the *ntrC* gene product. Therefore *glnII* appears to be under the control of a dual regulatory system responding to nitrogen starvation and oxygen limitation.

MATERIALS AND METHODS

Bacterial strains, plasmids, media and growth conditions. BJ110d is a small-colony derivative of *B. japonicum* 3I1b110 isolated as described (21). All other bacterial strains and recombinant plasmids are listed in Table 1.

The complex media were YEM (0.2% yeast extract, 1% mannitol, 3 mM K_2HPO_4, 0.8 mM $MgSO_4$, 1.1 mM NaCl) and YEMAN (YEM with 10 mM NH_4Cl, 0.5 mM KNO_3). To check growth on various nitrogen sources, a mineral salts base medium adapted from O'Gara and Shanmugan (36) was used which had the following composition per liter: KH_2PO_4, 0.3 g; Na_2HPO_4, 0.3 g; $MgSO_4.7H_2O$, 0.12 g. Trace elements were added (36) and the pH was adjusted to 6.8. After autoclaving, 1 ml of a filter-sterilized vitamin soluion (21), 0.05 g $CaCl_2$ and 7.5 ml of filter-sterilized 20% (wt/vol) D-xylose were added per liter. For growth observations, the various

Table 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
B. japonicum strains		
BJ110d	Wild-type	(21)
BJ27147	*ntrC::nptII*, null mutant; Km^r	This study
BJ3028	*ntrC::nptII*, truncated NtrC; Km^r	This study
Plasmids (phages)		
M13mp18		(54)
M13*glnA*	391 bp *Sal*I fragment from *B. japonicum glnA* promoter (8) region cloned into M13mp18	(2)
M13*glnII*	2.1 kbp *Sal*I fragment from *B. japonicum glnII* promoter (7) region cloned into M13mp18	(2)
pBN386	Ap^r; 1.7 kbp *Eco*RI fragment containing *B. parasponiae ntrC* in pSP64CS (18)	B. T. Nixon
pRJcos4-84	Tc^r; cosmid clone (in pLAFRI [20]) containing *B. japonicum ntrC* DNA	This study
pBJ262	Ap^r; 4.6 kbp *Bgl*II *B. japonicum ntrC* fragment cloned into the *Bgl*II site of pIC19R (32)	This study

continued . . .

Table 1 (continued)

pBJ312	Ap^r; 5.1 kbp *XhoI B. japonicum ntrBC* fragment cloned into the *XhoI* site of pIC19R (32)	This study
pBJ271	Ap^r, Km^r; 1.7 kbp *SalI B. japonicum ntrC* fragment cloned into the *XhoI* site and 1.3 kbp *EcoRI B. japonicum ntrC* downstream fragment cloned into the *EcoRI* site of pKC7 (39)	This study
pBJ302	Ap^r, Km^r; 2.7 kbp *B. japonicum ntrC* fragment cloned into the *EcoRI* site of pBR322-S (see below). *nptII* gene contained in cassette CK.1 (19) inserted into the *SalI* site of *ntrC*	This study
pBR322-S	pBR322 with the *SalI* site removed	J. S.-Craig
pDS4101	Ap^r; derivative of ColK	(53)
pRK2013	Km^r; carries conjugal transfer genes from RK2	(14)

nitrogen sources were added at 0.5 mM from filter-sterilized stock solutions. For analyzing gene expression in aerobic cultures the mineral salts basal medium was used with either 10 mM glutamate added (XG) or 10 mM glutamate plus 10 mM NH_4Cl (XGA).

For microaerobic growth experiments ten-liter cultures of *B. japonicum* were grown in Microferm fermentors (New Brunswick Scientific) agitated at 200 r.p.m. and sparged with 0.2% O_2 and 99.8% N_2 at a rate of 500 ml min-1. Gas flow rate was controlled using thermal mass flowmeters (Brooks Instruments, model 5850 C). Each 10 liter culture was inoculated with 50 ml of a stationary-phase aerobic culture in YEM medium. All *B. japonicum* cultures were grown at 30°C.

DNA biochemistry. Southern hybridizations, colony hybridizations and isolation of plasmid DNA were carried out as described (3). Total genomic DNA from *B. japonicum* was purified by phenol extraction (31). A *B. japonicum* cosmid library constructed in pLAFR1 (20) and described by Adams et al. (3) was screened using a ^{32}P-labeled 1.7 kbp *Eco*RI fragment purified from the recombinant plasmid pBN386 (Table 1; pBN386 was generously provided by B. T. Nixon). This 1.7 kbp *Eco*RI fragment contains 1.4 kbp of the *B. parasponiae ntrC* gene (35).

Gene-directed mutagenesis. Recombinant plasmids were manipulated in vitro and in *E. coli* to construct mutant alleles of *ntrC* (Table 1). The plasmid (pBJ302) used in the construction of BJ3028 was formed by first inserting the 2.7 kbp *Eco*RI fragment from pBJ262 into the *Eco*RI site of pBR322-S (Table 1). Into the *Sal*I site of *ntrC* in this plasmid

was inserted a 1.35 kbp *Sal*I fragment containing the *nptII* gene from *Tn*5. This *nptII*-containing *Sal*I fragment was obtained by inserting the cassette C.K1 (19) with *Bam*HI ends into the *Bam*HI site of L.HEH1 (19) and then removing the cassette as a *Sal*I fragment.

The plasmid (pBJ271) used in the construction of BJ27147 was formed by inserting the 1.7 kbp *Sal*I fragment from pBJ312 into the *Xho*I site of plasmid pKC7 (39). A 1.3 kbp *Eco*RI fragment from downstream of *ntrC* was inserted into the *Eco*RI site of the pKC7 recombinant plasmid but this fragment was not involved in the homologous recombination event resulting in BJ27147.

Each mutant plasmid was transformed into HB101 carrying pDS4101 for use as the donor strain in a conjugal mating with *B. japonicum*. The mating procedure was as described previously (21) using *B. japonicum* 110d as the recipient, HB101(pRK2013), and either HB101(pBJ302, pDS4101) or HB101(pBJ271, pDS4101) as the donor. The cells were spread on YEM plates supplemented with 10 mM glutamine. After incubation for 4 days at 30^{o}C, the cells were removed from the plates by suspension in 10 ml 0.01% Tween 80 and 0.2 ml was plated on YEM plates containing kanamycin (150 ug/ml) and chloramphenicol (30 ug/ml). Colonies from the selection plates were screened by colony hybridization. Southern hybridizations of restriction endonuclease digested genomic DNA were used to confirm the constructions.

RNA biochemistry. *B. japonicum* cells (1 g) were suspended in 10 ml of 4.0% sarkosyl, 0.1 M Tris-HCl, pH 8.0 and passed two times through a French pressure cell (Aminco; Silver Spring, MD) at 12,000 psi. For

each milliliter of broken cell solution 1 g CsCl was added. RNA was isolated by discontinuous CsCl gradient centrifugation as described previously (3, 10). The RNA pellet was drained thoroughly, redissolved in H_2O and then extracted twice with phenol and four times with diethyl ether. Following ethanol precipitation, the RNA was resuspended in sterile H_2O and stored in aliquots at -70°C.

Quantitative S1 nuclease protection assays. Levels of transcript were quantitated by the S1 nuclease protection method adapted from Berk and Sharp (5) and described previously (2) in which both *glnA* and *glnII* mRNAs could be quantified in a single reaction. Five micrograms of total cellular RNA was used per lane. Single-stranded 5' end-labelled probes were synthesized by primer extension (2) using oligonucleotide primers described previously (2). The recombinant M13 phages used in synthesizing the single-stranded DNA hybridization probes are described in Table 1. The partially protected fragments from S1 analyses with each of the probes are 125 (*glnA*) and 170 (*glnII*) nucleotides long. Hybridization, S1 digestion, and identification of partially protected products were as described previously (2).

Immunoblotting. *B. japonicum* cells were harvested by centrifugation then resuspended in 10% sucrose, 20 mM Tris-HCl pH 7.5. Lysates were prepared by passing the cells twice through a French pressure cell at 16,000 psi. Cell debris was pelleted by centrifugation and discarded. Proteins in the cleared lysate were precipitated by adding 2 vol of saturated ammonium sulfate, chilling on ice 30 min and centrifuging for

10 min at 12,000 g. The proteins were separated by SDS-PAGE (27) and then transferred to cellulose nitrate paper (47). The immunoblot development procedure was a modification of the method of Tsang et al. (48) as described by Darr et al. (11) except for the following changes: 3% non-fat dry milk was used instead of BSA as the blocking agent, the concentration of Tween-20 in the wash buffer was 0.05% (vol/vol), and the bound immunoglobulin was detected using a 1 ug/ml solution of *Staphylococcus aureus* protein A-alkaline phosphatase conjugate (Sigma). The method of Leary et al. was used for the color development (28). Polyclonal antiserum prepared against the *E. coli ntrC* (*glnG*) gene product (40) was generously provided by L. Reitzer and B. Magasanik.

Plant inoculation, growth conditions and nodule bacteria isolation. Soybean seeds (*Glycine max* [L.] Merr. cv. Amsoy 71) were sterilized as described previously (21) 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 (52) using the nitrogen-free medium of Johnson et al. (26). The plants were grown with a 16 hr daily light period, day/night temperatures of 28/25^{o}C, a relative humidity of 60%, and a photon fluence rate of 300 umol.m-2.sec-1. All plants were harvested at 28 days and the nodules were removed, rinsed with H_2O, counted, weighed and either used in acetylene reduction assays or immediately frozen at -70^{o}C for later RNA purification. *B. japonicum* cells were isolated from soybean nodules as described previously (1). The cells were used to isolate RNA as described above.

RESULTS

Isolation of the *B. japonicum ntrC* gene. To determine whether *B. japonicum* contains an *ntrC*-like gene, total BJ110d DNA was examined by Southern hybridization using a ^{32}P-labeled 1.7 kbp *Eco*RI fragment from pBN386 (this *Eco*RI fragment contains 1.4 kbp of the *B. parasponiae ntrC* gene; 35). A strong hybridization signal was detected at 5.1 kbp when BJ110d DNA was digested with *Xho*I (Figure 1, lane 1). The probe was therefore used to screen a *B. japonicum* cosmid library described previously (3). Five cosmids were identified that contained *ntrC*-homologous sequences (pRJcos4-84, pRJcos5-90, pRJcos9-9, pRJcos10-62, and pRJcos11-89). These cosmids were found to share a common 5.1 kbp *Xho*I fragment that was subsequently determined to span the entire *ntrBC* operon. One cosmid, pRJcos4-84, was used to subclone a 4.6 kbp *Bgl*II fragment (pBJ262) and the 5.1 kbp *Xho*I (pBJ312) for use in restriction mapping and in the construction of mutant alleles. A partial restriction map of the *ntrC*-homologous region in BJ110d is shown in Figure 2.

In *E. coli*, *Klebsiella pneumoniae*, *R. meliloti*, and *B. parasponiae* the nitrogen regulatory gene responsible for NtrC phosphorylation, *ntrB*, is situated directly upstream of the *ntrC* gene (29, 30, 35, 45). To determine whether an *ntrB* lies upstream of the *B. japonicum ntrC* gene a partial DNA sequence was determined for the *ntrC*-homologous region and a region one kilobase upstream of the presumed *ntrC* gene (data not shown). The 400 bp sequenced from the *B. japonicum ntrC* region was 86% homologous to *B. parasponiae ntrC* (35) and 61% homologous to *R. meliloti ntrC* (45) at the nucleic acid level. The 350 bp sequenced from the

Figure 1. Hybridization of *B. japonicum* genomic DNA to *B. parasponiae ntrC* DNA. Left: Ethidium bromide stained 0.7% agarose gel of *Xho*I digested total DNA. Right: Autoradiogram obtained after Southern transfer and hybridization with the 1.7 kbp *Eco*RI fragment from pBN386 corresponding to *ntrC* (35). Lanes: 1, BJ110d (wild-type); 2, BJ27147 (*ntrC* mutant); and 3, BJ3028 (*ntrC* mutant).

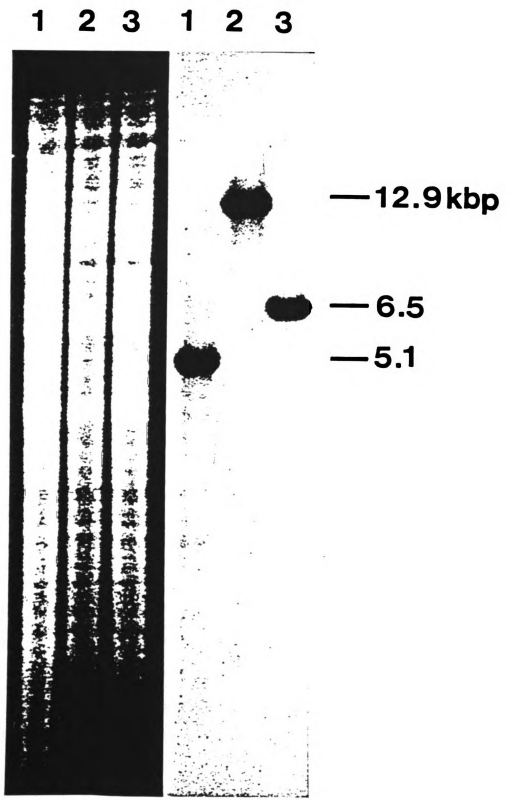

Figure 1.

region upstream of the *B. japonicum ntrC* was 87% homologous to *B. parasponiae ntrB* (35) and 75% homologous to the comparable region in *R. meliloti ntrB* (45). These data along with several conserved restriction sites with *B. parasponiae* confirmed the presence of an upstream *ntrB* and allowed us to localize the *B. japonicum ntrBC* operon within the isolated sequence (Figure 2).

Generation of *B. japonicum ntrC* mutants. The recombinant plasmids pBJ271 and pBJ302 (see Materials and Methods) were conjugated into wild-type *B. japonicum* (BJ110d) and recombined into the BJ110d genome. Two independent mutants, BJ27147 and BJ3028, were isolated and confirmed by Southern hybridization (Figure 1, lanes 2 and 3; Figure 2).

The mutant BJ3028 was formed as the result of a double crossover event such that the *ntrC* genomic region in this mutant now carries the *nptII* gene inserted into a *Sal*I site in the opposite orientation to the *ntrC* gene (Figure 2).

The mutant BJ27147 was formed as the result of a single crossover event in the *Sal*I 1.7 kbp fragment that was used in the construction of the recombinant plasmid pBJ271. Since this *Sal*I fragment is completely internal to the *ntrBC* operon the single crossover event resulted in the partial duplication of the operon (Figure 2). BJ27147 thus carries a copy of the operon that has a 3'-truncated *ntrC* gene and a copy that has a 5'-truncated *ntrB* gene. Since the *B. japonicum ntrC* is probably transcribed from a promoter located upstream of *ntrB*, I expected little, if any, NtrC protein to be produced in this mutant (see next section).

