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CHARACTERIZATION OF THE GENES ENCODING GLUTAMINE SYNTHETASE I AND GLUTAMINE SYNTHETASE II FROM BRADYRHIZOBIUM JAPONICUM

By

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ABSTRACT

CHARACTERIZATION OF THE GENES ENCODING
GLUTAMINE SYNTHETASE I AND GLUTAMINE SYNTHETASE II
FROM BRADYRHIZOBIUM JAPONICUM

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Bacteria of the genera Rhizobium and Bradyrhizobium are able to fix nitrogen in symbiotic association with leguminous plants. The repression of bacterial glutamine synthetase (GS) allows newly fixed nitrogen to be exported from the symbiotic bacteroids to the host plant. Therefore, the regulation of GS is an important part of the development of an effective symbiosis. In this dissertation, I describe the isolation and characterization of the genes encoding the two GS isozymes, GSI and GSII, from Bradyrhizobium japonicum, the soybean symbiont. The gene encoding GSI, glnA, was identified in a genomic library using a fragment of the Escherichia coli glnA gene as a hybridization probe. The B. japonicum glnA gene was found to have extensive homology to E. coli glnA, and when expressed in E. coli, produced large amounts of a GS with properties identical to that of GSI of B. japonicum. S1 nuclease protection experiments indicate that glnA is constitutively transcribed from a single promoter. The gene encoding GSII, glnII, was identified in a cosmid library using a mixed oligonucleotide hybridization probe based on a partial amino acid sequence determined from purified GSII. Amino acid sequence comparisons revealed extensive homology between GSII and eucaryotic glutamine synthetases, suggesting that GSII evolved as the result of a eucaryote to procaryote gene transfer event. Quantification of glnII mRNA in

nutrient limited cultures demonstrates that glnII is transcriptionally activated in response to nitrogen starvation or nitrogen limited growth. The site of transcriptional initiation upstream of glnII was mapped by S1 nuclease protection. The glnII promoter has no homology to the glnA promoter but has homology to B. japonicum nif promoters in the RNA polymerase binding region. Promoter structure and gene expression are discussed with respect to possible models of glnII and nif regulation during symbiotic nitrogen fixation.

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CHAPTER 1

Introduction

Bacteria of the genera Rhizobium and Bradyrhizobium are unique in their ability to fix nitrogen in symbiotic association with leguminous plants. During the development of this symbiosis, the bacteria undergo metabolic and morphologic transformations to become bacteroids, the intracellular "organelles" where nitrogen fixation occurs. Concurrent with this bacterial cell differentiation, root cells at the site of infection develop to form nodules, the multicellular organs which provide the specialized environment required for rhizobial nitrogen fixation. The legume-rhizobium symbiosis is species specific and is presumably coordinated by a series of complex interactions between the procaryotic and eucaryotic symbionts. The formation of effective nodules, therefore, can be viewed as a developmental pathway requiring the regulation of many genes and enzymes in both the bacterium and the host plant. A complete understanding of nodule development is necessary in order to develop fully the tremendous potential of the rhizobium-legume symbiosis. Because of the bacteroid's role in nitrogen fixation, the regulation of plant and bacterial nitrogen metabolism is of special interest.

In the bacteroid, atmospheric nitrogen is reduced to ammonia by nitrogenase, a complex enzyme system consisting of component I, which contains the active site of dinitrogen reduction, component II, which is involved in electron transport, and a set of nitrogenase specific cofactors. The coordinated expression of at least 18 genes (designated as nif genes) is required for the synthesis of nitrogenase and its associated cofactors in Klebsiella pneumoniae (Roberts & Brill, 1981) and presumably also in Rhizobium and Bradyrhizobium species. All known nitrogenases are oxygen labile. Bacteria which fix nitrogen in aerobic environments have developed various mechanisms for protecting their nitrogenase from O_2 inactivation. Rhizobia depend on the plant encoded, oxygen binding protein leghemoglobin, which maintains a very low concentration of free oxygen in the nodule, thus protecting bacteroid nitrogenase from O_2 inactivation. Breaking the triple bond of dinitrogen is a very energy expensive process, requiring approximately 12 molecules of ATP and six electons for each N_2 fixed (Mortenson & Thorneley, 1979). The plant provides a respirable energy source for the nitrogen fixing bacteroids in the form of dicarboxylic acids (Ronson et al., 1981). The oxygen required for bacteroid respiration is transported through the nodule by facilitated diffusion using leghemoglobin as a carrier protein (Bergersen, 1980). Thus, leghemoglobin serves the dual function of protecting the oxygen labile nitrogenase and transporting oxygen for bacteroid respiration.

In order for rhizobium to function as an effective nitrogen fixing symbiont, it is necessary for the bacteroid to export fixed nitrogen for utilization by the host plant. Isotopic tracing studies indicate that as much as 94% of the nitrogen fixed by bacteroids in legume root

nodules is exported to the plant (O'Gara & Shanmugam, 1976). The primary pathway of ammonia assimilation is by the coordinated activity of glutamine synthetase (GS) and glutamate synthase (GOGAT; Tyler, 1978). GS catalyzes the production of glutamine from glutamate and ammonia. GOGAT produces two molecules of glutamate from glutamine and 2-ketoglutarate. During the development of nitrogen fixing bacteroids, GOGAT activity remains relatively constant but GS activity is repressed in concert with the derepression on nitrogenase (Brown & Dilworth, 1975; Robertson et al., 1975; Upchurch & Elkan, 1978). As a result, the ammonia formed by the nitrogenase complex is exported from the bacteroid to the plant cell where it is assimilated by a very abundant, nodule specific glutamine synthetase (Brown & Dilworth, 1975; Cullimore et al., 1983; Evans & Crist, 1984; Lara et al., 1983; McParland et al., 1976; O'Gara & Shanmugam, 1976; Upchurch & Elkan, 1978). Because GS is the first enzyme of the ammonia assimilation pathway, its regulation is critical in the control of overall nitrogen metabolism. Some bacteria use glutamate dehydrogenase (GDH), which catalyzes the formation of glutamate from 2-ketoglutarate and ammonia, to assimilate ammonia present at high concentrations (Tyler, 1978). In many strains of rhizobia, however, GDH is only used for glutamate catabolism and GS/GOGAT is the only pathway of ammonia assimilation (Howitt & Gresshoff, 1985; Ludwig, 1976; O'Gara & Shanmugam, 1976; Vairinhos et al., 1983).

The regulation of GS in rhizobia is unusual when compared to non-symbiotic nitrogen fixing bacteria, such as Klebsiella pneumoniae, which activate both GS and nitrogenase during nitrogen starvation. It has been suggested that the ability to simultaneously activate ammonia

production (nitrogenase) and repress ammonia assimilation (GS) is an adaptation of rhizobial nitrogen metabolism necessary for symbiotic nitrogen fixation (Ludwig & Signer, 1977; O'Gara & Shanmugam, 1976). Although the export of ammonia by bacteroids is logical in terms of their role as symbiotic nitrogen fixing "organelles," it is not immediately apparent how rhizobia could have evolved this apparently altruistic behavior. Kahn et al. (1985) have suggested that bacteroid nitrogen fixation is not altruistic but rather is necessary for self feeding. According to their model, nitrogen in the nodule is used as a carrier of carbon into the bacteroids, entering as amino acids and excreted as ammonia. The purpose of nitrogen fixation is to replace the nitrogen that has been utilized by the plant to ensure a continual flow of carbon into the bacteroid. Thus, nitrogen fixation is a response to carbon rather than nitrogen starvation.

The excretion of nitrogen is not unusual among diazotrophic bacteria. Some species of Bradyrhizobium are able to fix nitrogen ex planta, but the reduced nitrogen is excreted into the culture medium and not utilized for growth (Evans & Crist, 1984; Ludwig, 1980a; O'Gara & Shanmugam, 1976; Upchurch & Elkan, 1978). Some actinomycetes can fix nitrogen in a symbiotic association with non-leguminous plants similar to the rhizobium-legume symbiosis (Akkermans & Roelofsen, 1980). The filimentous blue-green algae Anabaena fix nitrogen in terminally differentiated cells termed heterocysts (Haselkorn, 1978). The heterocysts "sacrifice" themselves in order to fix nitrogen for the benefit of other cells in the culture. The development of specialized nitrogen fixing cells is characteristic of nitrogen exporting diazotrophs. In contrast to rhizobial bacteroids which repress GS and export ammonia, Anabaena

heterocysts produce large amounts of GS and export fixed nitrogen in the form of glutamine (Thomas et al., 1977). This observation supports the conclusion that the regulation of GS is an important component of bacteroid metabolism.

The study of GS regulation in rhizobia is complicated by the presence of two species of GS, designated GSI and GSII (Darrow & Knotts, 1977). GSI, in all respects, is typical of procaryotic glutamine synthetases. It has 12 subunits of 59,000 daltons and is post translationally modified by a regulatory system similar to that of E. coli GS in which any or all of the 12 subunits can be repressed by the adenylylation of a specific tyrosine residue (Darrow, 1980; Darrow & Knotts, 1977; Ludwig, 1980b). Furthermore, GSI will cross-react with antibodies raised against E. coli GS (Tronick et al., 1973). Although the synthetic activity of GSI is regulated by adenylylation, the enzyme is produced at a relatively constant level (Ludwig, 1980b). The gene encoding GSI, termed glnA, has been isolated from Rhizobium meliloti and is homologous to the glnA gene of E. coli (Somerville & Kahn, 1983).

Whereas GSI is similar to glutamine synthetases from all bacteria, GSII is found only in the Rhizobiaceae family, which includes the genera Rhizobium, Bradyrhizobium, Agrobacterium, and Phyllobacterium (Fuchs & Keister, 1980). GSII is encoded by a gene distinct from the gene encoding GSI (Darrow, 1980; Somerville & Kahn, 1983). The structure of GSII (8 subunits of 36,000 daltons) is characteristic of eucaryotic glutamine synthetases (DeVries et al., 1983). DeVries et al. (1983) demonstrated that GSII of Rhizobium leguminosarum will cross-react with antibodies raised against Pisum satuvum GS and suggested that the gene encoding GSII may be of eucaryotic origin. However, this hypothesis was

not confirmed by Cullimore and Miflin (1984) who found that Rhizobium phaseoli GSII would not cross-react with antibodies raised against Phaseolus vulgaris GS and that a partial cDNA clone of the gene encoding P. vulgaris GS had no detectable homology to R. phaseoli genomic DNA. Thus the origin of GSII remains a mystery.