Figure 2. Physical map of the *B. japonicum ntrBC* region in wild-type BJ110d and the *ntrC* mutants BJ27147 and BJ3028. The locations of the *ntrB* and *ntrC* genes are indicated and transcription is from right to left. The insertion location and orientation of the *nptII* gene is indicated for strains BJ27147 and BJ3028. The cross-hatched region in the map of BJ27147 represents 4.1-kbp of pKC7 vector DNA and the bold-lined regions indicate the approximate boundaries of the DNA that was carried on pBJ271 (see Materials and Methods). Restriction endonuclease sites: B, *Bgl*II; E, *Eco*RI; N, *Nru*I, S, *Sal*I; X, *Xho*I.

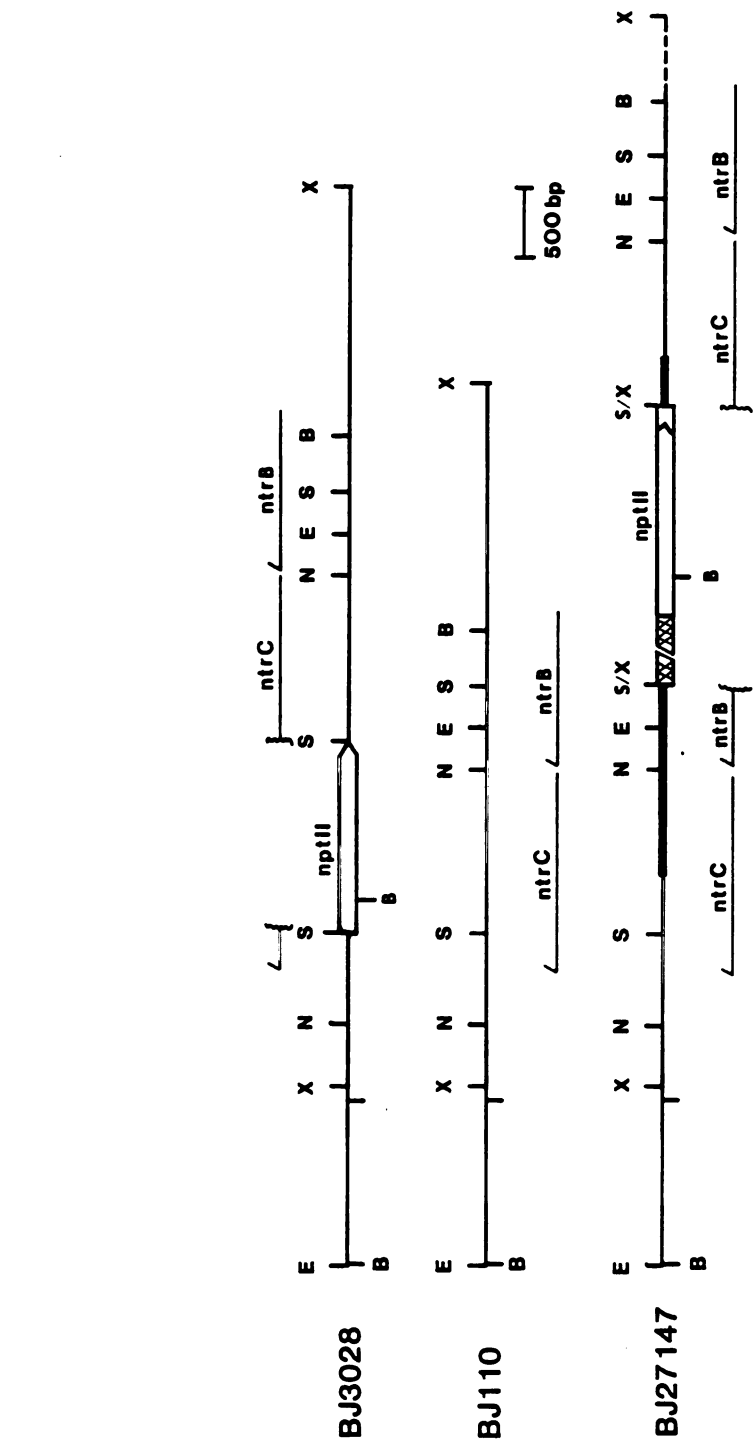

Figure 2.

Detection of the NtrC protein by immunoblotting. Since both BJ27147 and BJ3028 carried an insertion near the 3' end of the *ntrC* gene it was possible that a truncated NtrC protein might still be produced. To investigate this possibility an antiserum raised against the *E. coli* NRI (NtrC) protein was used in immunoblot experiments. Extracts were prepared from cells grown in aerobic minimal media cultures that were either nitrogen-starved (XG) or nitrogen-surfeited (XGA), the proteins were separated by SDS-PAGE, and transferred to cellulose nitrate paper. In BJ110d (wildtype) a band that migrated with an apparent molecular weight of 59 kd reacted with the NtrC antiserum (Figure 3, lanes 5 and 6). (*B. parasponiae* NtrC is 53 kd based on DNA sequence, [35]). No strongly reacting protein to the NtrC antiserum was detected in the mutant BJ27147 (Figure 3, lanes 3 and 4). The mutant BJ3028 produced a protein that reacted with the *E. coli* NtrC antiserum but this protein had a smaller apparent molecular weight (57 kd) than that of wild-type *B. japonicum* (Figure 3, lanes 1 and 2). Although both BJ27147 and BJ3028 carried insertions at the same point in the *ntrC* gene, the *nptII* cassettes were from different origins and thus the cassette sequence directly adjoining the *ntrC* sequence was different in each mutant (Figure 2). Apparently this difference is responsible for the formation of a stable truncated protein in one mutant (BJ3028) but not in the other (BJ27147). Finally, a band at approximately 56 kd was detected in all strains and thus did not appear to be associated with the *B. japonicum ntrC* locus reported here.

Measurements of the intracellular concentration of NtrC protein in *E. coli* using immunological techniques have revealed 70 molecules of NtrC

Figure 3. Immunoblot analysis of the wild type and the two *ntrC* mutants. Samples of cell extracts from *B. japonicum* grown under nitrogen-excess (+) or nitrogen-starved (-) conditions were separated by SDS-PAGE, transferred to cellulose nitrate and probed with antiserum prepared against the *E. coli ntrC* gene product. Each lane contains 200 ug of total protein. Lanes: 1 and 2, BJ3028; 3 and 4, BJ27147; 5 and 6, BJ110d.

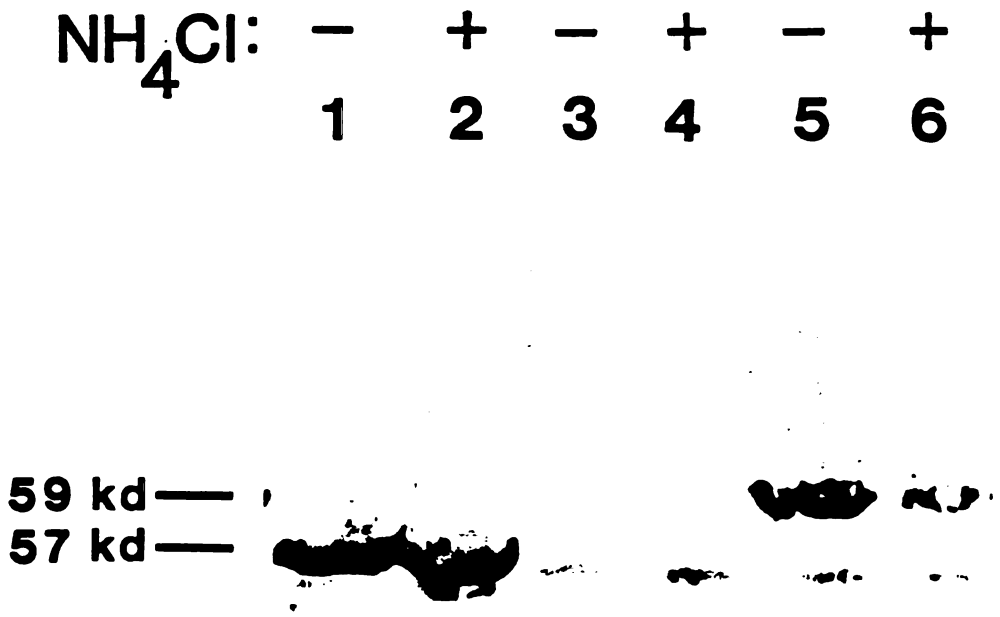

Figure 3.

dimers per cell in nitrogen-limited cultures and only 5 molecules per cell in nitrogen-excess medium (40). This ratio of 14:1 agrees with gene fusion data (44). My analysis of the BJ110d wild-type NtrC levels also detected a substantial increase in protein abundance under nitrogen-limiting conditions (Figure 3, lanes 5 and 6). However, this increase was not seen in the BJ3028 mutant. Rather, an NtrC abundance roughly equal to the wild-type nitrogen-limited cultures was detected in both nitrogen-excess and nitrogen-starved cultures of the BJ3028 mutant (Figure 3, lanes 1 and 2).

In summary, the data presented in this section indicate that BJ27147 is a null mutant lacking NtrC and that BJ3028 is a constitutive producer of a truncated NtrC protein.

Growth of *B. japonicum ntrC::nptII* mutants on various nitrogen sources. It has been reported in several other species that NtrC is required for the utilization of nitrate. (43, 45, 46). Both BJ27147 and BJ3028 were unable to utilize potassium nitrate as the sole nitrogen source. The growth rates of BJ27147 and BJ3028 were the same as wild-type *B. japonicum* when grown in minimal medium with ammonium, glutamine, glutamate, proline, arginine, histidine or aspartate as the sole nitrogen source. These results indicate that the *B. japonicum ntrC* is similar to the *ntrC* genes reported for *R. meliloti* and *A. tumefaciens* and is unlike the *A. caulinodans ntrC* and the *Enterobacteriaceae ntrC* which are required for the utilization of several amino acids as sole nitrogen sources (30, 38).

Transcription of *B. japonicum glnA* and *glnII* genes in *ntrC::nptII* mutants grown in aerobic cultures. Previous work in our laboratory indicated that in aerobic cultures *glnII*, but not *glnA*, was transcriptionally regulated in response to nitrogen availability (9). To determine the role, if any, of the *B. japonicum ntrC* gene in this regulation, S1 nuclease protection anaylses were carried out using primer extended probes specific to *glnA* and *glnII*. RNA was purified from cells grown in aerobic minimal media cultures as those described for the immunoblot analyses. As expected from earlier work (9), the *glnA* transcript level did not vary significantly in response to nitrogen availability (Figure 4). In contrast, in wild-type *B. japonicum*, the *glnII* transcript abundance was barely detectable in nitrogen-excess cultures but was induced about 40-fold in nitrogen-limited cultures (Figure 4). In strain BJ27147, no *glnII* transcript was apparent in either nitrogen-limited or nitrogen-excess cultures. This result indicated that the *ntrC* gene product is involved in the transcriptional activation of *glnII* under aerobic nitrogen-limited conditions. In strain BJ3028, *glnII* transcript was present under both nitrogen-limited and nitrogen-excessculture conditions indicating that constitutive expresssion of NtrC affects *glnII* expression (Figure 4; see discussion).

Transcription of *B. japonicum glnA* and *glnII* genes in *ntrC::nptII* mutants grown in nitrogen-excess microaerobic cultures. Earlier studies in our laboratory with *B. japonicum* demonstrated that in nitrogen-excess cultures (YEM plus 10 mM KNO3 or YEM plus 10 mMKNO3, 10 mM NH4Cl) *glnII* transcription could be induced by decreasing the oxygen concentration to

Figure 4. Expression of *B. japonicum glnA* and *glnII* genes in aerobic cultures. Samples of RNA purified from BJ110d, BJ27147 or BJ3028 grown under nitrogen-excess (+) or nitrogen starved (-) aerobic cultures were hybridized to ^{32}P-labeled single-stranded DNA probes specific for the 5' ends of *glnA* or *glnII* and analysed by the S1 nuclease protection method (see Material and Methods). The migration positions of the protected fragments (P_{glnA} and P_{glnII}) are indicated. The other radioactive bands represent residual undigested probe DNA. Lanes: 1 and 2, BJ110d; 3 and 4, BJ3028; 5 and 6, BJ27147.

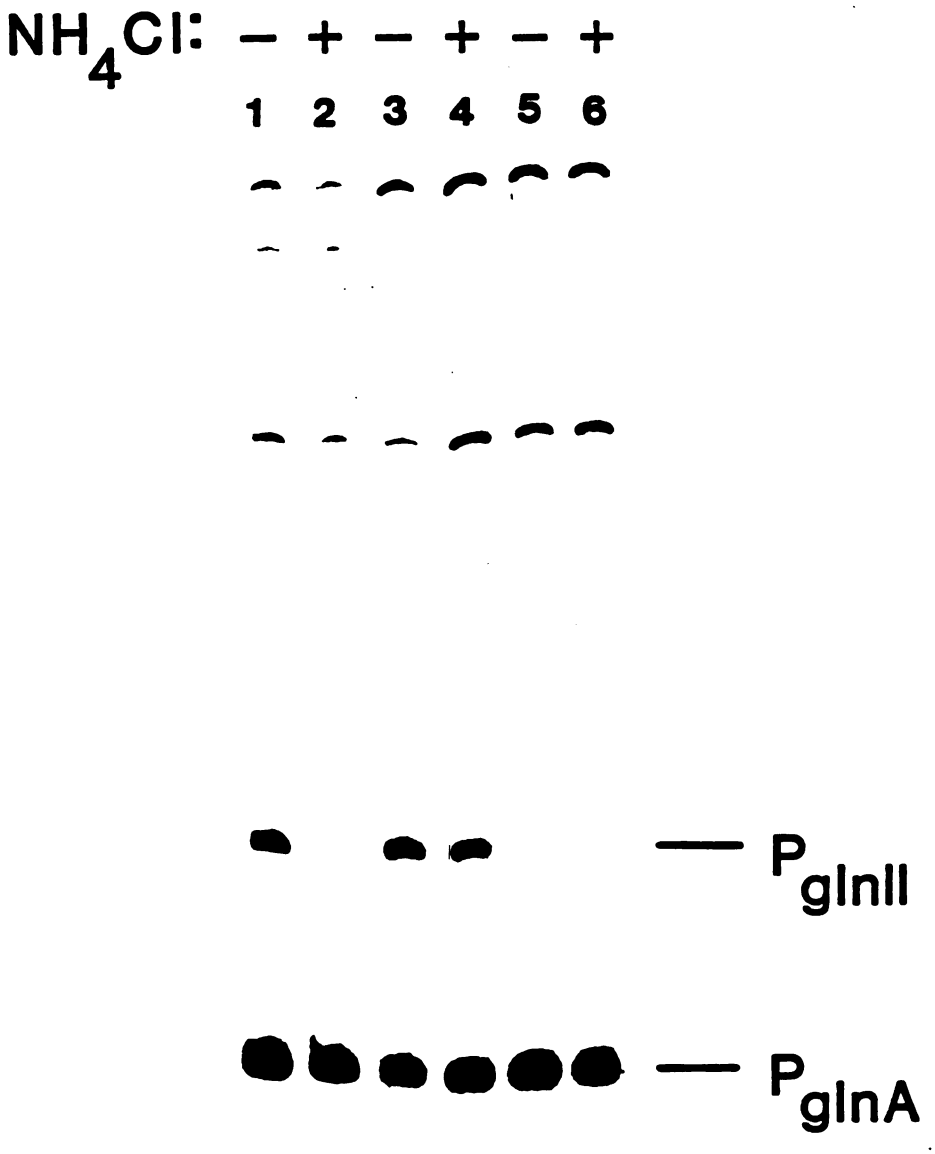

Figure 4.