GSI and GSII are differentially regulated in response to a number of different stimuli, including nitrogen source, carbon source, oxygen concentration, and symbiotic development (Cullimore et al., 1983; Darrow et al., 1981, Fuchs & Keister, 1980; Ludwig, 1980b; Rao et al., 1978). GSI activity is regulated by the adenylylation cascade system. In contrast, there is no known regulation of GSII by post translational modification. Therefore, changes in total cellular GSII activity are presumably due to the regulation of transcription of the gene encoding GSII or translation of its mRNA.

It is not yet clear what function GSII serves in rhizobial nitrogen metabolism. A R. meliloti glnA deletion strain, which has no GSI, grows normally, indicating that GSII alone is able to satisfy all of the ammonia assimilation needs of the cell (Somerville & Kahn, 1983). This is in contrast to a report by Ludwig (1980b) who found that GSII did not function in ammonia assimilation. Darrow et al. (1981) have suggested that GSII provides extra ammonia assimilatory capacity during nitrogen limited growth. This conclusion is based on the observation that GSII is induced in B. japonicum when grown on poor nitrogen sources, such as amino acids, or good carbon sources, such as succinate and gluconate. The concentration of oxygen in the culture medium also seems to affect the regulation of GSII. Rao et al (1978) found that, at O_2 concentrations below 0.40%, GSII levels decline dramatically in concert with the

adenylylation of GSI. The repression of GSII at low oxygen concentrations may be especially significant since similar conditions exist in root nodules. In all cases that have been examined, GSII repression is concurrent with an increase in GSI adenylylation. The simultaneous repression of GSI and GSII suggests the presence of a general nitrogen regulatory system which can control both gene transcription and protein modification.

The regulation of bacterial nitrogen metabolism has been most extensively studied in the enteric bacteria Klebsiella pneumoniae and Escherichia coli (Alvarez-Morales et al., 1984; Magasanik, 1982). In these bacteria, GS and other enzymes, responsible for the catabolism of various nitrogen sources, are under the control of a complex regulation system, termed Ntr. Ntr regulated genes include argT (arginine uptake), hisJQHP (histidine uptake), hutUH (histidine utilization) and nif (nitrogen fixation). This group of coordinately regulated, unlinked genes is sometimes referred to as the Ntr regulon. All genes activated by the Ntr system require the Ntr specific sigma factor, σ^{60}, which substitutes for the normal sigma factor and directs RNA polymerase binding and transcriptional initiation at Ntr promoters (Hirschman et al., 1985; Hunt & Magasanik, 1985). σ^{60} is the product of the rpoN gene (formerly designated glnF and ntrA). All Ntr activated genes have DNA sequence homology in the region 10 to 30 base pairs (bp) upstream of the RNA start site. The consensus sequence (-27 CTGGCAC-N_5-TTGCA -10) defines the Ntr promoter (Dixon, 1984b; Ow et al., 1983).

RNA polymerase and σ^{60} alone are insufficient for transcription from Ntr promoters. An additional nitrogen regulatory protein, NRI, is required. NRI is the product of the ntrC gene (also designated glnG;

Reitzer & Magasanik, 1983). NRI is a dimeric protein which binds double stranded DNA at the sequence 5'-TGCACC$\overset{AAAA}{TTTT}$TGGTGCA-3' (Ames & Nikaido, 1985). The mechanism by which NRI activates Ntr promoters is not completely understood. It has been suggested that the partial homology between the NRI binding sequence and the Ntr consensus promoter (TGCA) is responsible for the direct interaction of NRI with the DNA at the RNA polymerase binding site (Hunt & Magasanik, 1985). In addition, Reitzer and Magasanik (1986) have shown that NRI binding sites upstream of glnA are required for Ntr promoter activation. The function of these upstream activating sequences is independent of their exact position and orientation relative to the RNA polymerase binding site. Position independence is unusual for cis-acting regulatory sequences in procaryotes but is typical of eucaryotic enhancer elements.

The key to the control of the Ntr system seems to be regulating the amount and activity of NRI (Bueno et al., 1985). The gene encoding NRI, ntrC, is in the complex glnA-ntrBC operon, located downstream from glnA (the gene encoding GS), and ntrB (also termed glnL) which encodes another nitrogen regulatory protein, NRII. This operon is transcribed from three differentially regulated promoters (Alvarez-Morales et al., 1984). Upstream of glnA is an NRI dependent Ntr promoter and a standard promoter which is regulated by catabolite repression (Dixon, 1984b; Reitzer & Magasanik, 1985). In the intergenic region between glnA and ntrB is another standard promoter which can be repressed by the DNA binding activity of NRI (Hawkes et al., 1985; Reitzer & Magasanik, 1983). Thus NRI autoregulates its own production by both promoter activation and repression. NRI has also been implicated in gene regulation by antitermination (Ames & Nikaido, 1985). The conversion of NRI

from an activating to a repressing form is central to the control of the entire Ntr system (Bueno et al., 1985). This conversion is carried out by NRII, perhaps by a covalent modification of NRI. NRII, in turn, is regulated by two other proteins, a uridylyltransferase and the PII protein, encoded by the glnD and glnB genes respectively. The activity of these proteins is controlled by the intracellular ratio of glutamine to 2-ketoglutarate which is an indicator of the cell's nitrogen nutritional status (Bueno et al., 1985). The reversible uridylylation of PII also regulates the adenylyltransferase responsible for the modification of GS (Stadtman & Ginsburg, 1974). Thus the Ntr system is able to regulate nitrogen metabolism at both the transcriptional and post translational levels.

One of the operons induced by the K. pneumoniae Ntr system is nifLA. The products of the nifL and nifA genes are regulatory proteins with homology to NRII and NRI respectively, and are responsible for the regulation of the nif genes (Buikema et al., 1985; Dixon, 1984a; Drummond et al., 1986). nif promoters, like Ntr promoters, require the Ntr specific sigma factor and have the consensus Ntr promoter sequence (Benyon et al., 1983; Ow et al., 1983). nif promoters, however, require the product of the nifA gene instead of NRI as an additional activating factor (Ow & Ausubel, 1983). All nif genes also have a distinct upstream activating element (5'-TGT-N_{10}-ACA-3'; Alvarez-Morales et al., 1986; Buck et al., 1986) Thus, there are two classes of σ^{60} dependent promoters, Ntr and nif. Both classes share the σ^{60} RNA polymerase binding site. Differences in the Ntr and nif upstream activating sequences presumably account for their differential regulation.

The molecular mechanism of nitrogen regulation in rhizobia is not as well understood as the Ntr system of the enteric bacteria. The nif genes from both Rhizobium meliloti and Bradyrhizobium japonicum have been isolated and were found to have promoter structures characteristic of Klebsiella pneumoniae nif promoters, including σ^{60} binding sites and upstream activating sequences (Adams & Chelm, 1984; Alvarez-Morales, et al., 1986; Ausubel, 1984; Buck et al., 1986; Fuhrmann & Hennecke, 1984). These observations indicate that rhizobia have a nif regulatory system homologous to K. pneumoniae. A number of rhizobial mutations that block nif expression also alter the regulation of GSI and GSII, suggesting that the regulation of nitrogen fixation is tied in with general nitrogen metabolism (Donald & Ludwig, 1984; Kondorosi et al., 1977; Ludwig, 1980a; Morett et al., 1985). To date, no nitrogen regulated promoters, other than nif, have been characterized from rhizobia.

In this dissertation, I describe the isolation and characterization of the genes encoding GSI and GSII (glnA and glnII respectively) from Bradyrhizobium japonicum, the soybean symbiont. These genes were chosen because the regulation of GS is central in the control of nitrogen metabolism. Furthermore, the presence of two differentially regulated enzymes which catalyze identical reactions despite extensive structural differences is unprecedented. Thus a comparison of the glnA and glnII genes and their regulatory elements will be of interest. Finally, these genes will serve as molecular probes for the characterization of GS expression. Differences between GS regulation in B. japonicum and other bacteria may reflect adaptations of nitrogen metabolism necessary for symbiotic nitrogen fixation.

CHAPTER 2

Characterization of the Gene Encoding Glutamine Synthetase I (*glnA*) from *Bradyrhizobium japonicum*

Introduction

In free-living *Bradyrhizobium japonicum*, ammonia is assimilated primarily by the coordinate activity of glutamine synthetase (GS) and glutamate synthase (Brown & Dilworth, 1975; Vairinhos *et al.*, 1983). However, in bacteroids, the differentiated symbiotic form of these bacteria, GS activity is repressed in concert with the derepression of nitrogenase activity (Brown & Dilworth, 1975; Upchurch & Elkan, 1978). The ammonia formed by the nitrogenase enzyme complex is then exported to the plant cell cytoplasm, where it is incorporated by plant assimilatory enzymes (Brown & Dilworth, 1975; Evans & Crist, 1984; Lara *et al.*, 1983; O'Gara & Shanmugam, 1976; Upchurch & Elkan, 1978). Therefore, the regulation of the rhizobial genes involved in nitrogen metabolism is an important part of the bacterial developmental process which leads to symbiotic nitrogen fixation.

The study of GS regulation in rhizobia is complicated by the presence of two GS species, designated GSI and GSII (Darrow & Knotts, 1977). GSI is very similar to the single GS enzyme found in most other

Gram negative bacteria. It is a polymeric enzyme consisting of 12 identical subunits of 59,000 daltons, is relatively heat stable, and is regulated by a reversible adenylylation cascade system (Darrow, 1980; Darrow & Knotts, 1977). In contrast, GSII has eight subunits of 36,000 daltons, is heat labile, and is not known to be modified after translation (Darrow, 1980; Darrow & Knotts, 1977). These proteins are products of different genes (Darrow, 1980; Somerville & Kahn, 1983) and are differentially regulated in response to changes in nitrogen source (Ludwig, 1980b), carbon source (Darrow et al., 1981), and oxygen concentration (Darrow et al., 1981; Rao et al., 1978).

The mechanisms by which rhizobia regulate GSI and GSII activities are not well understood. In a variety of rhizobial species, glutamine auxotrophs have been isolated which have very low GS activity and are ineffective in symbiotic nitrogen fixation (Donald & Ludwig, 1984; Kondorosi et al., 1977; Ludwig, 1980a). The complex pleiotrophic phenotypes of these strains suggest that they have mutations in a general nitrogen regulation system. Such regulatory systems have been described for other bacteria (Magasanik, 1982). To study directly the mechanism of GS regulation during nodule development, I isolated the genes encoding GSI and GSII from B. japonicum, the soybean symbiont. With cloned genes it will be possible to characterize those factors involved in the regulation of GS expression. In this chapter, I describe the characterization of the gene encoding GSI, designated glnA. I determined that the rhizobial glnA gene is highly homologous to the E. coli glnA gene and is constitutively transcribed from a single promoter. The sequence of the glnA promoter has weak homology to the E. coli consensus promoter and is distinct from the differentially regulated nif

promoters of the same organism. The isolation and characterization of this gene has been reported (Carlson et al., 1983; Carlson et al., 1985).

Materials and Methods

Bacterial Strains and Media. The E. coli K-12 strain ED8654 (galK galT trpR metB $hsdR_k$ supE supF) was used for routine plasmid construction and maintenance. The phage lambda recombinant library was constructed and amplified on E. coli K802 (lacY metB galT $hsdR_k$ galK supE). Plaque hybridization, complementation, and GS assay studies used the glutamine auxotroph E. coli ET8051 Δ(rha-glnA) $hutC_k$ rbs Nal^r, which contains a deletion of the entire glnA region (Fisher et al., 1981). E. coli strains were grown at 37°C in either LB medium (Davis et al., 1980) or M9 medium (Miller, 1972) with 0.4% glycerol. B. japonicum strain USDA 110 was grown at 30°C in minimal salts arabinose medium (MA) (Ludwig & Signer, 1977) with trace elements (O'Gara & Shanmugam, 1976); modified Bergersen's medium (Bergersen, 1961) with 0.2% xylose and 10 mM of the designated nitrogen source (MBX); formate medium (Manian & O'Gara, 1982) with 2.5 mM $(NH_4)_2SO_4$; or anaerobic yeast extract xylose medium (YEX; Adams et al., 1984) with 10 mM KNO_3.

Materials. Genomic DNA from B. japonicum was purified by phenol extraction (Marmur & Doty, 1962). Plasmid DNA was isolated from E. coli by CsCl ethidium bromide equilibrium centrifugation (Clewell & Helinski, 1972). Phage particles were prepared by two rounds of CsCl block

density gradient sedimentation (Davis et al., 1980), and phage DNA was then extracted by formamide treatment (Davis & Thomas, 1974). DNA restriction endonuclease fragments to be used for cloning, DNA sequencing, S1 nuclease protection analyses, and hybridization probes were isolated by separation on and elution from polyacrylamide gels by the method of Maxam and Gilbert (1980). Unless otherwise stated, recombinant plasmids are derivatives of pBR322 (Bolivar et al., 1977).

B. japonicum Genomic Library Construction. B. japonicum DNA, partially digested with the restriction endonuclease MboI, was ligated to the BamHI sites of the cloning vector λBF101 (Maniatis et al., 1982). The vector DNA had been digested with SalI in addition to BamHI to cleave the stuffer fragment and thus lower the background of intact vector in the library. The ligated mixture was packaged into lambda phage particles (Hohn, 1979) and plated on E. coli K802; 1.4×10^5 plaques were obtained. Assuming an estimated genome size of 10,000 kilobase pairs (kbp) for B. japonicum USDA 110 and a minimum insert size of 6 kbp, a complete representation of the genome (P=0.99) would be expected in less than 7,700 recombinant phage.

Hybridization Methods. Lambda library plaques were prepared by standard procedures (Maniatis et al., 1982) with E. coli ET8051 grown in LB plus 3 mM glutamine, 10 mM $MgSO_4$ and 0.2% maltose. Phage DNA from plaques was transferred to cellulose nitrate sheets as described by Benton & Davis (1977). Hybridization with nick-translated DNA probes were carried out for 24 h at 45^oC in 40% formamide, 2x Denhardt solution (Denhardt, 1966), 5x SSPE (1x SSPE is 180 mM NaCl, 10 mM $NaPO_4$ [pH 7.7],

1.0 mM EDTA), and 0.2 mg of sheared and denatured salmon sperm DNA per ml. The filters were then washed at room temperature twice for 15 min in 2x SSPE, 0.1% sodium dodecyl sulfate (SDS) followed by two 15 min washes in 0.25x SSPE, 0.1% SDS. Southern transfers of restriction endonuclease fragments separated by agarose gel electrophoresis (Southern, 1975), were hybridized and treated in a similar fashion. Hybridization signals were detected by autoradiography at -70°C.

Plasmid Complementation and GS Assays. Plasmid complementation of glutamine auxotrophy was tested in E. coli ET8051 with various plasmids constructed from the expression vector pUC8 (Vieira & Messing, 1982). The cells were streaked on M9 glycerol defined medium agar plates with 0.5 mM isopropylthio-β-D-galactoside (IPTG), which is included as a synthetic inducer of the lac promoter (Vieira & Messing, 1982). This medium contains NH_4^+ as the sole nitrogen source. GS activity was determined on cell-free extracts of B. japonicum USDA 110 grown to the late log phase in MA medium or E. coli grown to the stationary phase in LB broth plus 1 mM IPTG, 80 ug of ampicillin per ml, and 3 mM glutamine. Cell-free extracts were prepared by the procedure of Tronick et al. (1973), with the following minor modifications. Cells were suspended in 5 volumes of grinding buffer (10 mM imidazole-hydrochloride [pH 7.0], 1.0 mM $MnCl_2$) and disrupted by two passes through a French pressure cell at 12,000 lb/in^2. The streptomycin sulfate precipitation was omitted. To separate GSI and GSII, cell-free extracts were loaded over a continuous gradient of 5% to 20% (wt/vol) sucrose prepared in grinding buffer and centrifuged for 4 h at 45,000 rpm in a Beckman SW 50.1 rotor at 4°C (Darrow, 1980). The gradient fractions were assayed for total glutamine

synthetase activity by the γ-glutamyl transferase assay as described by Shapiro & Stadtman (1970), with Mn^{2+} as the activating cation to measure GSII and both adenylylated and non-adenylylated forms of GSI (Darrow & Knotts, 1977). Heat inactivation was carried out by incubation of the crude extract at 58°C for 30 min. One unit of enzyme produces 1 umol of γ-glutamylhydroxamate in 1 min at 37°C. Protein concentrations were determined by a modification of the Lowry procedure (Markwell et al., 1978) with bovine serum albumin as a standard.

Nodule Bacteria Isolation. The total bacterial population from frozen soybean nodules was prepared as described previously (Adams & Chelm, 1984). This preparation can be separated into three developmental fractions by centrifugation through a discontinuous sucrose gradient (Ching et al., 1977). This procedure has been adapted in our laboratory for use with a zonal ultracentrifuge rotor as follows. The total bacterial fraction from 50 g of nodules was loaded on top of a discontinous sucrose gradient and centrifuged at 40,000 rpm for 4 h at 4°C in a Beckman 14 Ti zonal ultracentrifuge rotor. The gradient had been prepared by sequentially loading 90, 140, 160, and 275 ml of 45%, 50%, 52%, and 57% sucrose (wt/vol in 50 mM KPO_4, [pH 7.5]), respectively, from the outer edge of the zonal rotor. The fractions containing each of the bacterial forms from three separate gradients were combined, and each of the three combined fractions was rerun through a second similar gradient. The peak fractions from these second gradients constituted the doubly purified nodule bacteria, transforming bacteria, and bacteroids.

S1 Nuclease Protection Analysis. Transcriptional initiation sites were mapped by a modification of the S1 nuclease protection technique of Berk and Sharp (1977). The 360 basepair (bp) SalI fragment, subcloned in pBJ93 was purified and 5' end-labeled with T4-polynucleotide kinase (Maniatis et al., 1982). The two labeled strands were separated by boiling in 80% formamide for 5 min followed by electrophoresis for 2 days at 4°C on an 8% polyacrylamide gel (Maniatis et al., 1982). The slower-migrating fragment on this gel was determined to be the coding strand by DNA sequence analysis. The 5' end-labeled coding strand DNA was precipitated with 20 ug of RNA, suspended in 10 ul of hybridization buffer (Adams & Chelm, 1984), boiled for 10 min, and allowed to hybridize for 3 h at 58°C. The sample was then added to 0.3 ml of S1 digestion buffer (Adams & Chelm, 1984) containing 40 units of S1 nuclease (PL Biochemicals) and incubated for 30 min at 37°C. The digestion was stopped with 75 ul of 2.5 M ammonium acetate, 50 mM EDTA and precipitated with 0.8 ml of ethanol. The pellet was washed in 80% ethanol and suspended in electrophoresis sample dye (Maxam & Gilbert, 1980). The resultant DNA fragments were separated on denaturing 6% polyacrylamide gels (Maxam & Gilbert, 1980).

Results

B. japonicum Library Screening. A genomic library, constructed in the E. coli phage lambda vector λBF101, was screened for recombinant phage carrying B. japonicum GS genes by plaque hybridization with an E. coli GS probe. A 600 bp BamHI-EcoRI restriction endonuclease fragment

from the middle of the *E. coli glnA* gene was subcloned from p811 (Fisher *et al.*, 1981) to yield pBJ6 and used to probe cellulose nitrate transfers of lambda library plaques formed on *E. coli* ET8051. This strain carries a deletion of the *glnA* gene and thus eliminates background hybridization of the *E. coli* probe to the *E. coli* chromosomal DNA within the plaques (Fisher *et al.*, 1981). Several copies of the library were screened; two different phage, λ6IA and λ6IG, were isolated. Restriction endonuclease mapping of these two phage indicated that they contain a 6.5 kbp overlapping region of rhizobial DNA with the cloned fragments in opposite orientation with respect to the lambda vehicle, indicating that, at least in the region of overlap, they contain a contiguous portion of the *B. japonicum* genome. The restriction map of the combined 11.8 kbp region of the cloned DNA is shown in Figure 1.

Localization of the glnA Homology. To localize the region of λ6IA and λ6IG that is homologous to the *E. coli glnA* probe, DNA from the recombinant phage λ6IA was purified and analyzed by Southern blot hybridization. Three restriction endonuclease fragments of the *E. coli glnA* gene, representing the 5' end, middle, and 3' end of the gene hybridized with approximately equal efficiency to λ6IA DNA. Each probe, however, hybridized to different regions of the cloned DNA (Figure 2). The location and direction of transcription of the *B. japonicum* GS gene as shown in Figure 2 is inferred from these results by analogy to the structure of the *E. coli glnA* gene (Backman *et al.*, 1981).

Complementation Analysis. To confirm the presence of a GS gene in these recombinant phages, several subclones that contained the entire

Figure 1. **Restriction Endonuclease Map of the B. japonicum glnA Region.** EcoRI (E), BamHI (B), SstI (S), XhoI (X), and BglII (Bg) restriction endonuclease sites are indicated. The hatched bars show the regions of the B. japonicum genome isolated in the recombinant phage λ6IA and λ6IG. The bars at the bottom of the figure indicate the regions that were subcloned into the expression vector pUC8, with the arrows indicating the direction of transcription from the lac promoter. The position of the glnA gene is indicated by the cross hatched arrow.