5.0% or less (2). Adams and Chelm (2) suggested this oxygen-limited *glnII* induction may be mediated through an Ntr-like system. To test if the *B.* japonicum *ntrC* gene is involved in the oxygen-limited response of *glnII*, microaerobic (0.2% O_2) cultures were grown that contained excess nitrogen (10 mM NH_4Cl, 0.5 mM KNO_3). RNA was purified from these cells and S1 protection analyses were conducted as described for the aerobic culture experiments. As observed previously (2), *glnII* expression was induced in the wild-type BJ110d strain under microaerobic conditions even though excess nitrogen is present (Figure 5). The *ntrC* null mutant, BJ27147, also expressed *glnII* under these conditions in contrast to the aerobic cultures of this strain (Figure 5). This establishes that the *ntrC* gene product is not required for the microaerobic induction of *glnII*. In BJ3028, *glnII* transcript abundance was similar to BJ27147.

Symbiotic phenotype of *B. japonicum ntrC::nptII* mutants. Both BJ27147 and BJ3028 formed approximately the same number of nodules on soybean plants as the wild-type strain. In addition, the average nodule fresh weight per plant, average weight per nodule, and average plant dry weight from *ntrC* mutant-incited symbioses were not significantly different from a wild-type incited symbiosis at 4 weeks after inoculation. Moreover, nodules incited by *ntrC* mutants showed wild-type levels of acetylene reduction activity. These results indicate that, as in *R. meliloti* (45), the *B. japonicum ntrC* gene is not required for initiating effective nodulation or for activating genes essential to nitrogen fixation during symbiotic growth.

Figure 5. Expression of *B. japonicum glnA* and *glnII* genes in nitrogen-excess, microaerobic (0.2% O_2) cultures. Samples of RNA purified from BJ110d, BJ27147, or BJ3028 grown in nitrogen-excess cultures that were maintained at 0.2% oxygen, 98.2% nitrogen were analysed by the S1 nuclease protection method. Lanes: 1, BJ110d; 2, BJ27147; and 3, BJ3028.

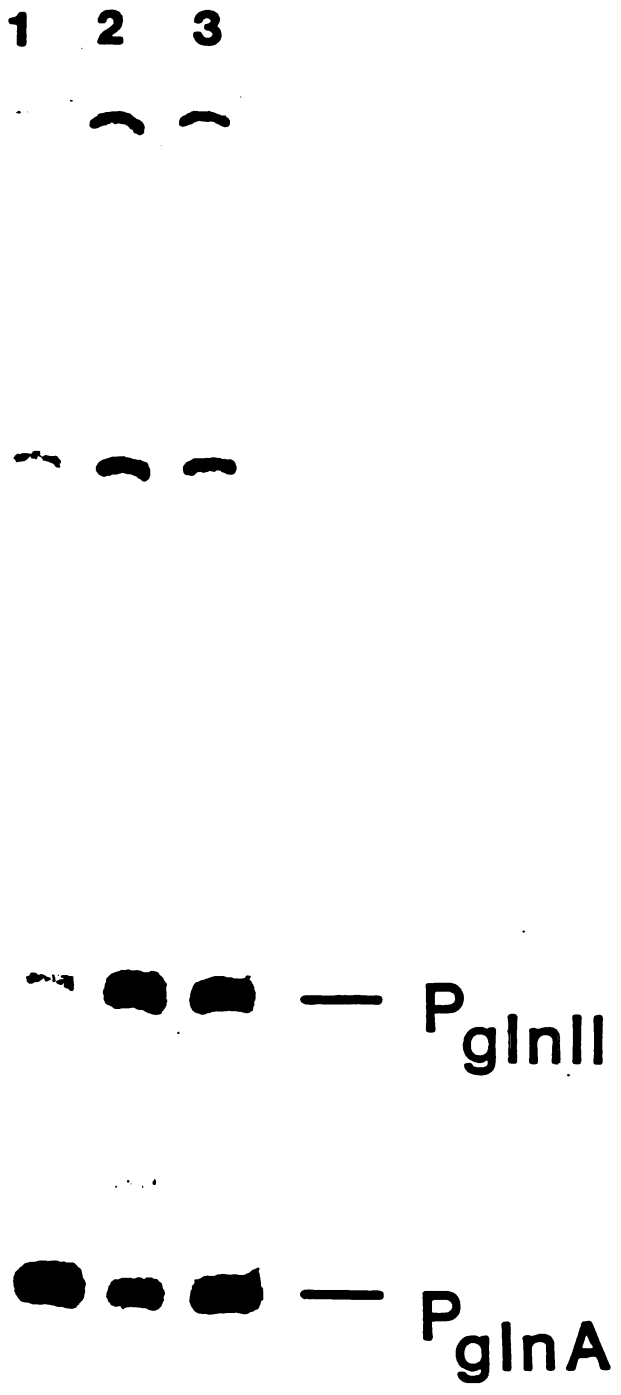

Figure 5.

Figure 6. Expression of *B. japonicum glnA* and *glnII* genes in bacteria isolated from soybean nodules. Samples of RNA purified from *B. japonicum* cells isolated from nodules incited by BJ110d, BJ27147 or BJ3028 were analysed by the S1 nuclease protection method. Lanes: 1, BJ110; 2, BJ27147; 3, BJ3028; 4, BJ2513 (*glnII* strain [9]); 5, BJ2841 (*glnA* strain [9]).

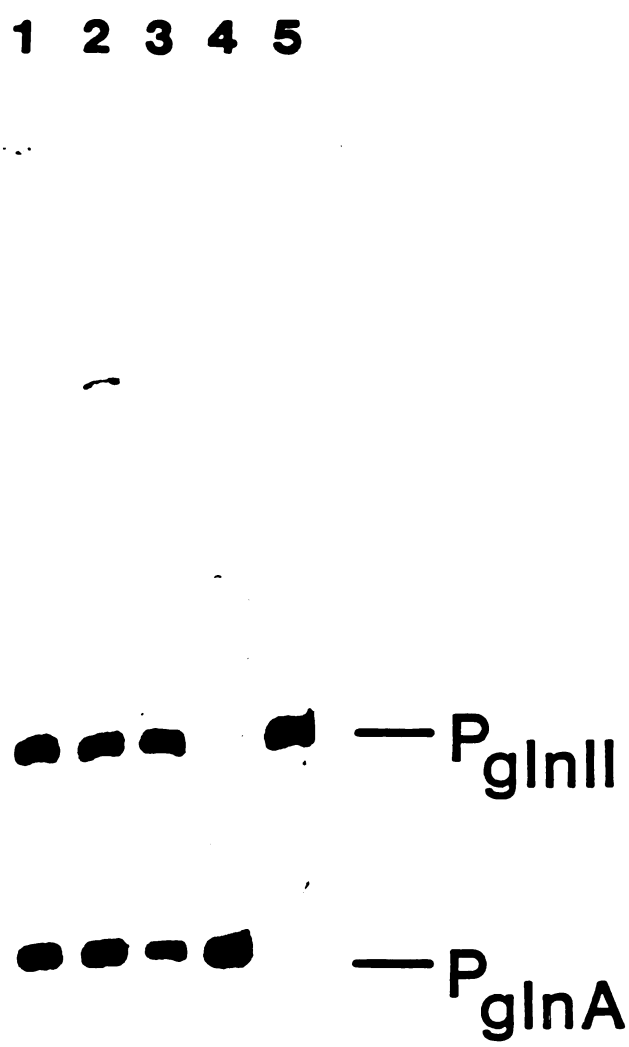

Figure 6.

Transcription of *B. japonicum glnA* and *glnII* genes in soybean nodules. Transcripts of both *glnA* and *glnII* are present in wild-type *B. japonicum* incited soybean nodules (2). To determine if *ntrC* is involved in the regulation during symbiosis of either of these genes, RNA was purified from bacteria that were isolated from 4 week old soybean nodules and used in S1 protection analyses (Figure 6). In both BJ27147 and BJ3028 *glnA* and *glnII* transcripts were present in levels similar to microaerobically grown cells. This indicates that the *B. japonicum ntrC* gene is not required for the expression of either glutamine synthetase gene during symbiosis.

DISCUSSION

I have isolated the *B. japonicum ntrC* gene and examined its role in regulating nitrogen assimilation. Several lines of evidence indicate that this locus is *ntrC*. First, only one homologous sequence was observed in the *B. japonicum* genome when the *B. parasponiae ntrC* probe was used (Figure 1). This homology was confirmed by comparing a *B. japonicum* partial DNA sequence with the *B. parasponiae ntrC* sequence. Secondly, a region upstream of the *B. japonicum ntrC* was found to be highly homologous to the *ntrB* genes reported upstream of the *ntrC* genes from *B. parasponiae* and *R. meliloti* (35, 45). Thirdly, consistent with *ntrC* genes in several other diazotrophs (43, 45, 46), the *B. japonicum ntrC* is required for the utilization of potassium nitrate as a sole nitrogen source. Finally, an NtrC antiserum from *E. coli* recognized the protein produced by the *B. japonicum ntrC* (Figure 3). The expression of

the *B. japonicum glnII* gene was induced when aerobic growth was limited by nitrogen depletion as expected if regulated by an Ntr-like system (Figure 4). Indeed, the absence of *glnII* transcript in the NtrC null mutant (BJ27147) indicates that in *B. japonicum* the control of *glnII* expression under aerobic nitrogen-limited growth is dependent on the *ntrC* gene and that this control is at the transcriptional level. This observation is consistent with two aspects of the upstream region of *glnII*. First, the -8 to -26 region of the *glnII* promoter is homologous to promoter regions of several *nif* genes that are thought to be recognized by RNA polymerase associated with NtrA (9, 37). Secondly, the upstream region of *glnII* contains two sequence elements (at -103 to -119 [9] and at -282 to -299 [5'-GGCACCAGCTTGGTGAC-3'; T. A. Carlson, Ph.D. dissertation, Michigan State University, E. Lansing, 1986]) that match the 5'-TGCACCnnnnTGGTGCA- 3' consensus NtrC binding site from the *Enterobacteriaceae* (4) in 10 of 13 conserved positions. In BJ3028, a truncated NtrC protein was produced under nitrogen-starved and nitrogen-excess conditions (Figure3). Also in BJ3028, in contrast to wild-type, *glnII* was transcribed under both nitrogen regimes. This indicates that the truncated NtrC protein remained functional as an activator of *glnII* expression, i.e. the portion of the NtrC protein that is missing was not required for *glnII* activation. The *B. parasponiae* NtrC has a region very near the carboxy-terminus that is characteristic of the helix-turn-helix motif thought to be involved in DNA binding (17). Assuming colinearity of the *B. japonicum* and *B. parasponiae* NtrC proteins over this region, it is possible that the truncated NtrC protein of BJ3028 lacks this putative DNA binding region. The observation that the

truncated NtrC retained function as an activator of *glnII* expression therefore raises the possibility that the helix-turn-helix region is not required for glnII activation. In *E. coli*, NtrC represses the transcription of the *ntrBC* operon under nitrogen-excess conditions by binding to a sequence upstream of the promoter (49). In *B. japonicum*, preliminary observations indicate that the constitutive production of the truncated NtrC in mutant BJ3028 is due to a loss of repressor activity at the promoter for the *ntrBC* operon. This suggests that the repressor activity is present in the carboxy-terminal region of the *B. japonicum* NtrC. Studies are underway to determine if *B. japonicum ntrC*-regulatable promoters have upstream regions that bind NtrC. The inability to utilize nitrate as a nitrogen source is a common *ntrC* phenotype having been reported in *R. meliloti* (45), *A. tumefaciens* (43), and *Azotobacter vinelandii* (46). Although BJ27147 and BJ3028 had different phenotypes for aerobic *glnII* regulation and NtrC production, neither was able to utilize potassium nitrate as a nitrogen source. Thus, the *B. japonicum ntrC* may also be involved in the induction of assimilatory nitrate reductase as has been demonstrated in *A. vinelandii* (46). The reason why the truncated-NtrC mutant is able to activate *glnII* but is unable to utilize nitrate is not clear. It is possible that the nitrate reductase promoter is "weaker" and requires NtrC binding activity present in the carboxy-terminus whereas the *glnII* promoter does not. Alternatively, the carboxy-terminal region of *B. japonicum* NtrC may be involved in repressing a repressor of nitrate reductase.

One of the most interesting aspects of the *B. japonicum ntrC* gene is its involvement in the differential regulation of *glnII* when cells are exposed to different oxygen conditions. The appearance of *glnII* transcript in the NtrC null mutant (BJ27147) when it was grown microaerobically (or symbiotically; Figure 5 and 6) indicates that another regulator of *glnII* may be active under conditions where oxygen becomes limiting. This oxygen-limited induction of *glnII* has been shown to require the *B. japonicum nifA* gene (T. H. Adams, Ph.D dissertation, Michigan State University, E. Lansing, 1986) although no Nif-specific sequence element is present upstream of *glnII* (9). Whether or not NifA protein interacts directly with an upstream *glnII* region requires further study.

In nature, *B. japonicum* exists as a free-living organism relying upon soil nitrogen or as a bacteroid where it fixes atmospheric nitrogen for itself and its soybean host. As a bacteroid, *B. japonicum* survives in a microaerobic environment necessitated by the oxygen lability of nitrogenase. The data presented in this paper support a model for the involvement of the *B. japonicum ntrC* gene in a dual regulatory system for *glnII* that responds to nitrogen or oxygen availability depending upon the developmental stage of the organism. Such a model has been postulated for the regulation of the *nifHD*, *nifB* and *fixABC* genes in *R. meliloti* (45). In this case the model posits that during free-living, nitrogen-starved growth in soil, *glnII* is transcriptionally activated by the *ntrC* gene product in concert with the NtrA sigma factor. However, under the microaerobic conditions encountered during symbiotic growth, *glnII* is activated by another regulatory mechanism involving the *nifA*

gene product in concert with NtrA. In light of the apparent dispensability of *glnII* (9), the significance of such dual regulation for this gene remains to be elucidated.

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CHAPTER 3

Bradyrhizobium japonicum ntrBC/glnA and *nifA/glnA* mutants: Evidence that separate regulatory pathways govern *glnII* expression in free-living and symbiotic cells.

SUMMARY

The presence in *Bradyrhizobium japonicum* of two glutamine synthetase genes, *glnA* and *glnII*, makes it difficult to study the physiological effects of possible regulatory genes that differentially control these genes. Here I have investigated effects of the products of the *B. japonicum ntrBC* operon and the *nifA* gene on *glnII* expression during the formation of nodules on soybean roots (*Glycine max*). Two *B. japonicum* strains with dual mutations were constructed by gene-directed mutagenesis. Each mutant carried 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*, and *nifA*, for the ability to grow on various nitrogen sources, nodulate soybeans, and fix atmospheric nitrogen. The *ntrBC/glnA* mutant, but not the *nifA/glnA* mutant, was unable to grow on nitrate or ammonia as the sole nitrogen source when grown in aerobic cultures. Both *ntrBC/glnA* and *nifA/glnA* mutant strains formed fewer nodules per plant than the wild-type strain or the single mutants and were altered in their ability to fix nitrogen. These observations are interpreted in the context of a model of separate regulatory networks controlling *glnII* expression in *B. japonicum*. The

networks involve the products of *ntrBC* and *nifA*, and respond to either nitrogen deprivation or a signal present in symbiotic cells.