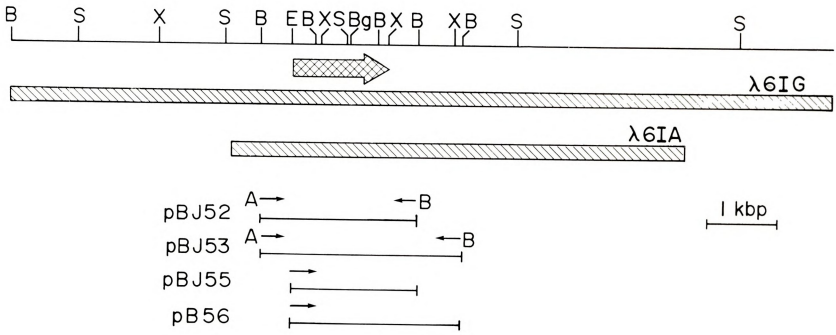
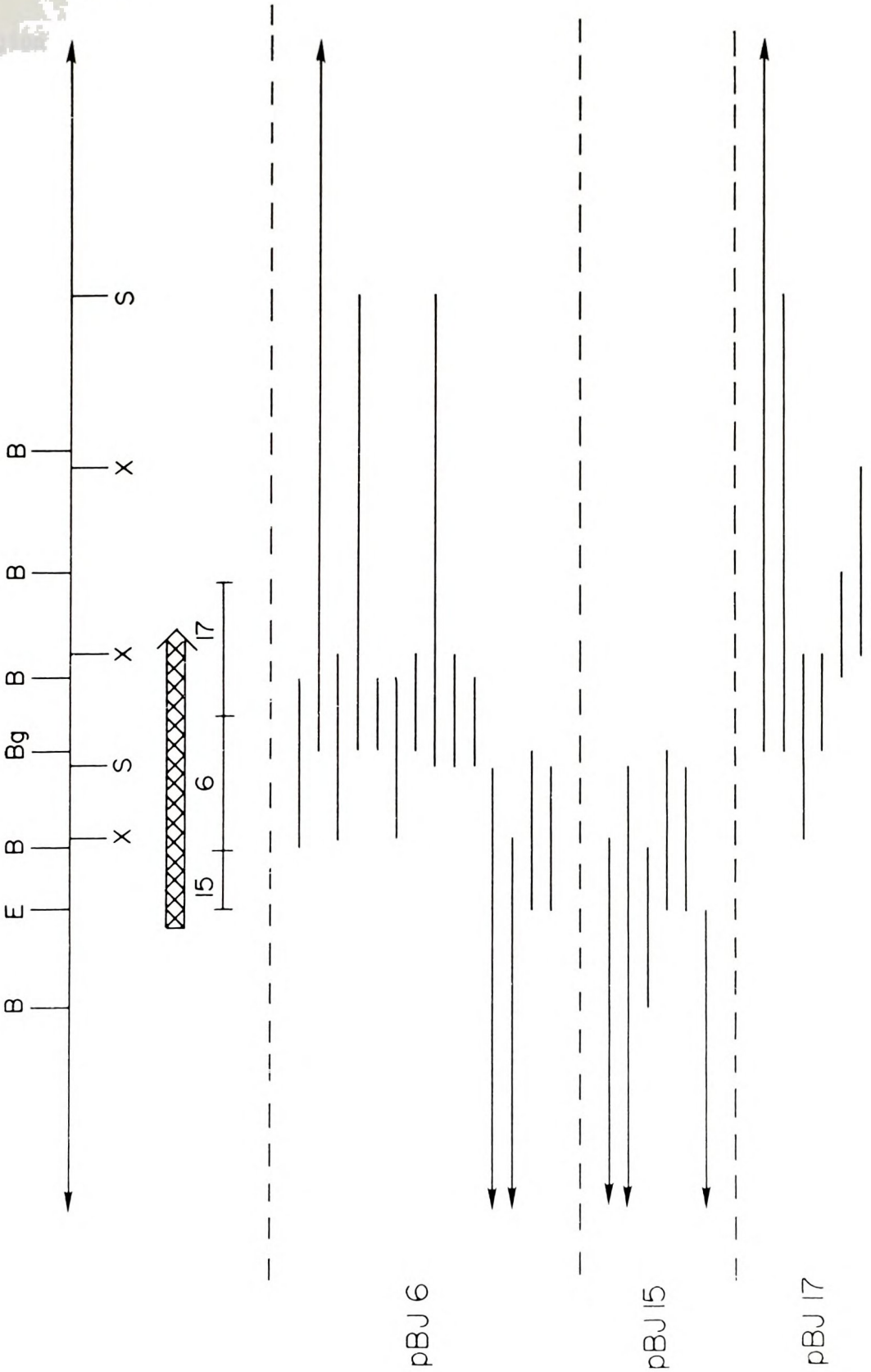

B S X S B E B X S B g B X B X B S S
λ6IG
λ6IA
pBJ52 A B
pBJ53 A B
pBJ55
pB56
1 kbp

Figure 2. **Localization of glnA by Heterologous Hybridization.** Restriction endonuclease sites for EcoRI (E), BamHI (B), BglII (Bg), SstI (S), and XhoI (X) are indicated. The top line is a restriction map of the isolated B. japonicum DNA. The lower portion of the figure indicates the B. japonicum DNA restriction fragments that hybridize to the probes from the 5' end, middle, and 3' end of the E. coli glnA gene shown above (pBJ15, pBJ6 and pBJ17 respectively; Carlson et al., 1983). The position glnA gene (hatched arrow) is aligned below the B. japonicum restriction map as predicted by the regions of homology to the three E. coli probes.


B
E
B
X
S
Bg
B
X
B
B
X
S
17
6
15
pBJ 6
pBJ 15
pBJ 17

region of homology were constructed in the E. coli expression vector pUC8 (Figure 1). These plasmids were transformed into E. coli ET8051 to test their ability to complement glutamine auxotrophy. A plasmid contianing the E. coli glnA gene, p811, and the cloning vector, pUC8, were also tested as positive and negative controls, respectively. The ability of these plasmids to relieve glutamine auxotrophy was determined by comparing the sizes of colonies on M9 defined medium agar plates (Table 1). ET8051(pBJ52A), ET8051(pBJ53A), ET8051(pBJ55), and ET8051-(pBJ56) all have the cloned gene in the correct orientation for lac promoter mediated transcription, and all yield colonies that are nearly the size of the positive control, ET8051(p811). However, ET8051(pBJ52B) and ET8051(pBJ53B) have the gene cloned in the opposite orientation. ET8051(pBJ52B) yields very small colonies detectable only under a dissection microscope. ET8051(pBJ53B) and ET8051(pUC8) do not yield visible colonies under these growth conditions. These results confirm that a B. japonicum GS gene is located as indicated in Figure 2 with the 5' end of the gene near the EcoRI restriction endonuclease site.

Measurements of GS Activity. To confirm further that the gene isolated on the basis of E. coli glnA homology encodes a functional GS and to determine which B. japonicum GS gene had been cloned, GS activity was measured in the complemented E. coli and compared with the GSI and GSII activities of B. japonicum. Cell-free extracts of ET8051(pBJ52A) contain high levels of GS activity. The GS specific activity in these cells is 5.9 units per mg protein as compared with 0.15 units per mg protein for the positive control, ET8051(p811). The negative control, ET8051(pUC8), had no detectable GS activity (less than 0.001 units per

Table 1. **Complementation of *E. coli* ET8051 *glnA* deficiency by plasmids carrying the *B. japonicum* *glnA* gene.**

Plasmid [a]	Relative Growth [b]
pBJ52A	+++
pBJ52B	+
pBJ53A	+++
pBJ53B	-
pBJ55	+++
pBJ56	+++
p811	++++
pUC8	-

[a] pBJ plasmids carry fragments of the *B. japonicum* *glnA* region as indicated in Figure 1. Control plasmids p811 and pUC8 are described in the text.

[b] + to ++++ indicates relative growth on M9-glycerol-IPTG agar plates; - indicates no growth.

mg protein). This clearly indicates that the isolated region of the B. japonicum genome encodes a GS enzyme. The high specific activity obtained in the cells complemented by the rhizobial gene is presumably due to unregulated overexpression of this gene from the lac promoter in the expression vector. The B. japonicum enzyme can be shown to be GSI by two criteria. Sucrose gradient sedimentation of B. japonicum cell-free extracts yields two peaks of GS activity, with the larger GSI sedimenting faster than the smaller GSII (Darrow, 1980). The GS encoded by the rhizobial DNA carried in ET8051(pBJ52A) cosediments with B. japonicum GSI (Figure 3). In addition, GSI can be distinguished from GSII by its relative heat stability (Darrow & Knotts, 1977). I find that the cloned rhizobial enzyme retains 100% of its activity when heat treated. These data are in agreement with the identification of this gene as the structural gene for GSI; because of its analogy with the E. coli gene, I term it glnA.

Promoter Sequence and Expression Analysis. The heterologous hybridization data and the complementation studies described above confirm that the 5' end of the gene is near the EcoRI site (Figure 2). I have characterized this region by sequencing the 554 bp region indicated in Figure 4. The coding region of the amino terminus of GSI was located by its homology to the previously sequenced glnA genes of E. coli (Covarrubias & Bastarrachea, 1983) and Anabaena sp. strain 7120 (Tumer et al., 1983). A single long open reading frame that encodes greater than 73 amino acid residues is indicated in Figure 4. The amino acid sequence of B. japonicum GSI, predicted from this reading frame starting with the first AUG initiation codon, has 43% and 55% homologies

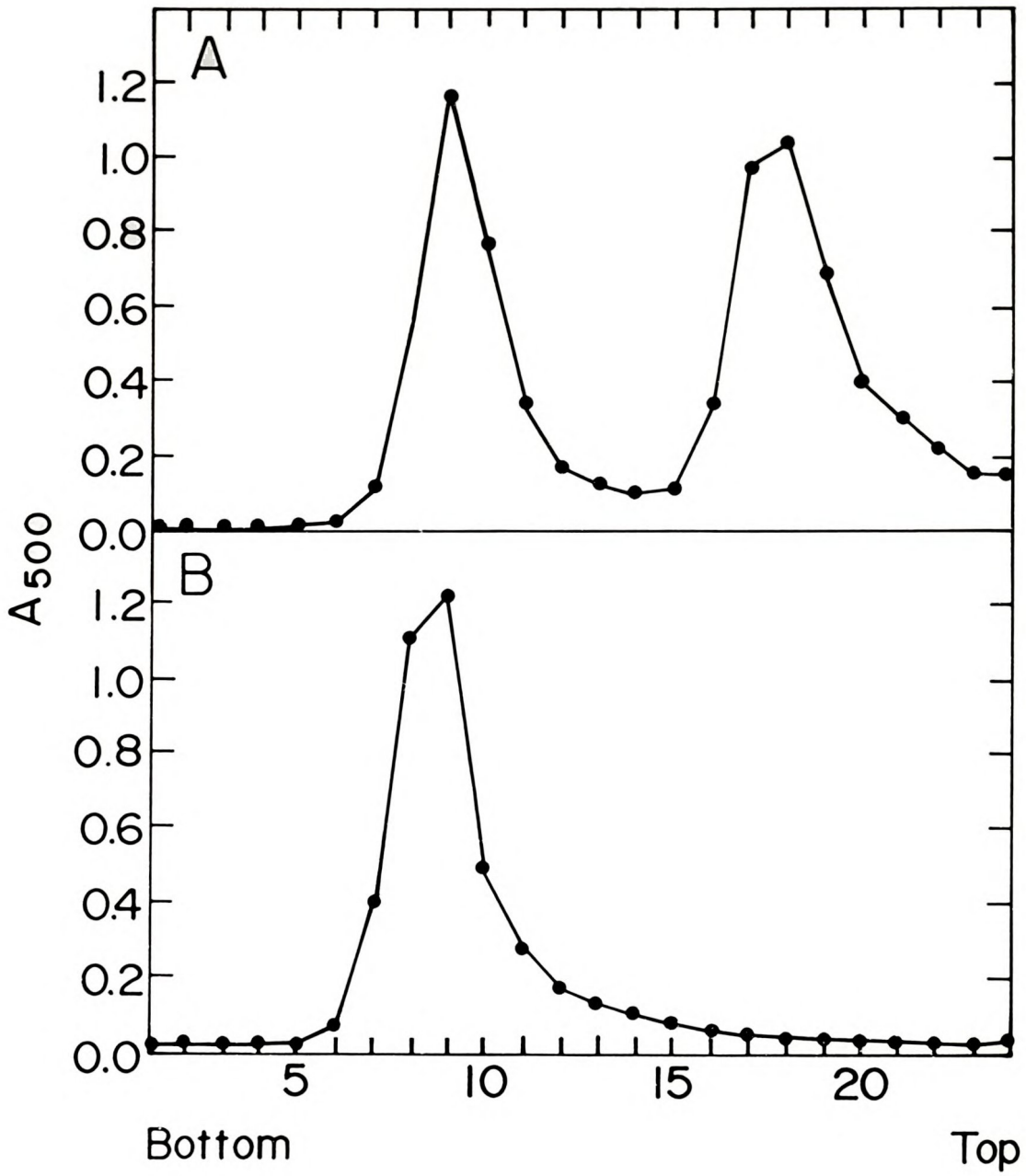
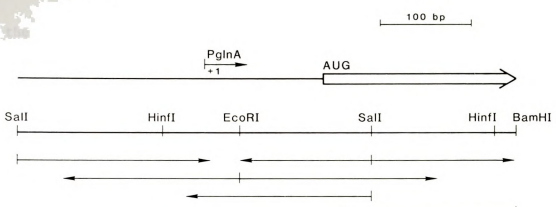


Figure 3. **Identification of GSI by Sedimentation Centrifugation.** Panel A shows the separation of GSI and GSII of B. japonicum. The larger GSI sediments further than the smaller GSII. Panel B shows that the GS from E. coli ET8051(pBJ52A) cosediments with GSI of B. japonicum.



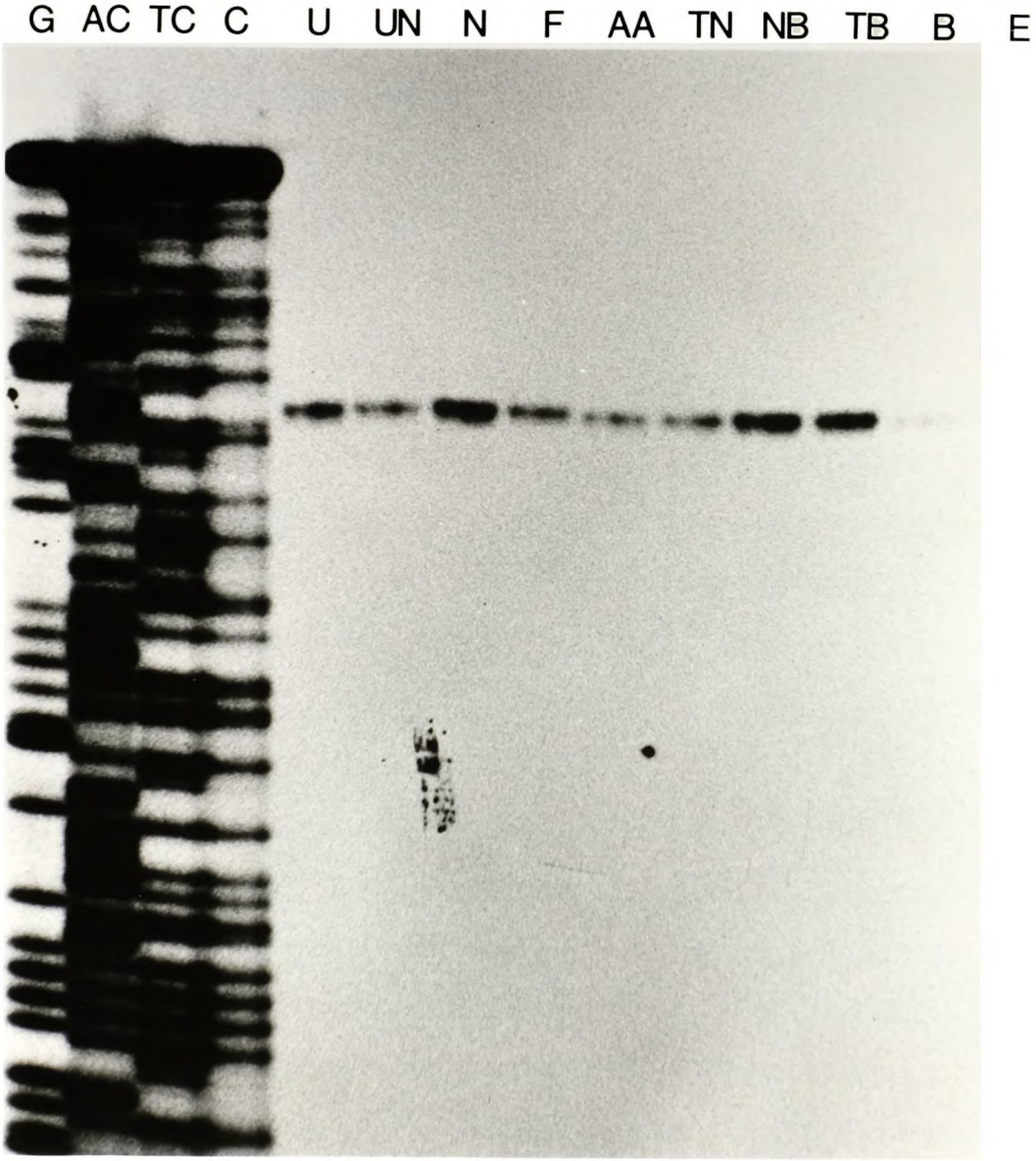

```
    -336
    GTCGAC TTCCTGCCCA AGGTGAAGAT CGAGATCGTG ATCGGCGACG ATCTGGTCGA
-280
GCGCGCCATC GACGCGATCC GCCGCGCTGC GCAGACCGGT CGCATCGGTG ACGGCAAGAT
-220
TTTCGTCTCC AACATCGAAG AGGCGATCCG CATCCGAACC GGCGAATCCG GGCTGGACGC
-160
TATCTGAGCC GGGTGCTATC CCGCATTTTG CGACACTCTG ACAAGAAATA AGGCTGCTTC
                          *
-100
GGCGGCCCGA ATTCGTTTGT GCGGTCGCGC GCGAACTCGC CAAAAGCGAG AATAATCCTG
-40
CTCATCTGGA AACCGACCCG CAGAGCCAAA AGGGGTATGC ATG AAG ACC GCC AAA GAC
                                             M   K   T   A   K   D

GTC CTG AAA TCG ATC AAG GAC AAC GAC GTC AAG TAC GTC GAC CTG CGC TTC
 V   L   K   S   I   K   D   N   D   V   K   Y   V   D   L   R   F

ACC GAT CCG CGC GGC AAG TGG CAG CAT GTG ACG TTC GAC GTC AGC ATG ATC
 T   D   P   R   G   K   W   Q   H   V   F   T   D   V   S   M   I

GAT GAA GAC ATA GGG GCC GAA GGG ACG ATG TTC GAC GGC TCC TCG ATC GCC
 D   E   D   I   G   A   E   G   T   M   F   D   G   S   S   I   A

GGC TGG AAG GCG ATC AAC GAG TCC GAC ATG TGC CTG ATG CCG GAT CC
 G   W   K   A   I   N   E   S   D   M   C   L   M   P   D   P
```

Figure 4. **The Sequence of the 5' End of the B. japonicum glnA Gene.** The open arrow shows the beginning of the glnA coding region as predicted from the translated open reading frame in the DNA sequence below. The arrows below the restriction endonuclease map illustrate the DNA sequencing strategy. The asterisk indicates the approximate location of the site of transcriptional initiation (PglnA). The 390 bp SalI fragment used for the S1 nuclease analysis was subcloned as pBJ93.

to the amino termini of E. coli and Anabaena GS, respectively.

A transcriptional start site upstream from the glnA coding region was identified by using the S1 nuclease protection technique of Berk and Sharp (1977). These experiments used the 5' end-labeled coding strand of the 390 bp SalI fragment cloned in pBJ93 (Figure 4), which contains the coding region for the first 19 amino acids of GSI plus 331 bp of DNA upstream of the initiation codon. RNA for these hybridizaton experiments was purified from B. japonicum grown on various nitrogen and carbon sources. In addition, RNA samples isolated from the total nodule bacteria preparation before further fractionation and from the doubly purified nodule bacteria, transforming bacteria, and bacteroid fractions were analyzed. The results of the S1 protection analysis are shown in Figure 5. All nine rhizobial RNA types tested produced a single protected fragment whose 5' end maps to 131±2 bp upstream from the initiation codon. There is no evidence of transcriptional initiation upstream of the region analyzed, as this would yield complete protection of the hybridization probe. From these data I conclude that glnA is constitutively transcribed from a single promoter, although minor modulations in the levels of transcription are observed. These hybridization reactions were carried out at conditions of probe excess, so that the amount of protection is proportional to the amount of input RNA. Thus, differences in the level of glnA mRNA are indicated by the relative amounts of the protected DNA fragments (Figure 5). I estimate that the glnA transcription varies no more than 3 to 5 fold in free-living cells grown with various nitrogen sources, and that the glnA gene in fully differentiated bacteroids is repressed 5 to 10 fold from the expression level in free-living cells.