INTRODUCTION

During free-living growth *Bradyrhizobium japonicum*, the endosymbiont of soybean, assimilates ammonia primarily by the coordinate activity of glutamine synthetase (GS) and glutamate synthase (4, 30). However, in bacteroids, the symbiotic form of these bacteria, GS activity decreases in concert with the derepression of nitrogenase activity (4). The regulation of GS during free-living growth, incipient nodule formation and in mature bacteroids is therefore an integral part of the developmental process in *B. japonicum*.

Bacteria of the *Rhizobiaceae* family, including *B. japonicum*, are unusual in that most members contain two GS enzymes GSI and GSII, encoded by *glnA* and *glnII*, respectively (7, 8, 9). The *B. japonicum glnA* gene has sequence similarity to the *E. coli glnA* gene and is not appreciably nitrogen-regulated (6). In contrast, the GS encoded by *glnII* has sequence similarity with eucaryotic GS's and is transcriptionally regulated in response to nitrogen availability (5, 7).

In *Klebsiella pneumoniae*, 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 (2). The first level, which is specific to nitrogen-fixation genes (*nif* genes) is mediated by the transcriptional activator encoded by *nifA* (NifA; 2). The second level of regulation is mediated by the centralized Ntr system, which controls the expression of a variety of nitrogen assimilation genes in the enteric bacteria (18). In the Ntr system the

gene product of *ntrC* (NtrC) functions as a transcriptional activator of many genes including *nifA* (14). Both *nifA* and *ntrC* homologous genes are present in members of the *Rhizobiaceae* and these genes have been isolated and characterized in several species (12, 20, 23, 27, 28, 29). These studies have found that the rhizobial *nifA* gene is expressed independently of the centralized Ntr system and is not dependent on NtrC for expression. *Azorhizobium caulinodans*, where some NtrC control over *nifA* has been reported, appears to be the exception (23).

I have shown previously that in *B. japonicum* NtrC is required for the transcriptional regulation of *glnII* under conditions of nitrogen-starved aerobic growth (20). Under microaerobic conditions and during symbiotic growth, however, *glnII* is expressed but does not require NtrC (20). Szeto et al. (28) have shown in *Rhizobium meliloti* that, under symbiotic conditions, NifA activates several genes that are regulated by NtrC during aerobic growth. I have suggested that the NtrC-independent expression of *glnII* during microaerobic and symbiotic growth also involves NifA control (20). 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.

In order to examine the physiological effects of specific regulatory proteins on the expression of *glnII*, I have developed double mutants that carry a *glnA* deletion along with a deletion in either *ntrBC* or *nifA*. By eliminating the interfering GS activity encoded by *glnA*, this strategy allowed us to assess the role of NtrB, 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 which probably involves oxygen limitation.

MATERIALS AND METHODS

Bacterial strains, plasmids, media and growth conditions. BJ110d is a small-colony derivative of *B. japonicum* 3I1b110 (13). All other bacterial strains and recombinant plasmids 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 (22) was used which had the following composition per liter: KH_2PO_4, 0.3 g; Na_2HPO_4, 0.3 g; $MgSO_4.7H_2O$, 0.12 g. Trace elements were added (22) and the pH was adjusted to 6.8. After autoclaving, 1 ml of a filter-sterilized vitamin solution (22), 0.05 g $CaCl_2$ and 7.5 ml of filter-sterilized 20% (wt/vol) D-xylose were added per liter. The various nitrogen sources were added at 1.0 mM from filter-sterilized stock solutions.

DNA biochemistry. Southern hybridizations and isolation of plasmid DNA were carried out as described previously (1). Total genomic DNA from *B. japonicum* was purified by phenol extraction (19). The isolation of *glnA* and the *ntrBC* operon has been described (6, 20). The *nifA* locus was isolated from a *B. japonicum* cosmid library using a 950 base pair *Pst*I-*Eco*RV fragment from the *Klebsiella pneumoniae nifA* gene as a probe (T. H. Adams, Ph.D. dissertation, Michigan State University, East Lansing, 1986).

Table 1. Bacterial strains and plasmids.

Strain or plasmid	Description	Source or reference
BJ110d	Wild type	(14)
BJ2841	*glnA*::spc; *glnA* mutant, Spc^r	(7)
BJ3263	*ntrBC*::*nptII*; *ntrBC* mutant, Km^r	This work
BJ2101	*nifA*::*nptII*; *nifA* mutant, Km^r	T. H. Adams
GM10-1	*glnA*::spc, *ntrBC*::*nptII*; *glnA/ntrBC* mutant, Spc^r, Km^r	This work
GM4-33	*glnA*::spc, *nifA*::*nptII*; *glnA/nifA* mutant, Spc^r, Km^r	This work
Plasmids		
pBJ284	Spc^r; *glnA* deletion allele cloned into pKC7	(7)
pBJ326	Km^r, Ap^r; *ntrBC* deletion allele cloned into pBR322	This work
pDS4101	Ap^r; derivative of ColK	(33)
pKC7	Km^r, Ap^r; vector	(27)
pBR322	Ap^r, Tc^r; vector	(3)

Gene-directed mutagenesis. Recombinant plasmids were manipulated in vitro and in *E. coli* to construct mutant alleles of the *glnA* and *ntrBC* loci (Table 1, Fig. 1). In both cases, sizable deletions within the genes were created and, for the purposes of *B. japonicum* strain construction, the deleted regions were replaced with antibiotic resistance genes. The plasmids carrying the mutant alleles were conjugally transferred from *E. coli* to *B. japonicum* by methods previously described (13). 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 (13). 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. The *nifA* mutant, BJ2101, was constructed using identical procedures to those described for the *glnA* and *ntrBC* mutants (T. H. Adams, Ph. D. dissertation). In the case of the double mutations, the confirmed *ntrBC* and *nifA* deletion mutants (BJ3263 and BJ2101) were processed through a second mating round using an *E. coli* strain carrying the plasmids pBJ284 and pDS4101 (see Table 1) to mutate the *glnA* gene.

Plant inoculation, growth conditions and acetylene reduction assays. Soybean seeds (*Glycine max* [L.] Merr. cv. Amsoy 71) were sterilized as described previously (13) 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 (31) using the

nitrogen-free medium of Johnson et al. (17). The plants were grown with a 16 hr daily light period, day/night temperatures of 28/25°C, a relative humidity of 60%, and a photon fluence rate of 300 umol.m-2.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 (15).

Strain verification of bacteria isolated from nodules. Nodules were chosen at random, immersed in 95% ethanol for 30 sec, then in 3% hydrogen peroxide for 4 min, and then rinsed six times in 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.

Electron Microscopy. Nodules were harvested from 4 week-old root systems, rinsed in deionized water, and sliced with a razor to pieces of approximately 1 mm in the largest dimension. The slices were then immediately fixed in 4% glutaraldehyde in 0.15 M sodium cacadylate, pH 7.2, for 2 hr under vacuum and then at 4°C overnight. Following fixation, the tissue was rinsed three times over a period of 3 hr at room temperature with Buffer A (21) 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 ultra low viscosity medium (Ladd Research Industries, Burlington, VT). Thin sections were cut on

an LKB Ultrotome III with a Dupont diamond knife. Sections were subsequently stained with uranyl acetate and lead citrate and examined in a Phillips EM300 electron microscope.

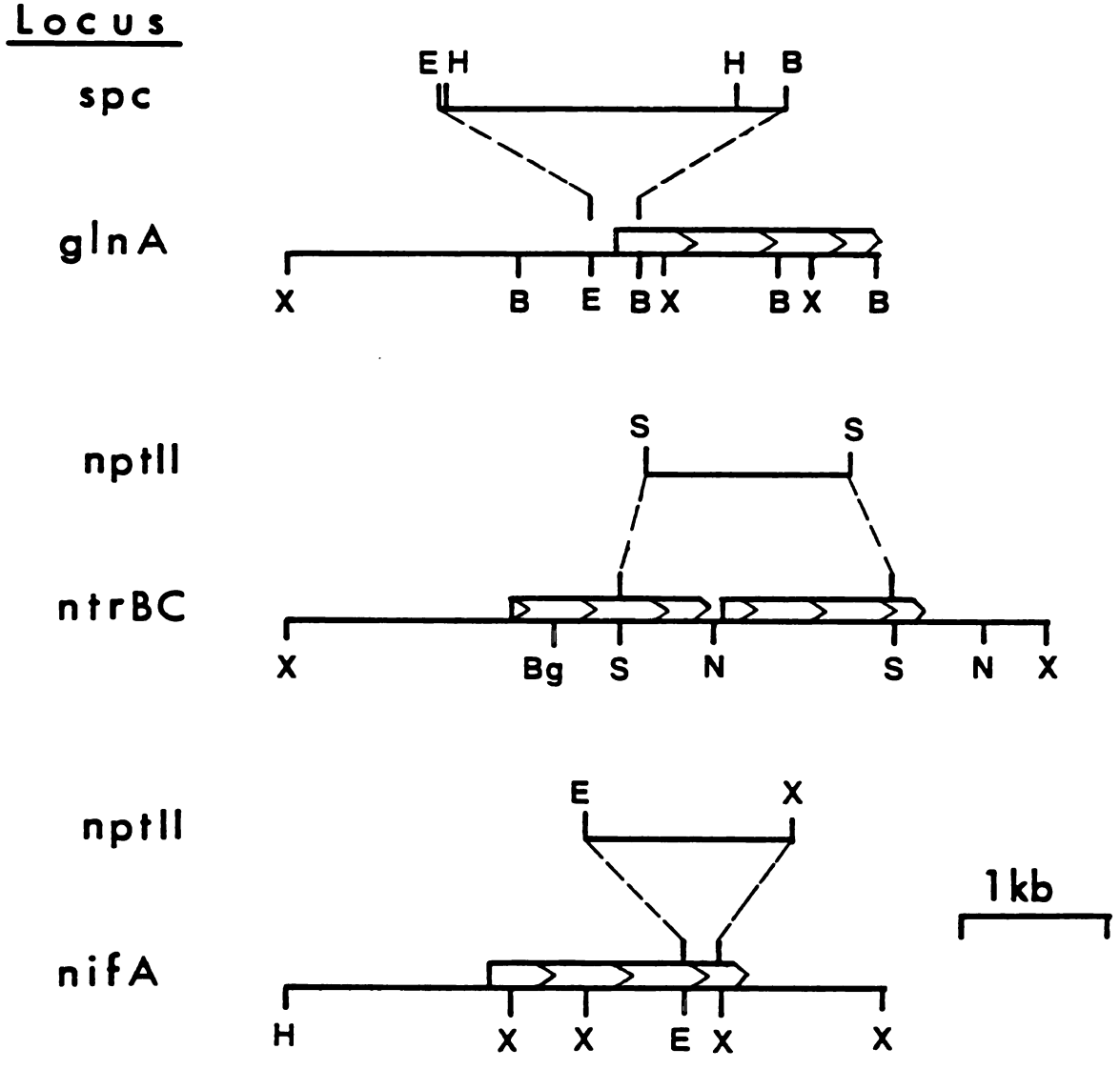
RESULTS AND DISCUSSION

Construction of *B. japonicum ntrBC/glnA* and *nifA/glnA* mutants.

We have reported the construction of a *glnA* mutant allele and its use in the generation of a *B. japonicum glnA* mutant (7). Here this allele was used in conjugal matings with *ntrBC* and *nifA B. japonicum* strains to create *ntrBC/glnA* and *nifA/glnA* mutants (Table 1, Fig. 1).

The mutant *glnA* allele was constructed by deleting the *glnA* 300 bp *Eco*RI-*Bam*HI region and replacing it with a *Hind*III fragment containing a spectinomycin resistance gene. The source of the fragment was a derivative of the plasmid pHC45 (25), to which *Eco*RI-*Hind*III and *Bam*HI-*Hind*III adaptors obtained from plasmid pKC7 (26) had been added (Fig. 1). The mutant *ntrBC* region was constructed by deleting the 1.8 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 (20). This cassette is homologous to the kanamycin resistance gene present on pKC7. The mutant *nifA* allele carried a 300 bp deletion which was replaced with a pKC7-derived *Eco*RI-*Xho*I 1.6 kb kanamycin cassette (T. Adams, Ph.D. dissertation). The double mutants were constructed by conducting a second round of mutagenesis as described in the Materials and Methods section. The mutations in all the regions were confirmed by Southern hybridizations of *Xho*I-digested total genomic DNA with pKC7 (Fig. 2), and with plasmids containing the

Figure 1. Physical maps of the *B. japonicum glnA*, *ntrBC* and *nifA* wildtype and mutant alleles. The coding regions of the genes are indicated by the boxed regions containing arrowheads, and transcription is from left to right. The insertion locations are indicated for the spectinomycin resistance gene (spc) and the kanamycin resistance gene (*nptII*). See Materials and Methods for details of constructions. Restriction endonuclease sites: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III; S, *Sal*I; N, *Nru*I; X, *Xho*I.


Locus
spc
glnA
nptII
ntrBC
nptII
nifA
E H
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X B E B X B X B
S S
X Bg S N S N X
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H X X E X X
1kb

Figure 1.

Figure 2. Hybridization analysis of *B. japonicum* genomic DNA from wildtype and mutant strains to pKC7. *Xho*I digests of total cellular DNA were transferred to cellulose nitrate and hybridized to radiolabeled pKC7. Lanes: 1, GM4-33 (*glnA/nifA* mutant); 2, BJ2101 (*nifA* mutant); 3, BJ2841 (*glnA* mutant); 4, BJ3263 (*ntrBC* mutant); 5, GM10-1 (*glnA/ntrBC* mutant); and 6, BJ110d (wildtype).

Figure 2.

appropriate gene region (data not shown). The presence of pKC7-homologous sequences in all of the constructions allowed us to use this vector to verify the presence of the mutant alleles in all strains (Fig. 2). These results confirmed that the mutant alleles had been recombined into the expected regions of the *B. japonicum* chromosome. The *glnA*, *ntrBC* and *nifA* mutant alleles were carried on 4.4 kb, 4.7 kb and 2.3 kb *Xho*I fragments, respectively (Fig. 2).

Free-living and symbiotic phenotypes of the *B. japonicum glnA*, *ntrBC* and *ntrBC/glnA* mutants.