Figure 5. **S1 Nuclease Protection Analysis of B. japonicum glnA.** G, A>C, T+C, and C indicate lanes with Maxam and Gilbert sequence reactions on the labeled coding strand of the 232 bp SalI-HinfI fragment (Figure 4). S1 nuclease protection experiments used the 390 bp SalI fragment with the following RNA types: MBX medium with glutamate (U), MBXglutamate-NH_4^+ (UN), MBX-NH_4^+ (N), formate-NH_4^+ (F), anaerobic YEX (AA), total bacterial population from nodules (TN), nodule bacteria (NB), transforming bacteria (TB), bacteroids (B), and E. coli ET8051 (E).


G AC TC C U UN N F AA TN NB TB B E

A comparison of the DNA sequence surrounding the B. japonicum glnA promoter to promoter sequences from B. japonicum and E. coli is shown in Figure 6. Weak homology between the B. japonicum glnA promoter and the E. coli consensus promoter (Hawley & McClure, 1983) can be detected. In contrast, no apparent homology is observed between the glnA promoter and the B. japonicum nif promoters (Adams and Chelm, 1984).

Discussion

The regulation of GS is of special interest in the Rhizobium-legume symbiosis, where repression of GS activity in the differentiated bacteroid allows for newly fixed nitrogen to be exported to the plant cytoplasm in the form of ammonia, where it is assimilated by a plant-encoded, nodule-specific GS (Lara et al., 1983). In order to initiate the study of rhizobial GS regulation on the molecular level, I have isolated a GS gene from B. japonicum USDA 110. I conclude that this gene, designated as glnA, encodes the GSI protein of B. japonicum based on several lines of evidence. B. japonicum glnA is homologous to E. coli glnA throughout a region of at least 1.35 kbp, a region larger than that necessary for a gene encoding the 36,000 dalton subunit of GSII, but in good agreement with the amount of DNA necessary to encode the 59,000 dalton subunit of GSI. The enzyme encoded by the rhizobial gene, when expressed in E. coli, cosediments with native B. japonicum GSI and exhibits the same relative stability to heat, a property specific to GSI (Darrow & Knotts, 1977). We do not know whether the rhizobial GSI can

Figure 6. **B. japonicum glnA Promoter Sequence Comparison.** The upper portion is a comparison between the E. coli consensus promoter (Hawley & McClure, 1983) and the B. japonicum glnA promoter. Uppercase letters in the E. coli sequence indicate highly conserved bases among E. coli promoters. Uppercase letters in the glnA sequence indicate homologies to the E. coli consensus promoter. The lower portion is a comparison between two B. japonicum nif promoters (Adams & Chelm, 1984). Uppercase letters in the nif sequences indicate homologies between the nifH and nifDK promoters. The asterisks indicate points of transcriptional initiation.

be adenylylated in E. coli as it can be in B. japonicum (Darrow & Knotts, 1977).

The S1 nuclease protection data show that, in all cases which we have examined, glnA is transcribed from a single promoter. However, RNA from cells grown under a variety of conditions yield different amounts of protected fragment (Figure 5). Under the hybridization conditions used here, the amount of protected fragment is proportional to the amount of total RNA in the hybridization reaction, indicating that the glnA probe is in excess over its transcript. Therefore, the amount of protected fragment is proportional to the abundance of glnA message in each of the RNA types. Assuming that the degradation rate of the 5' end of the mRNA does not vary significantly in the different cell types, it is possible to use these results to estimate the relative activity of the glnA promoter in cells grown under various conditions. Of particular interest is the activity of the glnA promoter in the three bacterial forms found in nodules, with the bacteroid RNA having less glnA message than the RNA from either transforming bacteria or nodule bacteria (Figure 5). These data suggest that the low bacterial GS activity in nodules is at least partially due to lowered amounts of glnA transcript in the nitrogen fixing bacteroids. The mechanism of this response is unknown, although it may be due to specific repression of the glnA promoter in the latter stages of differentiation. This transcriptional control would be similar to the response of the PII promoter of Anabaena glnA, which is also repressed during nitrogen fixation (Tumer et al.,1983).

In B. japonicum, total biosynthetic GS activity is dependent on the amount of GSI and GSII as well as on the adenylylation state of GSI

(Darrow, 1980; Darrow et al., 1981). I find that total GS activity is not necessarily coordinated with the regulation of glnA transcription. For example, cells grown on glutamate and NH_4^+ have a high concentration of glnA message (Figure 5), but total GS activity is low due to the adenylylation of GSI and the repression of GSII (Ludwig, 1980b). I conclude that the constitutive glnA promoter is responsible for providing a relative constant level of GSI and contributes little to the regulation of total GS activity in free living cells. This pattern of GS regulation can be compared to that found in other procaryotes. In E. coli, glnA is transcribed from two promoters. One provides a basal level of activity, and the other is induced during nitrogen starvation (Reitzer & Magasanik, 1985). In Anabaena, glnA is transcribed from several promoters in ammonia-grown cells, but a single nif-like promoter is specifically induced when cells are derepressed for nitrogenase (Tumer et al., 1983). A situation similar to this exists in Klebsiella pneumoniae (Dixon, 1984). Thus in E. coli, Anabaena and K. pneumoniae, a single GS gene is transcribed from two or more differentially regulated promoters. In B. japonicum, however, GS regulation has been divided between two genes. Since I detect little transcriptional control of glnA, I conclude that GSI activity is primarily regulated by adenylylation. Although GSII activity, like GSI, is dramatically regulated in response to oxygen concentration and nitrogen source (Darrow et al., 1981; Ludwig, 1980b; Rao et al.,1978), there is no known regulation of GSII by post-translational modification. It is therefore likely that GSII activity is controlled at the transcriptional level. A comparison of the promoter sequences of the two GS genes of B. japonicum will be of interest.

CHAPTER 3

Apparent Eucaryotic Origin of Glutamine Synthetase II from the Bacterium Bradyrhizobium japonicum

Introduction

The Rhizobiaceae family of bacteria is characterized by their ability to form cortical hypertrophies on plants, and by the fact that bacteria can be reisolated from these galls or nodules (Jordan, 1984). This family includes the genera Rhizobium, Bradyrhizobium, Agrobacterium and Phyllobacterium. Another unique feature of these bacteria is that they contain two forms of the enzyme glutamine synthetase, termed GSI and GSII (Darrow & Knotts, 1977; Fuchs & Keister, 1980). GSI is typical of procaryotic glutamine synthetases with respect to enzyme structure, the modulation of activity by post-translational modification, immunological cross-reactivity, and amino acid sequence (Darrow & Knotts, 1977; Tronick et al., 1973; Carlson et al., 1985).

By contrast, GSII is distinct from all other known procaryotic glutamine synthetases in structure and immunological reactivity, and is not known to undergo post-translational modification. In these respects GSII is similar to eucaryotic glutamine synthetases (DeVries et al., 1983). I have isolated and characterized the gene encoding GSII, which

I term glnII, from Bradyrhizobium japonicum, the soybean symbiont. In this chapter, I **show that the amino acid sequence of GSII, as inferred from the gene sequence, is highly homologous to plant glutamine synthetases, suggesting that this bacterial gene is of eucaryotic origin.**

Materials and Methods

GSII Purification and Protein Methods. Bradyrhizobium japonicum USDA 110 was grown to mid-log phase in 2 l of MBX medium (Chapter 2) with 10 mM glutamate. Cell extracts were prepared by the method of Tronick et al. (1973) using GS Buffer (GSB is 10 mM imidazole-HCl [pH 7.15] and 1.0 mM $MnCl_2$). GS activity is expressed in units of umoles of γ-glutamylhydroxamate per min as measured by the reverse transferase assay (Bender et al., 1977). Heat inactivation of GSII was carried out on undiluted extracts by incubation at 58°C for 30 min. In the extract used for purification, GSII accounted for 88% of the total GS activity. All purification steps were done at 4°C.

The extract was diluted with GSB to a protein concentration of 1 mg per ml and loaded on a 10 ml Affi-Gel Blue (Bio-Rad) column at a flow rate of 0.5 ml per min. The column was washed with 50 ml of GSB and eluted with a 50 ml linear gradient of 0 to 5 mM ATP in GSB. A broad peak of GS activity, including both GSI and GSII, eluted immediately upon the initiation of the ATP gradient. The fractions with the highest specific GS activity were combined and loaded onto a Bio-Rad Bio-Gel TSK DEAE-5-PW ion exchange HPLC column, (75 x 7.5 mm) equilibrated with GSB, at a flow rate of 1 ml per min. The column was eluted with a 40 ml

linear gradient of 0 to 500 mM KCl in GSB. The first and second peaks of GS activity corresponded to GSII and GSI respectively. One ml fractions for sequence determination were desalted on a 10 ml Bio-Rad P-6 column and lyophylized.

Total protein was assayed by the method of Lowry et al. (1951). SDS polyacrylamide electrophoresis gels (Laemmli, 1970) were stained with Coomassie Brilliant Blue. Protein sequencing was performed on an Applied Biosystems Model 470A gas phase sequencer at the University of Michigan protein sequencing facility, Ann Arbor, Michigan.

Oligonucleotide and DNA Methods. Oligonucleotide probes were synthesized by the phosporamidite method on an Applied Biosystems Model 380A DNA synthesizer. Oligonucleotides were purified by denaturing 12% polyacrylamide gel electrophoresis (Maxam & Gilbert, 1980). DNA was sequenced by the dideoxy nucleotide chain termination method (Sanger et al., 1977). Random fragments for sequencing were generated by sonication (Deininger, 1983) and subcloned into the SmaI site of M13-mp19 (Norrander et al., 1983). All regions were sequenced either on both strands or from three different fragments of the same strand. An ordered B. japonicum genomic library was constructed by cloning B. japonicum DNA, partially digested with EcoRI, into the cosmid cloning vector pLAFR1 (Adams et al., 1984; Friedman et al., 1982). Cultures containing individual cosmids were replicated onto cellulose nitrate sheets layered on LB plates. The resulting colonies were screened by hybridization (Grunstein & Hogness, 1975) with the 5' end radiolabelled mixed oligonucleotide probe (Whitehead et al., 1983; Wood et al., 1985).