Previous work in our laboratory indicated that NtrC is required for the transcriptional activation of *glnII* under aerobic culture conditions (20). I therefore predicted that, lacking either glutamine synthetase, the *ntrBC/ glnA* mutant would be deficient in the utilization of some nitrogen sources under aerobic conditions. I also predicted, based on the observation that a *glnA/glnII* mutant is Nod$^-$ (7), that the *ntrBC/glnA* mutant would be altered in its ability to form an effectve symbiosis with soybean. A potentially critical difference, however, existed between the *ntrBC/glnA* and the *glnA/glnII* mutants. In symbiotic cells of the *ntrBC/glnA* mutant, *glnII* should still be expressed in an NtrC-independent manner (see Introduction). Thus, if *ntrBC/glnA* mutant cells were able to invade root cells then it was expected that they would produce GSII and, as a result, be capable of establishing a normal symbiosis.

In order to detect nitrogen-utilization defects, I tested the *ntrBC*, *glnA*, and *ntrBC/glnA* mutants for growth on various nitrogen sources

(Table 2). It was reported for several rhizobial species, including *B. japonicum*, that NtrC is required for aerobic growth on nitrate as a sole nitrogen source (20, 27, 28). I found, as expected, that the *ntrBC* region was required for growth on nitrate; it was not required for growth on ammonia, glutamate or glutamine (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 exhibit no nitrogen-utilization defects, but that a *glnA/glnII* double mutant is a glutamine auxotroph (7). The fact that the *ntrBC/glnA* mutant was 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.

The effects of the *ntrBC*, *glnA*, and *ntrBC/glnA* mutations on soybean nodulation and nitrogen-fixation were determined by growing cultures of minimal medium supplemented with 1 mM glutamine, washing the cultures in unsupplemented medium, and inoculating soybean seeds in modified Leonard jars. I found that the *ntrBC* mutant, BJ3263, formed similar numbers of nodules on soybean as the wild-type strain (BJ110d; Table 3). In addition, the average plant dry weight and acetylene reduction rates with this mutant were similar to wild-type (Table 3). However, the *ntrBC/glnA* mutant, GM10-1, produced many fewer nodules than the wild-type or *ntrBC* strain and these nodules were of two distinct types (Table 3, Fig. 3C). The less common type were apparently normal (see below), leghemoglobin-containing nodules of a similar size as wild type nodules.

Table 2. Growth properties of *B. japonicum* strains[a]

Strain	Genotype	Nitrogen source[b]			
		Nitrate	Ammonia	Glutamate	Glutamine
BJ110	wild type	++	++	++	++
BJ2841	*glnA*	++	++	++	++
BJ3263	*ntrBC*	-	++	++	++
BJ2101	*nifA*	++	++	++	++
GM10-1	*glnA/ntrBC*	-	-	+	+
GM4-33	*glnA/nifA*	++	++	++	++

[a] Growth was determined in liquid minimal medium (see Materials and Methods), and is indicated as follows: ++ very good; + good; - none.

[b] Nitrogen sources were tested at 1.0 mM concentration. All nitrogen sources were added from freshly prepared, filter-sterilized stock solutions.

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

Strain	Genotype	Number of Nodules	Acetylene reduction (umoles per plant)	Plant dry weight (gm)
BJ110	wildtype	63.7 ± 17.6	19.8 ± 4.6	4.33 ± .28
BJ2841	*glnA*	82.3 ± 7.5	27.3 ± 0.6	3.20 ± .35
BJ3263	*ntrBC*	58.8 ± 9.6	21.3 ± 7.4	3.00 ± .38
BJ2101	*nifA*	151.5 ± 15.2	0	.94 ± .07
GM10-1	*glnA/ntrBC*	13.1 ± 5.3	.2 ± 0.1	.86 ± .16
GM4-33	*glnA/nifA*	29.5 ± 13.3	0	.93 ± .15

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

Figure 3. Comparison of symbiotic phenotypes of *B. japonicum* BJ110d and mutant strains BJ2101, GM10-1, GM4-33. Sterilized soybean seeds were inoculated with the appropriate *B. japonicum* strain and grown in modified Leonard jars (see Materials and methods). Plants were harvested four weeks after sowing. Panels: A, BJ110d (wildtype); B, BJ2101 (*nifA* mutant); C, GM10-1 (*glnA/ntrBC* mutant); D, GM4-33 (*glnA/nifA* mutant).

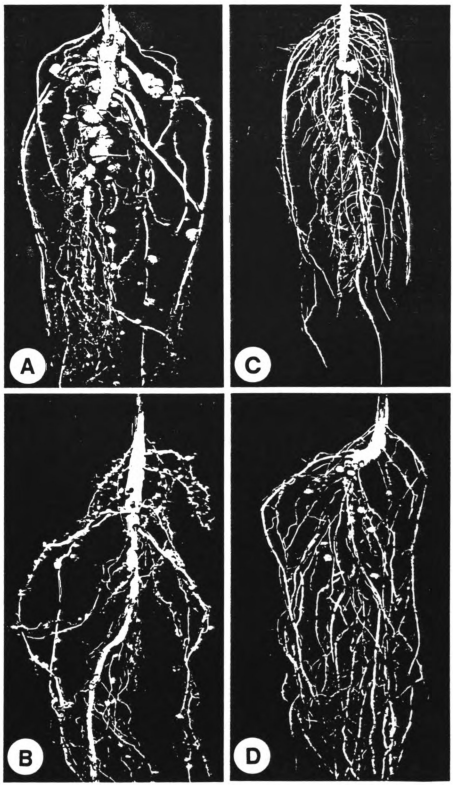

Figure 3.

The second, more common type, were smaller than wild-type nodules and were yellowish-white inside. Electron microscopy of the normal-appearing nodules revealed numerous mature bacteroids surrounded by well-formed peribacteroid membranes; in all respects the ultrastructure of these nodules was identical to wild type (BJ110d) nodule ultrastructure (Fig. 4A and 4D). Electron microscopy of the greenish-white nodules revealed very few bacteroids (Fig. 4C). To ensure that these nodules had been formed by GM10-1, bacteria were isolated from randomly chosen nodules of both types and these were checked for the presence of genetic markers. All of the 100 colonies tested were both kanamycin-resistant and spectinomycin-resistant indicating that all the bacteria present were mutant and that no reversion had occurred. 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 some wild- type nodules, however, suggests that when *ntrBC/glnA* mutant cells do invade soybean root hairs that *glnII* is expressed and a normal symbiosis is possible. The low rate of acetylene reduction detected from the GM10-1 nodules is apparently 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. A *B. japonicum glnA/glnII* mutant, also a glutamine auxotroph, is similarly defective in nodulation (7). An *Azorhizobium caulinodans* glutamine auxotroph also forms a defective symbiosis (10). It appears, however, that the inability of *B. japonicum* and *A. caulinodans* glutamine auxotrophs to form effective symbioses is not a general phenomenon in

Figure 4. Transmission electron micrographs of nodule tissue infected by *B. japonicum* BJ110d, and mutant strains GM10-1 and GM4-33. The tissue was harvested 4 weeks after seed inoculation and sowing. All micrographs were taken at the same magnification (5000X) with the bar in panel D representing 1 um. Panels: A, BJ110d; B, GM10-1 (yellowish-green nodule); C, GM4-33; D, GM10-1 (normal-type nodule).

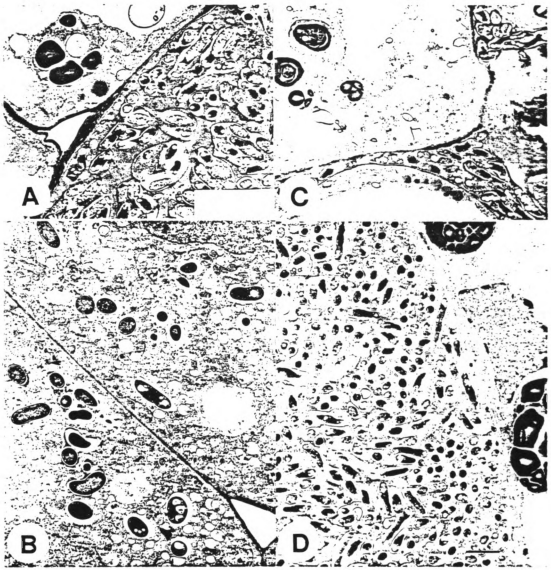

Figure 4.

rhizobial-plant associations. A *Rhizobium meliloti glnA/glnII* mutant is a glutamine auxotroph but is Nod^+ Fix^+ on *Medicago sativa* (11). 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 free-living rhizobial *nifA* mutants (12, 29) and no nitrogen-utilization defects were observed in a *B. japonicum nifA* mutant strain (BJ2101) in our laboratory (Table 2; T. Adams, Ph.D. dissertation). I did not expect that a *nifA/glnA* mutant would exhibit nitrogen-utilization defects in aerobic cultures, since *glnII* expression is activated by NtrC under these conditions (20). However, previous S1 protection experiments in our laboratory with RNA isolated from *B. japonicum nifA* bacteroids (BJ2101) revealed that NifA is required for the activation of *glnII* in symbiotic cells (T. H. Adams, Ph.D. dissertation). I expected, therefore, that *nifA/glnA* mutant cells inoculated onto soybean roots would rely on NtrC to activate *glnII* expression up until they encountered the symbiotic signal that normally transfers this control to NifA. In light of the pleiotropic nature of *B. japonicum nifA* mutants (12), I was particularly interested to determine at what developmental stage the loss of *glnII* expression would be manifested during nodulation.

As expected, I observed no nitrogen-utilization deficiencies in the *nifA/glnA* mutant, GM4-33, in free-living cultures (Table 2). This indicated that a normal level of GSII activity was present in this strain and that *nifA* therefore does not play an important role in activating *glnII* expression during aerobic, free-living growth.

B. japonicum nifA mutants fail to derepress many genes involved in an effective symbiosis including *nifH*, *nifDK*, *fixA*, and *fixBCX* (12, 16). In addition, Fischer et al. (12) reported that *B. japonicum nifA* mutants exhibit 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 (12, 24). I used an independently constructed *B. japonicum nifA* mutant (BJ2101) as a control in these experiments, and confirmed that more than double the number of nodules are observed on a *nifA* mutant-inoculated root system than are seen in a wild-type induced symbiosis (Table 3, Fig. 3B). In contrast, I found that the *B. japonicum nifA/glnA* mutant, GM4-33, formed significantly fewer nodules than the *nifA* mutant or the wild-type strain (Table 3, Fig. 3D). The nodules that were formed were larger than *nifA* mutant-induced nodules 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, albeit at a low level. These results were consistent with the notion that NifA activates *glnII* expression and, in addition, indicated that the glutamine synthetase activity encoded by *glnA* is

required for the formation of the numerous, small nodules seen in the *nifA* mutant-incited symbiosis. Electron microscopy of the *nifA/glnA*-incited nodules revealed bacteroid degradation and the presence of numerous intercellular bacteria (Fig. 4C). It is not clear why the *nifA/glnA* mutant-induced nodules were larger than the *nifA* mutant-induced nodules. 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 *nifA* mutant-induced nodules.

The *glnA* mutant, BJ2841, was also included in these experiments as a control and, as reported earlier (7), it formed slightly more nodules and had a higher acetylene reduction rate per plant than the wild-type incited symbiosis. A *B. japonicum glnII* mutant exhibits a similar phenotype (7). The significance of these observations is 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. The *ntrBC/glnA* mutant, according to this model, was defective in nodule formation because it lacked NtrC needed for the activation of *glnII* expression; it also lacked the ancillary GS activity encoded by *glnA*. An analogous *glnA/glnII* mutant is similarly ineffective in establishing a symbioisis with soybean (7). This defect probably results in an inability to maintain cell growth long enough to efficiently invade root hairs and thereby reach the microaerobic environment of the peribacteroid-membrane bound vesicle where NifA activation of *glnII* expression could occur. The *nifA/glnA* mutant-

induced symbiosis suffered from numerous defects caused by the absence of NifA and conclusions regarding the loss of *glnII* regulation by NifA are less clear. It can be concluded, however, that the inability of the *nifA/glnA* mutant 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 occurs. In addition, the inability to hypernodulate 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.

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CHAPTER 4

Bradyrhizobium japonicum glnB, a putative nitrogen regulatory gene, is regulated by NtrC at tandem promoters.

SUMMARY

The *glnB* gene from *Bradyrhizobium japonicum*, the endosymbiont of soybean (*Glycine max*), was isolated and sequenced and its expression under various culture conditions and in soybean nodules was examined. The *B. japonicum glnB* gene encodes a 12,237 dalton polypeptide that is highly homologous to the *glnB* gene products from *Klebsiella pneumoniae* and *Esherichia coli*. In contrast to the *glnB* genes from the enteric bacteria, *glnB* from *B. japonicum* is located directly upstream from *glnA*, the structural gene for glutamine synthetase. The *glnB* gene from *B. japonicum* is expressed from tandem promoters, which are differentially regulated in response to the nitrogen status of the medium. Expression from the downstream promoter involves the *B. japonicum ntrC* gene product (NtrC) in both free-living and symbiotic cells. Thus *glnB*, a putative nitrogen regulatory gene in *B. japonicum*, is itself Ntr-regulated, and NtrC is active in *B. japonicum* in the symbiotic state.

INTRODUCTION

Bradyrhizobium japonicum exists as a free-living organism, growing at the expense of soil nitrogen, or as a symbiont, reducing dinitrogen to ammonia for itself and its soybean host. Free-living *B. japonicum* cells assimilate ammonia primarily by the coordinate activities of glutamine

synthetase (GS) and glutamate synthase (12, 56). However, during the development of bacteroids, the symbiotic form of these bacteria, GS activity decreases in concert with the derepression of nitrogen-fixation activity (12, 56). The regulation of nitrogen assimilation pathways is therefore an integral part of the bacterial developmental process and the symbiotic interaction.

In the family *Enterobacteriaceae* many genes involved in nitrogen assimilation, including *glnA*, the glutamine synthetase gene, are regulated by the Ntr system (36, 40, and references below). This regulation involves both transcriptional and post-translational control exerted by the products of the *ntrA*, *ntrB*, *ntrC*, *glnB*, *glnD*, and *glnE* genes (NtrA, NtrB, NtrC, GlnB, GlnD, and GlnE, respectively; [8, 10, 14, 23, 28, 31, 32, 35]). NtrB and NtrC are bifunctional regulatory proteins that act in concert to either repress or activate transcription (35, 43, 47). The functional state of NtrB is modulated by GlnB and GlnD in response to the ratio of intracellular glutamine to 2-ketoglutarate (14). Depending on its state, NtrB is able to switch NtrC between the activating phosphorylated form and the repressing dephosphorylated form (33, 41). NtrA is an alternative sigma factor that confers specificity on core RNA polymerase for NtrA-specific promoter sequences (28, 31).

GlnB (P_{II} protein) plays a central role in coordinating the response of the Ntr system to combined nitrogen (13, 36, 37, 53). It is a tetramic protein with a subunit molecular weight of 12,387 daltons (29, 53) and exists in two interconvertible forms (13). Under nitrogen-deprived conditions, GlnB is uridylylated at a tyrosyl residue by GlnD,

a uridylyltransferase (UTase; [14, 53]). Under conditions of nitrogen excess GlnB is deuridylylated by GlnD (14). In the uridylylated form, GlnB stimulates the adenylyltransferase (ATase) activity of GlnE which mediates the deadenylylation (activation) of glutamine synthetase (GS; [4, 32]). In the deuridylylated form, GlnB acts in concert with GlnE to adenylylate (inactivate) GS (32). The deuridylylated GlnB also interacts with NtrB to dephosphorylate (inactivate) NtrC under conditions of nitrogen excess (23, 36). Thus, GlnB in the enteric bacteria is centrally involved in transmitting the nitrogen status of the cell to the GS modifying enzyme, GlnE, and the NtrC modifying enzyme, NtrB.

Expression of nitrogen assimilation capacity in *B. japonicum* is coordinated by separate regulatory networks that respond to nitrogen or oxygen limitation (2, 17, 39). The network responsive to nitrogen status shares many features with Ntr control in the *Enterobacteriaceae* (26, 36) and genes that have similar functional roles to the enteric *ntrC* and *ntrA* genes have been characterized from several rhizobial species (39, 44, 50, 51, 54). The regulatory network responsive to oxygen limitation is activated in symbiotic cells and probably relies on the positive regulator, NifA, to activate the genes controlled by this system (39).

Most members of the *Rhizobiaceae* contain two GS enzymes, GSI and GSII, encoded by *glnA* and *glnII* respectively (15, 16, 19, 24). In *B. japonicum* GSI is subject to adenylylation in response to ammonia whereas GSII is not known to be post-translationally modified (20, 24). Expression of *glnII* is controlled by both the nitrogen and oxygen

regulatory networks whereas *glnA* is constitutively expressed (39).

The adenylylation of GSI and the presence of NtrC suggest that a functional analog of the enteric *glnB* is present in *B. japonicum*. It was reported (18) that a region upstream from the *Rhizobium leguminosarum glnA* gene encodes a protein, part of which is highly homologous to a published ten-residue peptide sequence from the *E. coli glnB* gene product (49). Similar homologies were noted in published partial sequences upstream from the *glnA* genes from *Azospirillum basilense* and *B. japonicum glnA* (11, 16, 18).

Although the role of *glnB* in nitrogen regulation in the enteric bacteria is well characterized, its expression in response to environmental stimuli has not been studied. The possibility that a *glnB* gene exists in *B. japonicum* raises questions as to its role in this symbiont and how its expression may be integrated into rhizobial oxygen and nitrogen regulation. In this paper I report the isolation of the complete *B. japonicum glnB* gene, the *glnB* sequence, and the finding that the *B. japonicum glnB* is regulated by nitrogen status at tandem promoters. In addition, I show that *glnB* expression is regulated by a mechanism involving NtrC in both free-living and symbiotic cells.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions.

BJ110d is small-colony derivative of *B. japonicum* 3I1b110 (25). The *ntrC* mutants, BJ27147 and BJ3028, are derivatives of BJ110d with chromosomal insertions and have been described previously (39). Recombinant plasmids and phages are listed in Table 1. The complex

Table 1. Bacterial strains and plasmids.

Strain or plasmid	Description	Source or reference
B. japonicum strains		
BJ110d	Wild-type	(25)
BJ27147	*ntrC::nptII*, null mutant; Km^r	(37)
BJ3028	*ntrC::nptII*, truncated NtrC; Km^r	(37)
Plasmids (phages)		
M13*glnA*	391 bp *Sal*I fragment from *B. japonicum glnA* promoter (16) region cloned into M13mp18 (54)	(2)
M13*glnB*	1000 bp *Eco*RI - *Sst*I fragment from *B. japonicum glnB* region cloned into M13mp19 (54)	This study
λ61G	Recombinant phage carrying entire *glnB* and *glnA B. japonicum* genes in λBF101 (36)	(16)
pBJ47	Ap^r; 500 bp *EcoR*I - *Bam*HI fragment containing entire *B. japonicum glnB* cloned into pBR322 (36)	This study
pBJ299	Km^r, Ap^r; 2.3 kbp *Xho*I fragment containing the *B. japonicum glnB* and partial *glnA* from 61G cloned into pKC7 (44)	This study

medium YEMAN (YEM with 10 mM NH_4Cl and 0.5 mM KNO_3) has been described before (39). For analyzing gene expression in aerobic cultures, a mineral salts basal medium (39) was used supplemented with either 10 mM glutamate (XG) or 10 mM glutamate plus 10 mM NH_4Cl (XGA).

For microaerobic growth experiments, ten-liter cultures of *B. japonicum* in YEMAN were grown in Microferm fermentors (New Brunswick Scientific) agitated at 200 r.p.m., and sparged with 0.2% O_2 and 99.8% N_2 at a rate of 500 ml min^{-1}. The rate of gas flow was controlled using thermal mass flowmeters (Brooks Instruments, model 5850C). Each 10 liter culture was inoculated with 50 ml of a stationary-phase aerobic culture in YEM medium. All *B. japonicum* cultures were grown at 30°C. Isolation of bacteria from soybean nodules has been described previously (1).

DNA biochemistry. Construction of the *B. japonicum* lambda library in λBF101 and the isolation of recombinant phage λ6IG have been described (16). Phage particles were prepared by two rounds of cesium chloride block density gradient sedimentation (21) and phage DNA was extracted by formamide treatment. DNA restriction endonuclease fragments used in subcloning were isolated by separation on agarose gels and electroelution onto DE81 paper by the method of Dretzen (22). Standard procedures were used for restriction enzyme digestions, ligations and gel electrophoresis (38).

DNA sequencing strategy. The *B. japonicum glnB* locus is contained on the large insert of the recombinant lambda clone λ6IG that we have

described previously (Table 1, [14]). A 2.3 kbp *Xho*I fragment was subcloned from this lambda clone into pKC7 (46) to form pBJ299. The plasmid pBJ299 and a subclone, pBJ47, (Table 1) were used to determine the partial restriction endonuclease map shown in Fig. 1. The fragments indicated in Fig. 1 were subcloned into M13mp18 or M13mp19 and sequenced by the dideoxy method (52) using [^{35}S]dATP and either a primer hybridizing to the polylinker region (Bethesda Research Labs) or the primer, GM4, described below (S1 protection analysis). The sequence presented was determined from the analysis of both DNA strands.

RNA biochemistry. *B. japonicum* cells (1 g) were suspended in 10 ml of 4.0% sarkosyl, 0.1 M Tris-HCl, pH 8.0 and passed two times through a French pressure cell (Aminco; Silver Spring, MD) at 12,000 psi. For each milliliter of broken cell solution 1 g CsCl was added. RNA was isolated by discontinuous CsCl gradient centrifugation as described previously (3). The RNA pellet was drained thoroughly, redissolved in H_2O, and then extracted twice with phenol and four times with diethyl ether. Following ethanol precipitation, the RNA was resuspended in sterile H_2O and stored in aliquots at -70^{o}C.

S1 protection analysis. Levels of transcript were quantitated by the S1 nuclease protection method adapted from Berk and Sharp (9) and described previously (2) in which mRNAs transcribed from both *glnA* and *glnB* could be detected in a single reaction. Five micrograms of total cellular RNA was used per lane. Single-stranded, 5' end-labelled probes were synthesized by primer extension (2) using a *glnA*-specific

oligonucleotide primer described previously (2) or the *glnB*- specific primer GM4. The sequence of GM4 is as follows: 5'-TCCCGGCATTTCGATCA-3'. The recombinant M13 phages used in synthesizing the single-stranded DNA hybridization probes are described in Table 1. The protected fragment from S1 analysis with the *glnA* probe is 125 nucleotides long. Hybridization, S1 digestion, and identification of protected products were as described previously (2). The DNA sequence ladder used for sizing the *glnB* transcripts was produced by primer extension of GM4 using clone M13*glnB* (Table 1).

Computer analysis. The Pustell and Kafatos sequence analysis program (45) was used to search the upstream region for homologies to the *E. coli* consensus promoter, the Ntr consensus promoter, and the NtrC binding site consensus element. DNASIS and PROSIS (Hitachi Software Engineering Co.) were used to generate the predicted amino acid sequence and to optimize alignment of the amino acid sequences. Final alignment of the four amino acid sequences was done visually.

RESULTS

Nucleotide sequence of the *B. japonicum glnB* locus. The sequence of the 250 bp *Eco*RI-*Sal*I fragment that lies immediately upstream of *B. japonicum glnA* has been reported previously ([16]; Fig. 1). It was noted that this region contains an open reading frame (ORF) that shares sequence similarity with the *glnB* genes from several other species (18, 29). In order to complete the sequence of this ORF and to analyze the upstream region for promoter elements I subcloned and sequenced the

Figure 1. Physical map of the *B. japonicum glnB* region. The locations of the *glnB* and *glnA* genes are indicated, and transcription is from left to right. The arrows below the map indicate the sequencing strategy. The clone used for the synthesis of primer-extended probe (M13*glnB*) is shown along with the position of the oligonucleotide primer (GM4). The probe used for S1 mapping is indicated by a thin dashed line (---). Promoter regions: P_1, *glnB* promoter 1; P_2, *glnB* promoter 2; P_A, *glnA* promoter. Restriction endonuclease sites: B, *Bam*HI; E, *Eco*RI; S, *Sal*I; St, *Sst*I; X, *Xho*I.

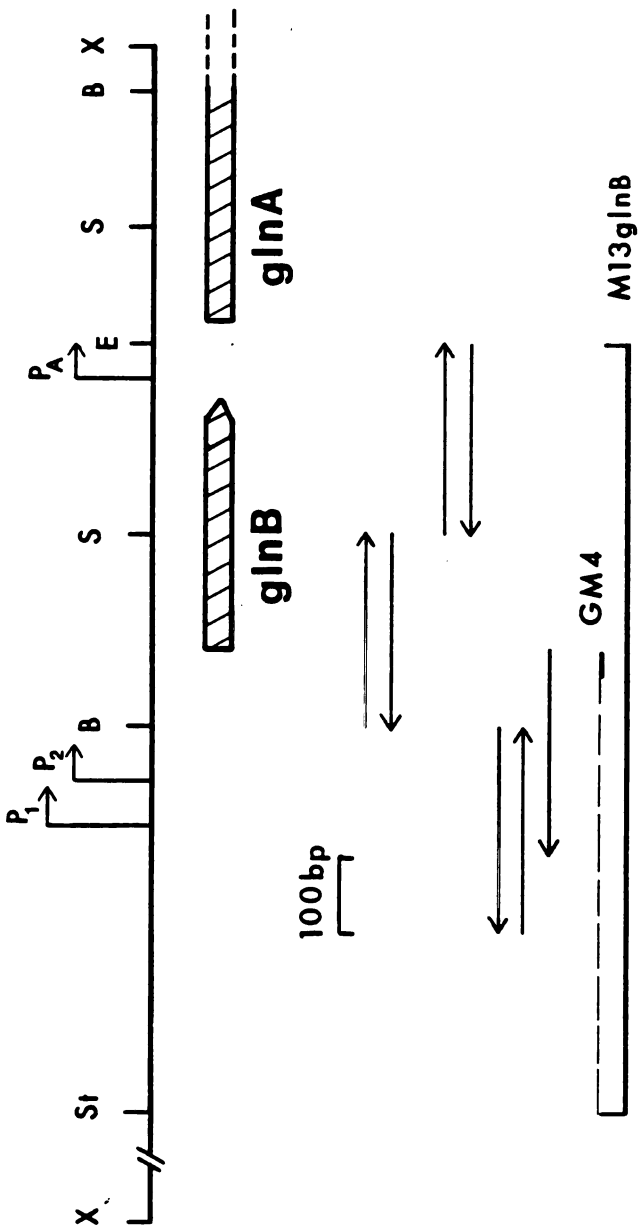

Figure 1.

fragments indicated in Fig. 1. The nucleic acid sequence of the complete ORF (Fig. 2) shows 67%, 66%, and 81% identity with the *glnB* genes from *K. pneumoniae*, *E. coli*, and *R. leguminosarum*, respectively (18, 29, 53). The GTG codon at position +168 was provisionally identified as the start codon of the ORF on the basis of its alignment with the start codons of *glnB* genes from the enteric bacteria (29, 53). Six base pairs upstream from this codon occurs a sequence with good homology to bacterial ribosome binding sites (Fig. 2; [34]). An ATG codon is present in the same reading frame six codons upstream from the designated GTG start codon, but it is not preceded by a characteristic ribosome binding site (Fig. 2). The stop codon of the ORF, a TGA, occurs at the same position as the TAA stop codon of the *glnB* genes from *K. pneumoniae* and *E. coli* if the *E. coli* sequence is modified as suggested by Holtel and Merrick (29, 53). The positioning of the stop codon here results in an overlap of the ORF with the postulated *B. japonicum glnA* promoter (Fig. 2; [16]). There is a 152 base pair intergenic region between the ORF and *glnA*.

Analysis of amino acid homologies. The protein that is predicted to be encoded by the ORF described above has extensive homology with the *glnB* products of *E. coli*, *K. pneumoniae* and *R. leguminosarum* (Fig. 3). The deduced molecular weight of the ORF product at 12,237 daltons is slightly smaller than the products of the enteric *glnB* genes (*K. pneumoniae*, 12,429 daltons, [29]; *E. coli*, 12,387 daltons; [29, 53]) and shows 70%, 69% and 83% identity with GlnB of *K. pneumoniae* (29), *E. coli* (29, 53), and *R. leguminosarum* (18), respectively (Fig. 3). If

Figure 2. Complete DNA sequence of the *B. japonicum glnB* gene and the 5' end of the *glnA* gene (16) with predicted amino acid sequences. The +1 denotes the first nucleotide of the transcript closest to the putative start codon. The likely ribosome binding sites (RBS) are denoted by overlining in front of the *glnB* and *glnA* start codons. The three transcriptional start sites are designated by asterisks (*). Homologies to the *E. coli* consensus promoter upstream of t_1 and t_{glnA}, and the Ntr consensus promoter upstream of t_2 are underlined. The position of the oligonucleotide primer, GM4, is indicated as is the region of homology to the consensus NtrC binding site (5, 47).