Complementation Analysis. Plasmids were tested for their ability to complement glutamine auxotrophy in Escherichia coli ET8051 [Δ(rha-glnA) $hutC_K$ rbs Nal^r] (Fisher et al., 1981) on M9 defined medium agar plates (Miller, 1972) with 0.2% glucose and 1 mM thiamine. GSI and GSII were separated by sucrose density gradient centrifugation as described previously (Carlson et al., 1985) except that the centrifugation was carried out at 50,000 rpm for 3 h.

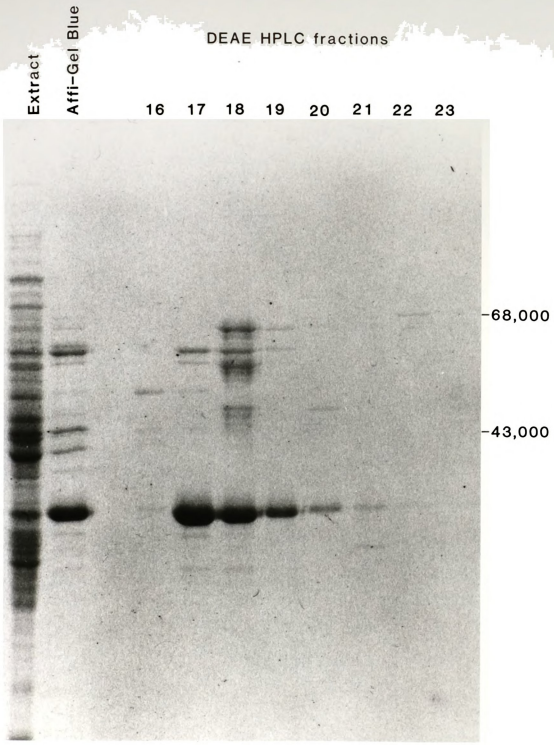
Results and Discussion

GSII of B. japonicum was purified as described in the materials and methods section. The results of the purification procedure are summarized in Table 2. This preparation was greater than 95% pure as indicated by SDS-polyacrylamide gel electrophoresis (Figure 7). The amino acid sequence of the amino terminus of GSII was determined by sequential Edman degradation and was used to design a mixed oligonucleotide probe with homology to the DNA which encodes the first six amino acids of GSII (Figure 8). An ambiguity at position 16 of the oligonucleotide was not included because two of the leucine codons, UUA and UUG, are infrequently utilized in B. japonicum. Two cosmids, pRjcos7-20 and pRjcos13-79, were isolated from a genomic library by hybridization to the mixed oligonucleotide probe. The region of hybridization in pRjcos7-20 was localized to 5.0 kbp EcoRI, 1.0 kbp BglII, and 2.1 kbp SalI fragments. These fragment sizes agree with those detected by hybridization to Southern transfers of total genomic DNA restriction digests.

Table 2. **GSII Purification**

Fraction	GSII Activity (units)	Total Protein (mg)	Specific Activity (units mg^{-1})	Purification Factor	Percent Yield
Extract	149	48	3.10	(1)	(100)
Affi-Gel Blue	39.1	0.89	43.9	14	26
DEAE HPLC fraction 17	9.92	0.12	82.7	27	6.7

Figure 7. **GSII Purification: SDS-Polyacrylamide Gel Electrophoresis Analysis.** Samples from the GSII purification procedure as described in the methods section were separated on a 10% gel. The major band in DEAE HPLC fractions 17-21 represents GSII. The large and small molecular weight markers are bovine serum albumin and ovalbumin, respectively. GSI eluted in fractions 22 through 25 and produced a single band on a similar polyacrylamide gel.


Extract
Affi-Gel Blue
DEAE HPLC fractions
16
17
18
19
20
21
22
23
68,000
43,000

```
                    5                  10                  15
  Met Thr Lys Tyr Lys Leu Glu Tyr Ile Trp Leu Asp Gly Tyr Thr
3',TAC TGG TTC ATG TTC GA 5',
       A   T   A   T
       T
       C

                    20            *  25            *   30  *
  Pro Thr Pro Asn Leu Arg Gly Lys Val Gln Ile Lys Glu His Ala Glu
```

Figure 8. **GSII Amino Terminus Sequence and Mixed Oligonucleotide Probe.** The first 31 amino acid residues of B. japonicum GSII, as determined by protein sequencing, are shown. The first 5 amino acids of the enzyme were used to design the structure of the mixed oligonucleotide probe as indicated. Asterisks indicate positions of discrepancies with the GSII amino acid sequence as determined from the DNA sequence.

The 2.1 kbp SalI fragment with homology to the oligonucleotide probe was subcloned in both orientations in pBR322. The resulting plasmids, pBJ196A and pBJ196B, were tested for their ability to complement glutamine auxotrophy in E. coli ET8051. ET8051(pBJ196A) grew well on defined medium plates with ammonia as the sole nitrogen source and yielded extracts with 0.82 units of GS activity per mg of protein. This GS activity was completely heat labile, a property specific to GSII, and cosedimented with the GSII activity of B. japonicum in sucrose density gradient centrifugation (Figure 9). These data indicate that the gene encoding B. japonicum GSII, glnII, is located entirely on the 2.1 kbp SalI fragment insert of pBJ196A. ET8051(pBJ196B), ET8051(pRjcos7-20), and the control strain, ET8051(pBR322), gave no detectable growth on defined medium plates and produced no detectable glutamine synthetase activity (less than 0.001 units per mg protein). Apparently the complementation observed with ET8051(pBJ196A) is due to glnII expression from the tet promoter of the vector, in agreement with the gene orientation as indicated by sequence determination.

In order to determine the precise location of the glnII gene, I sequenced the 2.1 kbp SalI fragment cloned in pBJ196A. The DNA sequence contains a single long open reading frame which encodes GSII (Figure 10). The subunit molecular weight of GSII predicted by the complete inferred amino acid sequence is 36,904 daltons, in good agreement with 36,000 daltons as determined by SDS-polyacrylamide gel electrophoresis (Darrow, 1980). The amino terminus of GSII matches the protein sequencing data in 28 of 31 positions (Figure 8). The discrepancies at positions 24, 29, and 31 are presumably due to inaccuracies in the final cycles of the protein sequencing.