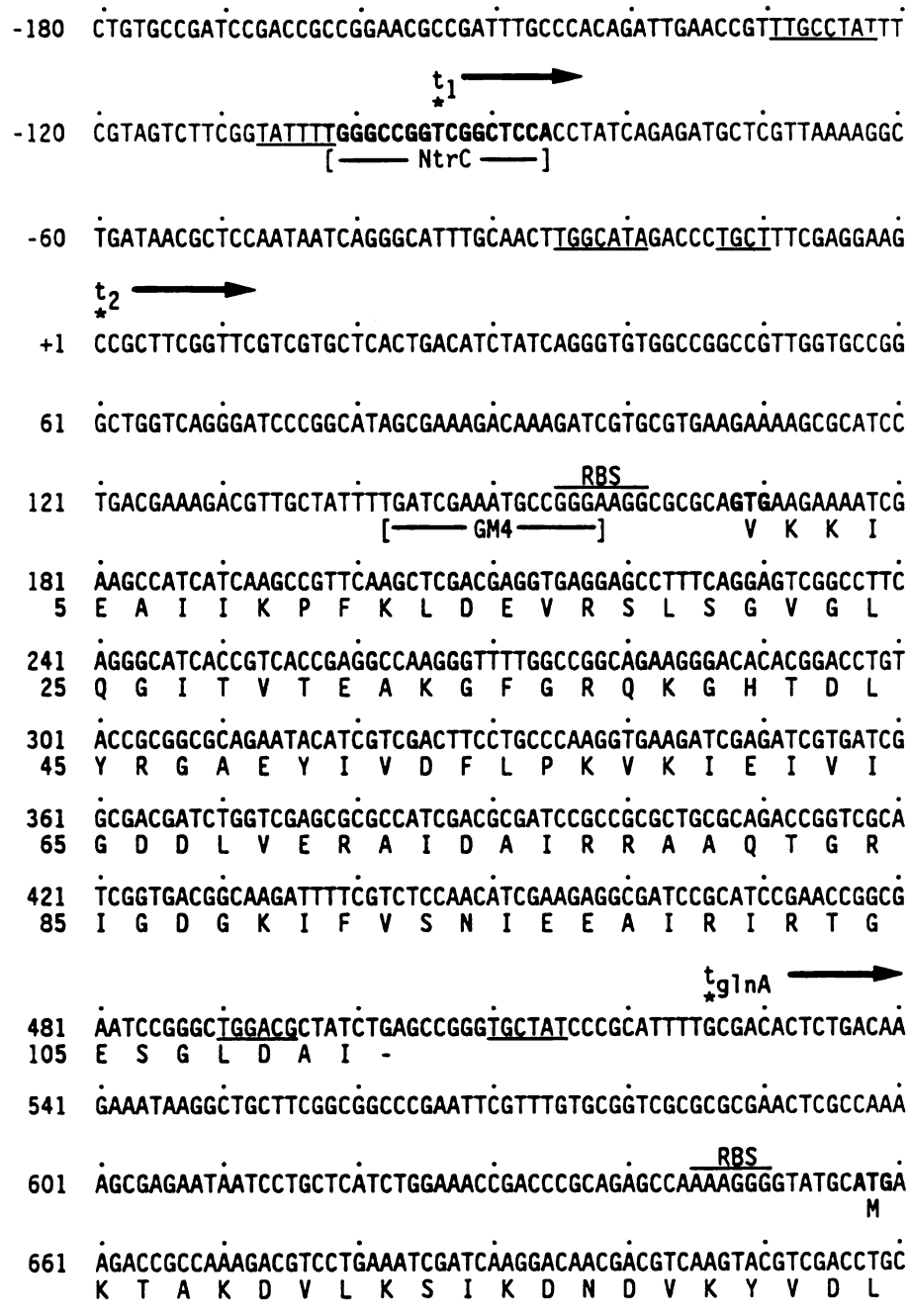
-180 CTGTGCCGATCCGACCGCCGGAACGCCGATTTGCCCACAGATTGAACCGTTTGCCTATTT
t1
-120 CGTAGTCTTCGGTATTTTGGGCCGGTCGGCTCCACCTATCAGAGATGCTCGTTAAAAGGC
[—— NtrC ——]
-60 TGATAACGCTCCAATAATCAGGGCATTTGCAACTTGGCATAGACCCTGCTTTCGAGGAAG
t2
+1 CCGCTTCGGTTCGTCGTGCTCACTGACATCTATCAGGGTGTGGCCGGCCGTTGGTGCCGG
61 GCTGGTCAGGGATCCCGGCATAGCGAAAGACAAAGATCGTGCGTGAAGAAAAGCGCATCC
RBS
121 TGACGAAAGACGTTGCTATTTTGATCGAAATGCCGGGAAGGCGCGCAGTGAAGAAAATCG
[—— GM4 ——] V K K I
181 AAGCCATCATCAAGCCGTTCAAGCTCGACGAGGTGAGGAGCCTTTCAGGAGTCGGCCTTC
5 E A I I K P F K L D E V R S L S G V G L
241 AGGGCATCACCGTCACCGAGGCCAAGGGTTTTGGCCGGCAGAAGGGACACACGGACCTGT
25 Q G I T V T E A K G F G R Q K G H T D L
301 ACCGCGGCGCAGAATACATCGTCGACTTCCTGCCCAAGGTGAAGATCGAGATCGTGATCG
45 Y R G A E Y I V D F L P K V K I E I V I
361 GCGACGATCTGGTCGAGCGCGCCATCGACGCGATCCGCCGCGCTGCGCAGACCGGTCGCA
65 G D D L V E R A I D A I R R A A Q T G R
421 TCGGTGACGGCAAGATTTTCGTCTCCAACATCGAAGAGGCGATCCGCATCCGAACCGGCG
85 I G D G K I F V S N I E E A I R I R T G
tglnA
481 AATCCGGGCTGGACGCTATCTGAGCCGGGTGCTATCCCGCATTTTGCGACACTCTGACAA
105 E S G L D A I -
541 GAAATAAGGCTGCTTCGGCGGCCCGAATTCGTTTGTGCGGTCGCGCGCGAACTCGCCAAA
RBS
601 AGCGAGAATAATCCTGCTCATCTGGAAACCGACCCGCAGAGCCAAAAGGGGTATGCATGA
M
661 AGACCGCCAAAGACGTCCTGAAATCGATCAAGGACAACGACGTCAAGTACGTCGACCTGC
K T A K D V L K S I K D N D V K Y V D L

Figure 3. Analysis of amino acid homologies between the *B. japonicum glnB* gene product (Bj) and the *glnB* gene products from *R. leguminosarum* (Rl; 18), *K. pneumoniae* (Kp; 29) and *E. coli* (Ec; 53). Identical residues are indicated by asterisks (*) in adjacent polypeptides and by colons (:) in non-adjacent polypeptides. The predicted translation product of the *E. coli glnB* has been modified following Holtel and Merrick (29). The site of uridylylation of the *E. coli glnB* gene product, a tyrosyl residue (53), is indicated by an arrow.

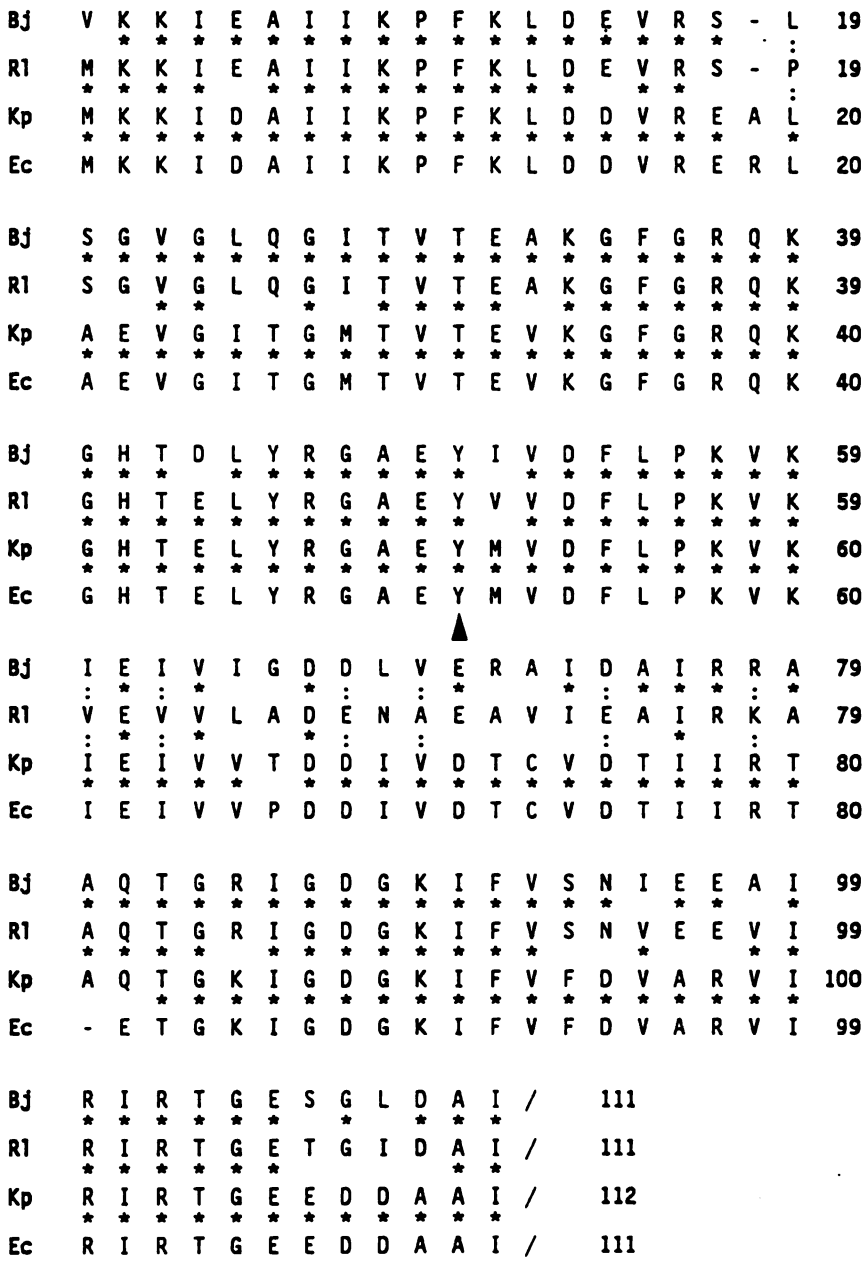
Bj V K K I E A I I K P F K L D E V R S - L 19
R1 M K K I E A I I K P F K L D E V R S - P 19
Kp M K K I D A I I K P F K L D D V R E A L 20
Ec M K K I D A I I K P F K L D D V R E R L 20
Bj S G V G L Q G I T V T E A K G F G R Q K 39
R1 S G V G L Q G I T V T E A K G F G R Q K 39
Kp A E V G I T G M T V T E V K G F G R Q K 40
Ec A E V G I T G M T V T E V K G F G R Q K 40
Bj G H T D L Y R G A E Y I V D F L P K V K 59
R1 G H T E L Y R G A E Y V V D F L P K V K 59
Kp G H T E L Y R G A E Y M V D F L P K V K 60
Ec G H T E L Y R G A E Y M V D F L P K V K 60
Bj I E I V I G D D L V E R A I D A I R R A 79
R1 V E V V L A D E N A E A V I E A I R K A 79
Kp I E I V V T D D I V D T C V D T I I R T 80
Ec I E I V V P D D I V D T C V D T I I R T 80
Bj A Q T G R I G D G K I F V S N I E E A I 99
R1 A Q T G R I G D G K I F V S N V E E V I 99
Kp A Q T G K I G D G K I F V F D V A R V I 100
Ec - E T G K I G D G K I F V F D V A R V I 99
Bj R I R T G E S G L D A I / 111
R1 R I R T G E T G I D A I / 111
Kp R I R T G E E D D A A I / 112
Ec R I R T G E E D D A A I / 111

Figure 3.

conserved amino acid substitutions are taken into account the *B. japonicum* similarities are: 87%, 86%, and 94%. In GlnB from *E. coli*, the tyrosyl residue at position 51 of the protein sequence is the amino acid that is uridylylated by GlnD (53). A tyrosyl residue occurs at this same position in the ORF product of *B. japonicum* and in the GlnB of the other two species (Fig. 3). The residues surrounding the tyrosine are also highly conserved in all the species. From this evidence I conclude that the 333 base pair ORF encodes a protein that could function as does GlnB in the enteric bacteria. I will refer to this ORF as *glnB*.

Ntr-regulated expression of *B. japonicum glnB*. S1 protection experiments were conducted in order to investigate the expression of *glnB* under different culture conditions (Fig. 4). A primer-extended probe specific to the 5' end of *glnB* (Fig. 1) was used to protect RNA isolated from *B. japonicum* 110d cells that were grown under conditions of nitrogen-deficiency (in XG medium) or nitrogen-sufficiency (in XGA medium). I observed two different *glnB* transcripts, t_1 and t_2, depending on the nitrogen status of the cultures (Fig. 4, lane 1, 2). The t_1 transcript was not present in glutamate-grown (XG) cells but appeared in ammonia-grown (XGA) cells. The shorter transcript, t_2, appeared in glutamate-grown cells but was much less abundant in ammonia-grown cells. A *glnA* specific probe, which was included in these experiments as a control, protected a relatively constant amount of RNA (Fig. 4).

Figure 4. Expression of the *B. japonicum glnB* and *glnA* genes in free-living cultures and in bacteria isolated from nodules. Samples of RNA purified from BJ110d (lanes 1, 2, 7 and 9), BJ27147 (lanes 3, 4 and 8), BJ3028 (lanes 5, 6) or yeast tRNA (lane 10) were hybridized to ^{32}P-labeled single stranded probes specific for the 5' ends of *glnB* or *glnA* and analyzed by the S1 nuclease protection method (see Materials and Methods). The migration positions of the protected *glnB* fragments (t_1 and t_2) and the *glnA* protected fragment are indicated. The topmost bands represent residual undigested probe of *glnB* (top band) and *glnA* (second band from the top). Lanes: 1, 3 and 5, XG (nitrogen-deficient cultures); 2, 4 and 6, XGA (nitrogen-sufficient cultures); 7 and 8, bacteria isolated from soybean nodules; 9, YEMAN, microaerobic cultures (nitrogen-sufficient, 0.2% O_2); 10, yeast tRNA.

Figure 4.

The differential expression of the t_2 transcript in response to nitrogen status is typical of regulation mediated by the activator form of NtrC. I therefore looked at *glnB* expression in response to nitrogen status in two *B. japonicum ntrC* mutants, BJ27147 and BJ3028 (Table 1). Regardless of the culture nitrogen status, both strains produced only the longer transcript, t_1 (Fig. 4, lanes 3-6). Thus, in aerobic, nitrogen-starved cultures of free-living *B. japonicum*, NtrC is required for expression of the t_2 transcript, and apparently represses t_1.

Previous observations in our laboratory, have indicated that separate regulatory systems responsive to nitrogen or oxygen status control gene expression in free-living and symbiotic cells (39). I therefore wished to determine how *glnB* expression was controlled in symbiotic cells. S1 experiments indicated that the t_2 transcript was the predominant transcript in wild type (BJ110d) bacteria isolated from soybean nodules (Fig. 4, lane 7). However, in nodules infected with the NtrC null mutant, BJ27147, there was a marked decrease in the t_2 transcript and an increase in the t_1 transcript, similar to the pattern of expression observed with this strain under aerobic conditions (Fig. 4, lane 8). These observations indicate that expression of the *glnB* t_2 transcript in bacteroids involves NtrC activation similar to its expression in aerobic, nitrogen-deprived cultures. In addition, I found that t_1 was the predominant transcript in microaerobic, ammonia-grown cultures of wild-type *B. japonicum*, as it was in aerobic, ammonia-grown cultures (Fig. 4, lane 9). These results indicate that oxygen regulation is probably not involved in expression of the *glnB* t_2 transcript under microaerobic or symbiotic conditions. Rather, expression of this

transcript appears primarily responsive to nitrogen status.

The position of *glnA* downstream from *glnB* in *B. japonicum* raises the question of whether the two genes may, under some conditions, be co-transcribed. If this were the case, then a significant fraction of the *glnA* probe used in S1 experiments (Fig. 4) should be fully protected. In fact, the abundance of full-length *glnA* probe never exceeded that observed in the control digestion (Fig. 4, lane 10).

Promoter mapping and analysis. The two 5' ends of the *B. japonicum glnB* transcripts were mapped by S1 protection experiments (Fig. 5) using the GM4 primer-extended probe depicted in Fig. 1. The transcripts, t_1 and t_2, were mapped to positions 261 bp and 157 bp, respectively, upstream of the putative start codon (Fig. 2, 5). Sequence analysis of the upstream region surrounding these transcriptional start sites revealed a characteristic *E. coli* consensus-type promoter sequence (27) preceding the t_1 start site and a typical Ntr promoter sequence (6) preceding the t_2 start site (Fig. 6). I designate the upstream promoter *glnB*p1 and the downstream promoter *glnB*p2. In addition, a region was found between nucleotides -86 to -102 that matches the consensus NtrC binding site (5'-TGCACCnnnnTGGTGCA-3') from the *Enterobacteriaceae* in 8 of the 13 conserved positions (5, 47; Fig. 2). Interestingly, this sequence overlaps the transcriptional start site of t_1, suggesting that the observed NtrC-dependent repression of t_1 under conditions of nitrogen starvation may be the direct result of NtrC binding to this region.

Figure 5. Determination of the 5' ends of the two *glnB* transcripts. A single stranded probe specific to the *glnB* 5' end was generated by primer extension of the oligonucleotide GM4 using M13*glnB* as a template (see Materials and Methods). This probe was hybridyzed with total bacterial RNA isolated from *B. japonicum* 110d cells that were grown in glutamate (XG) or ammonia (XGA) cultures. Transcripts t_1 and t_2 are indicated. The DNA sequence ladder was produced by primer extension of GM4. Lanes: 1 through 4 show products of the G, A, T, and C sequencing reactions, respectively; 5, BJ110d (XG); 6, BJ110d (XGA).

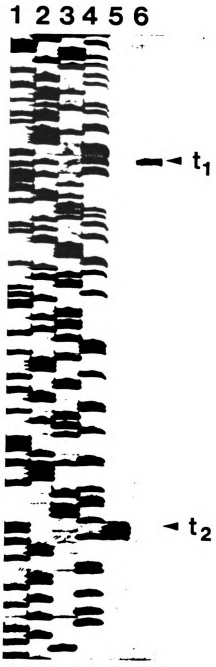

Figure 5.

Figure 6. Analysis of the promoters of the *B. japonicum glnB* gene. A.) The *glnB*p1 promoter is aligned to maximize homology with the *B. japonicum glnA* promoter (16) and the *E. coli* consensus promoter (27). Upper case letters in the *E. coli* sequence indicate highly conserved bases among *E. coli* promoters, and upper case letters in the *B. japonicum* sequences indicate homologies to the *E. coli* consensus promoter or to the other *B. japonicum* promoter. **B.)** The *glnB*p2 promoter is aligned to maximize homology with the Ntr consensus promoters from the enteric bacteria (6), *B. japonicum* (17) and the *glnII* promoter from *B. japonicum* (17). The asterisks (*) indicate points of transcriptional initiation; R denotes a purine, Y a pyrimidine, and N any nucleotide.

A.

E. coli consensus	TTGACat -N_{10}- t-tg-TAtAaT-------*
Bj *glnA*	TgGACgct -N_{10}- g-TG-TATccc-------*
Bj *glnB*p1	**TTGcCtAT -N_9- T-cG-TATttT-------***

B.

Bj Ntr consensus	---CC-TGGCACGCNAGTTGCT-AT------***
Enteric Ntr consensus	-----CTGGYAYRNNNNTTGCA—N_{6-11}—*
Bj *glnII*	------TGGCACGCtAaaTGCTtgT------*
Bj *glnB*p2	**----C-TGGCATAgacccTGCT—N_{10}———***

DISCUSSION

Several lines of evidence indicate that the open reading frame I have described encodes the *B. japonicum glnB* gene. First, the nucleic acid sequence is very similar to the sequences of the *glnB* genes from *E. coli* and *K. pneumoniae*. Second, the amino acid sequence is very highly conserved, particularly in regions that play a functional role in the GlnB protein from *E. coli*. Finally, a requirement for a GlnB protein in the rhizobia may be inferred from the presence of several components of an Ntr system. Members of the *Rhizobiaceae* contain functional homologs to the regulatory genes *ntrA*, *ntrB*, *ntrC*, and *B. japonicum* GSI is adenylylated in response to excess nitrogen in the medium (20, 24, 39, 50, 54).

The physiological role of GlnB in *B. japonicum* remains to be established. It should be possible to clarify the function of this protein by studying a *B. japonicum* strain that has a *glnB* insertional mutation. I have constructed various mutant *glnB* alleles *in vitro* by inserting drug resistance markers into the unique *Sal*I site of the open reading frame. However, despite numerous attempts with an established conjugative mating system, I have been unable to identify a recombinant that resulted from a double cross-over event. The difficulty in constructing a mutant may indicate that the GlnB protein plays an essential role in the regulation of nitrogen assimilation or some other process in *B. japonicum*.

A transcriptional analysis of the *glnB* gene indicated that transcription is initiated from two closely spaced, but independently regulated promoters. One promoter, *glnB*p2, is expressed under

conditions of nitrogen-deficiency, and its expression is NtrC-dependent. The other promoter, *glnB*p1, is expressed under conditions of nitrogen-sufficiency, and its repression is NtrC-dependent. The *glnB*p1 promoter is similar to an *E. coli* consensus promoter (27; Fig. 6). The *glnB*p2 promoter is similar to an Ntr-regulated promoter consensus derived from several nitrogen-regulated *K. pneumoniae* genes (6; Fig. 6). Transcription start sites have not been determined for the same region from *R. leguminosarum*, but a similar Ntr promoter consensus sequence is present (18). The regulation of the *glnB* genes from *K. pneumoniae* and *E. coli* has not been investigated (29, 53).

It is unclear what physiological significance may be attached to the dual regulation of *glnB*. The transcript abundance of *glnB* remains relatively constant regardless of which promoter is active (Fig. 4). It is possible that the two promoters are required simply to maintain a constant level of *glnB* product under different nitrogen conditions. This would be necessary if, for example, GlnB is required during growth on a good source of nitrogen, since under these conditions the *B. japonicum* Ntr promoter is only weakly activated (Fig. 4).

Tandem promoters are commonly observed in *E. coli* when a gene is expressed constitutively and under some global control, or when regulated by two global systems (30). For example, the *glnA* gene from *E. coli* is regulated by tandem promoters (47). The *glnA* upstream promoter, p1, is a consensus-type promoter and is repressed by NtrC under conditions of carbon deficiency and nitrogen excess (47). The *glnA* downstream promoter, p2, is a characteristic Ntr promoter and requires NtrC for expression under conditions of nitrogen limitation

(47). The structure and expression of the *E. coli glnA* tandem promoters is thus very similar to the *B. japonicum glnB* promoters. The presence of the *glnB* gene upstream of *glnA* in *B. japonicum*, however, apparently does not have physiological significance since little, if any, co-transcription can be detected. Moreover, the *B. japonicum glnA* has its own promoter and the transcript abundance of *glnA* does not change dramatically in response to nitrogen deprivation as it does in *E. coli* (17, 47). Rather, the NtrC-activated induction of *glnA* in *E. coli* in response to nitrogen limitation, is replaced in *B. japonicum* by the NtrC-activated expression of *glnII* (39).

The sequence upstream of *glnB* has two potential NtrC-binding sites. One, overlaps the *glnB* t_1 start site and is similar to a known NtrC binding sequence that overlaps the start site of *ntrB* in *E. coli* (55). A similar consensus element overlaps the *E. coli glnA*p1 start site (47). Since both *ntrB* and *glnA*p1 promoters are repressed by NtrC it seems reasonable to postulate that the NtrC binding consensus element overlapping the *B. japonicum glnB*p1 start site is also repressed by NtrC under nitrogen deprived conditions. The appearance in the *ntrC* mutants, BJ27147 and BJ3028, of transcript t_1 under nitrogen-deprived conditions, in contrast to wild-type *B. japonicum*, supports this conjecture (Fig. 4). BJ27147 does not produce NtrC protein, while BJ3028 constitutively produces an NtrC that is truncated at the carboxy-terminus (39). This truncated NtrC is thought to lack the repressor DNA binding domain (39). Another less symmetrical NtrC binding sequence element occurs at position +42 to +58 (Fig. 2). This may be indicative of multiple NtrC binding sites upstream of *glnB* that function in increasing the local

concentration of NtrC, similar to those surrounding the *E. coli glnA* promoters (48).

It was surprising to find that NtrC participated in the regulation of *glnB* in *B. japonicum* bacteroids isolated from soybean nodules. Other genes shown to be regulated by NtrC in free-living cells of *R. meliloti* or *B. japonicum* are not so regulated in symbiotic cells (39, 54). This would make sense if the intracellular nitrogen status of bacteroids is high as is generally thought to be the case (12). This supposition is based on the results of previous workers who found a low specific activity of glutamine synthetase in extracts from rhizobial cultures grown with excess ammonia or extracts from bacteroids, but a high specific activity in extracts from nitrogen-starved cultures (12). They concluded from these observations that intracellular nitrogen in symbiotic cells is not limiting. The observation that NtrC is involved in the expression of *glnB* in nodules raises two contrasting possibilities. First, if the intracellular nitrogen status is indeed high in bacteroids, then the involvement of NtrC indicates that this regulatory protein can be active in this nitrogen-sufficient environment, in contrast to its inactivity in nitrogen-sufficient free-living cells (39, 54). It would follow from this that in nodules *ntrC* may be expressed (or NtrC protein may be activated) in response to a signal independent of the intracellular nitrogen status. A second possibility is that intracellular nitrogen status is not high in nodules as has been thought. If this were the case then *ntrC* could be expressed, as it is in free-living cells, in response to nitrogen deprivation. The report that at least 94% of ammonia is exported from

bacteroids (42) would support the notion that bacteroid cells experience nitrogen deprivation.

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SUMMARY

The major conclusions of this dissertation are illustrated in Figure 1 and will be summarized here.

1. Two glutamine synthetase enzymes are present in *Bradyrhizobium japonicum* but either one alone provides glutamine prototrophy and enables the bacterium to establish an effective symbiosis with soybean.

2. Separate regulatory networks exist in *Bradyrhizobium japonicum* for activating expression of *glnII* in free-living and symbiotic cells. The *ntrC* gene product (NtrC) is involved in *glnII* transcription in aerobic free-living cells while another positive regulator, probably NifA, fulfills this role in oxygen-starved and symbiotic cells.

3. NtrC is also involved in nitrate utilization. Whether this is by direct regulation of the nitrate reductase promoter (P_{nar}) or indirectly via a repressor is unknown.

4. The *Bradyrhizobium japonicum glnA* gene is not regulated in response to nitrogen status and, more specifically, is not under NtrC or NifA control.

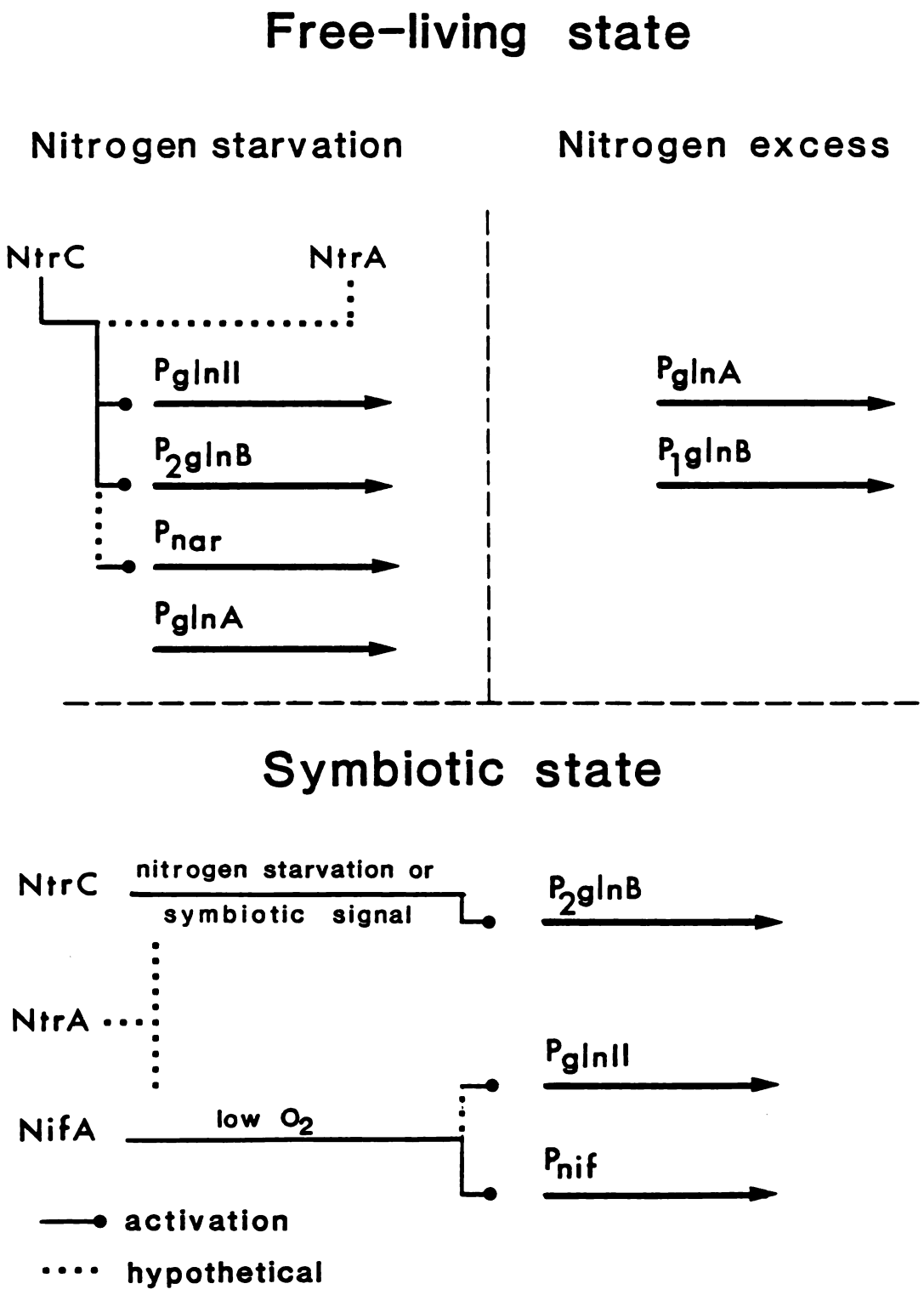

Figure 1. Model of nitrogen regulation (Ntr) in Bradyrhizobium japonicum.

5. A gene sharing sequence similarity with the enteric nitrogen regulatory gene, *glnB*, is present in *Bradyrhizobium japonicum* and is regulated at tandem promoters in response to nitrogen status. This regulation involves both NtrC repression (at promoter P_1) and activation (at promoter P_2).

6. The *glnB* gene is also activated at promoter P_2 by NtrC in soybean nodules. The involvement of NtrC in nodule gene expression raises the possibility that, in contrast to prevailing views, bacteroids do not sense excess nitrogen.