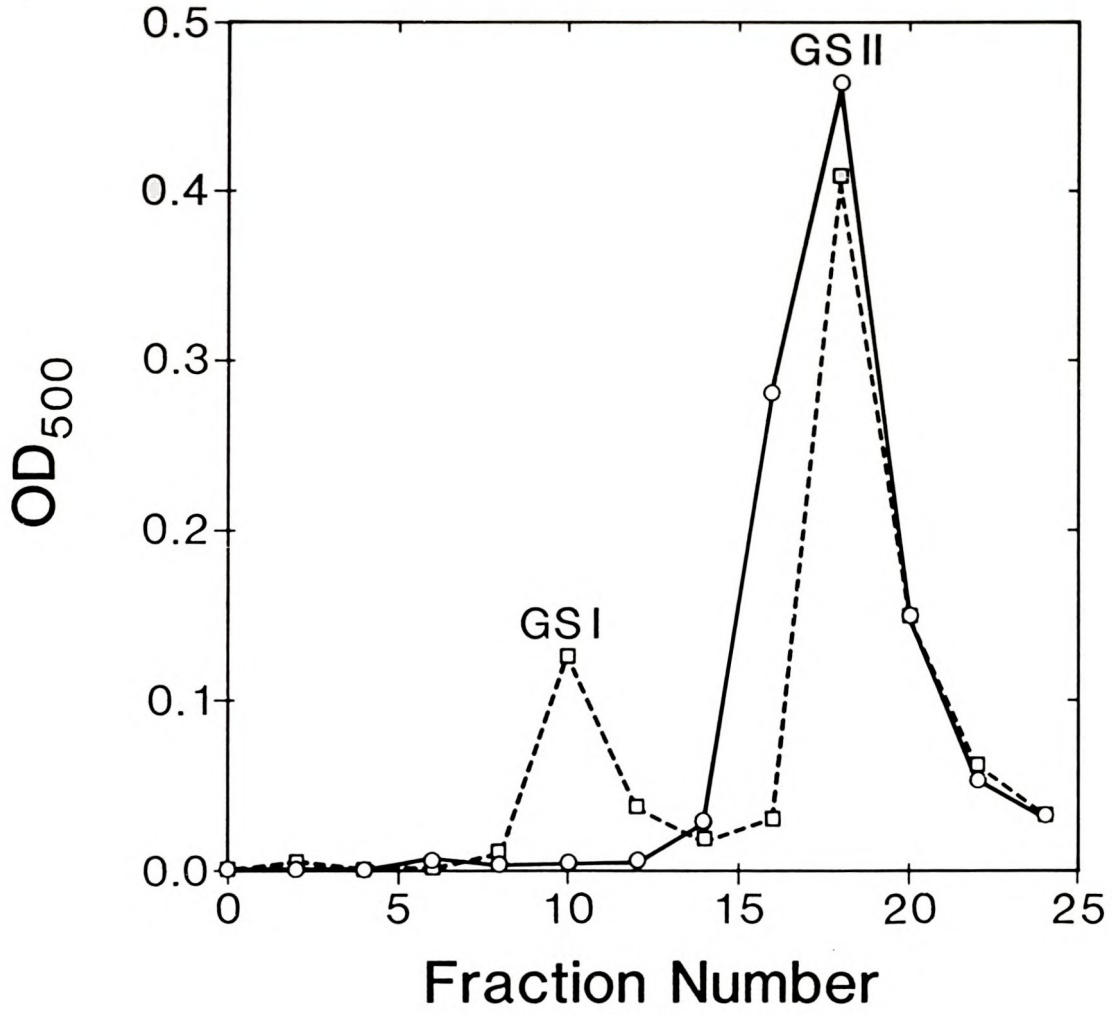


Figure 9. **Identification of GSII by Sedimentation Centrifugation.** The dashed line shows the separation of GSI and GSII of B. japonicum. The larger GSI (fraction 10) sediments further than the smaller GSII (fraction 18). The solid line shows that GS from E. coli ET8051(pBJ196A) cosediments with GSII of B. japonicum

```
                *           *           *           *           *           *           *   75
ATG ACC AAG TAT AAG CTC GAG TAC ATC TGG CTC GAC GGA TAT ACG CCG ACT CCG AAC TTG CGC GGC AAA ACT CAG
 M   T   K   Y   K   L   E   Y   I   W   L   D   G   Y   T   P   T   P   N   L   R   G   K   T   Q

    *           *           *           *           *           *           *           *  150
ATC AAG GAA TTC GCG TCG TTC CCG ACG CTC GAG CAC GTT CCG CTC TGG GGC TTC GAT GGC TCC TCC ACC CAG CAG
 I   K   E   F   A   S   F   P   T   L   E   H   V   P   L   W   G   F   D   G   S   S   T   Q   Q

                *           *           *           *           *           *           *  225
GCC GAA GGC CAC AGC TCT GAT TGC GTG CTG AAG CCA GTC GCC GTC TTC CCG GAC GCC GCG CGC ACC AAC GGC GTG
 A   E   G   H   S   S   D   C   V   L   K   P   V   A   V   F   P   D   A   A   R   T   N   G   V

    *           *           *           *           *           *           *           *  300
CTC GTG ATG TGC GAA GTC ATG ATG CCC GAT GGC AAG ACC CCG CAC GCC TCC AAC AAG CGC GCC ACC ATT CTC GAC
 L   V   M   C   E   V   M   M   P   D   G   K   T   P   H   A   S   N   K   R   A   T   I   L   D

                *           *           *           *           *           *           *  375
GAC GCC GGC GCC TGG TTC GGC TTC GAG CAG GAA TAC TTC TTC TAC AAG GAC GGC CGT CCG CTC GGC TTC CCG ACC
 D   A   G   A   W   F   G   F   E   Q   E   Y   F   F   Y   K   D   G   R   P   L   G   F   P   T

    *           *           *           *           *           *           *           *  450
TCC GGT TAT CCG GCG CCG CAG GGC CCG TAC TAC ACC GGC GTC GGC TTC TCG AAC GTC GGC GAC GTC GCC CGC AAG
 S   G   Y   P   A   P   Q   G   P   Y   Y   T   G   V   G   F   S   N   V   G   D   V   A   R   K

                *           *           *           *           *           *           *  525
ATC GTC GAA GAG CAT CTC GAC CTC TGC CTC GCG GCC GGC ATC AAC CAT GAA GGC ATC AAC GCG GAA GTC GCG AAG
 I   V   E   E   H   L   D   L   C   L   A   A   G   I   N   H   E   G   I   N   A   E   V   A   K

    *           *           *           *           *           *           *           *  600
GGC CAG TGG GAA TTC CAG ATC TTC GGC AAG GGC TCC AAG AAG GCC GCT GAC GAA ATG TGG ATG GCC CGC TAC CTG
 G   Q   W   E   F   Q   I   F   G   K   G   S   K   K   A   A   D   E   M   W   M   A   R   Y   L

                *           *           *           *           *           *           *  675
ATG CTG CGC CTG ACC GAG AAG TAC GGC ATC GAC ATC GAG TTC CAC TGC AAG CCG CTT GGC GAC ACG GAC TGG AAC
 M   L   R   L   T   E   K   Y   G   I   D   I   E   F   H   C   K   P   L   G   D   T   D   W   N

    *           *           *           *           *           *           *           *  750
GGC TCC GGC ATG CAC GCC AAC TTC TCG ACC GAG TAC ATG CGC ACG GTC GGC GGC AAG GAG TAC TTC GAG GCG CTG
 G   S   G   M   H   A   N   F   S   T   E   Y   M   R   T   V   G   G   K   E   Y   F   E   A   L

                *           *           *           *           *           *           *  825
ATG GCT GCC TTC GAC AAG AAC CTG ATG GAC CAC ATC GCC GTC TAC GGC CCG GAC AAC GAC AAG CGT CTG ACC GGC
 M   A   A   F   D   K   N   L   M   D   H   I   A   V   Y   G   P   D   N   D   K   R   L   T   G

    *           *           *           *           *           *           *           *  900
AAG CAC GAG ACC GCG CCG TGG AAC AAG TTC AGC TAC GGC GTG GCC GAC CGC GGC GCT TCG ATC CGC GTG CCG CAC
 K   H   E   T   A   P   W   N   K   F   S   Y   G   V   A   D   R   G   A   S   I   R   V   P   H

                *           *           *           *           *           *           *  975
TCC TTC GTC AAC AAC GGC TAC AAG GGC TAT CTG GAA GAC CGT CGC CCG AAC TCG CAG GGC GAC CCA TAC CAG ATC
 S   F   V   N   N   G   Y   K   G   Y   L   E   D   R   R   P   N   S   Q   G   D   P   Y   Q   I

    *           990
GTT CGC AGA TCC TGA
 V   R   R   S   -
```

Figure 10. **DNA and Amino Acid Sequence of glnII and GSII.** The DNA sequence of glnII is shown starting with the ATG initiation codon and ending with the TGA termination codon. The predicted amino acid sequence of GSII is shown with single letter amino acid codes.

The comparisons of the B. japonicum GSII amino acid sequence with Phaseolus vulgaris root GS (Gebhardt et al., 1986) and Anabaena 7120 GS (Tumer et al., 1983) are shown as homology matrixes in Figure 11 and Figure 12. These matrixes were generated using the analysis program of Pustell and Kafatos (1984) with parameters set so that each letter within the matrix represents a match of 47% or greater over a span of 23 amino acids. It can be seen that GSII has only limited homology to the bacterial GS but extensive homology to the plant GS.

GSII was compared to a variety of glutamine synthetases using the method of Lipman and Pearson (1985) which optimizes the alignment between amino acid sequences and quantitates the significance of the similarity (Table 3). Despite extensive homology among most procaryotic glutamine synthetases, as indicated by the ability of their genes to cross hybridize, the homology between GSII and a typical bacterial glutamine synthetase of Anabaena is only marginally significant. In contrast, GSII has extensive homology with all eucaryotic glutamine synthetases that we have examined. Because all suggested cases of convergent evolution result in similarities of enzyme function without extensive sequence homology (Bannister & Parker, 1985), and because GSII is found only in the Rhizobiaceae (Fuchs & Keister, 1980), we conclude that the B. japonicum glnII gene is the result of a eucaryote to procaryote gene transfer event. We suggest that a plant served as the source of the progenitor glnII gene because of the plant pathogenic nature of the Rhizobiaceae.

The presence of the glnII gene in the Rhizobiaceae is the first evidence of gene transfer to symbiotic bacteria from the eucaryotic host. Another suggested example of eucaryote to procaryote gene

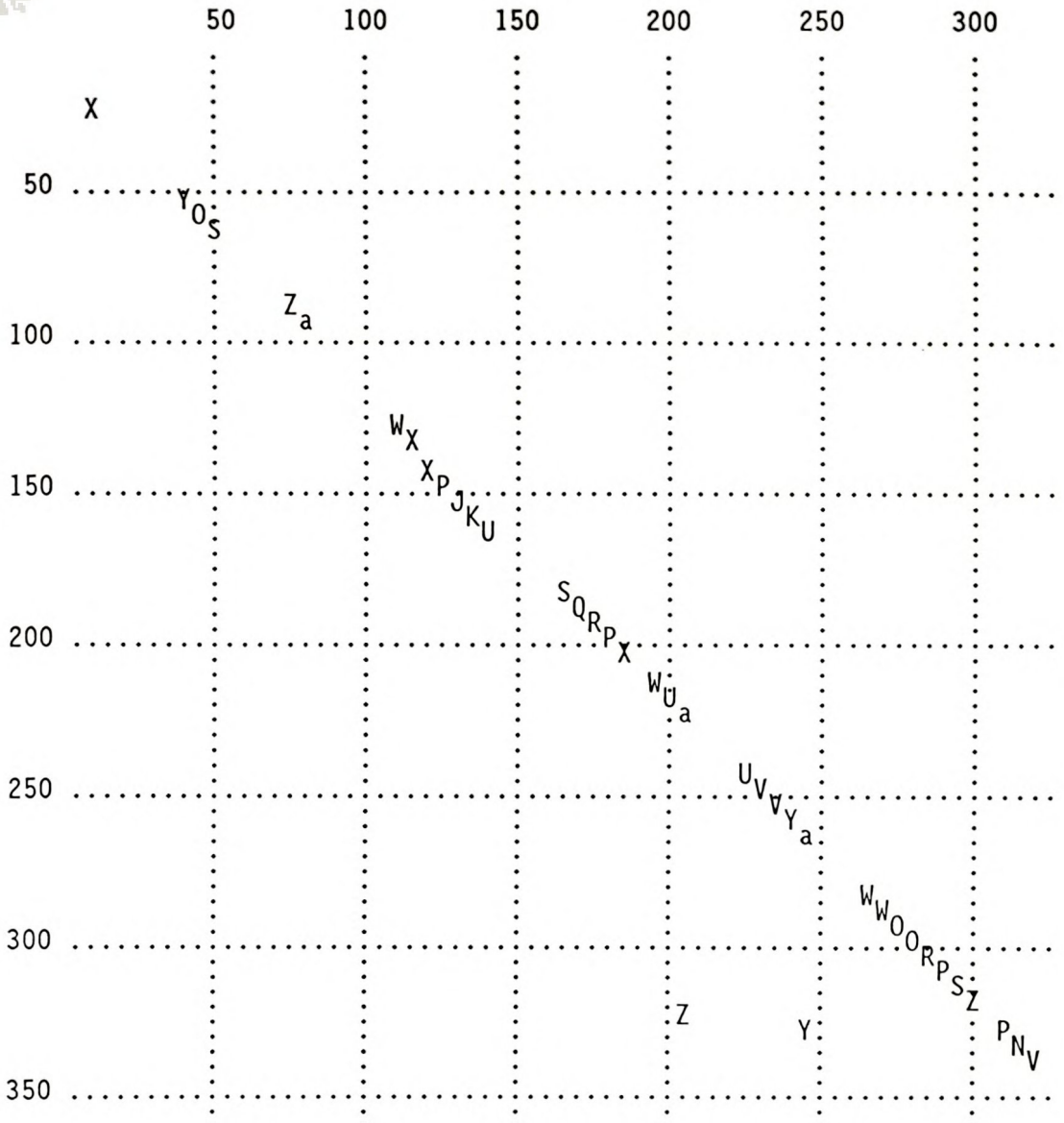


Figure 11. **Homology Matrix Comparing GSII With Phaseolus vulgaris Root GS.** The B. japonicum GSII amino acid sequence is plotted along the x axis and is compared to the amino acid sequence of Phaseolus vulgaris root GS plotted along the y axis. The homology matrix was plotted using the following parameters: range=11, scale factor=0.75, minimum value plotted=47%, compression=5. Each letter represents a 2% range of homology for that region of the matrix (A=100%-99%, B=98%-97%,... Z=50%-49%, a=48%-47%).

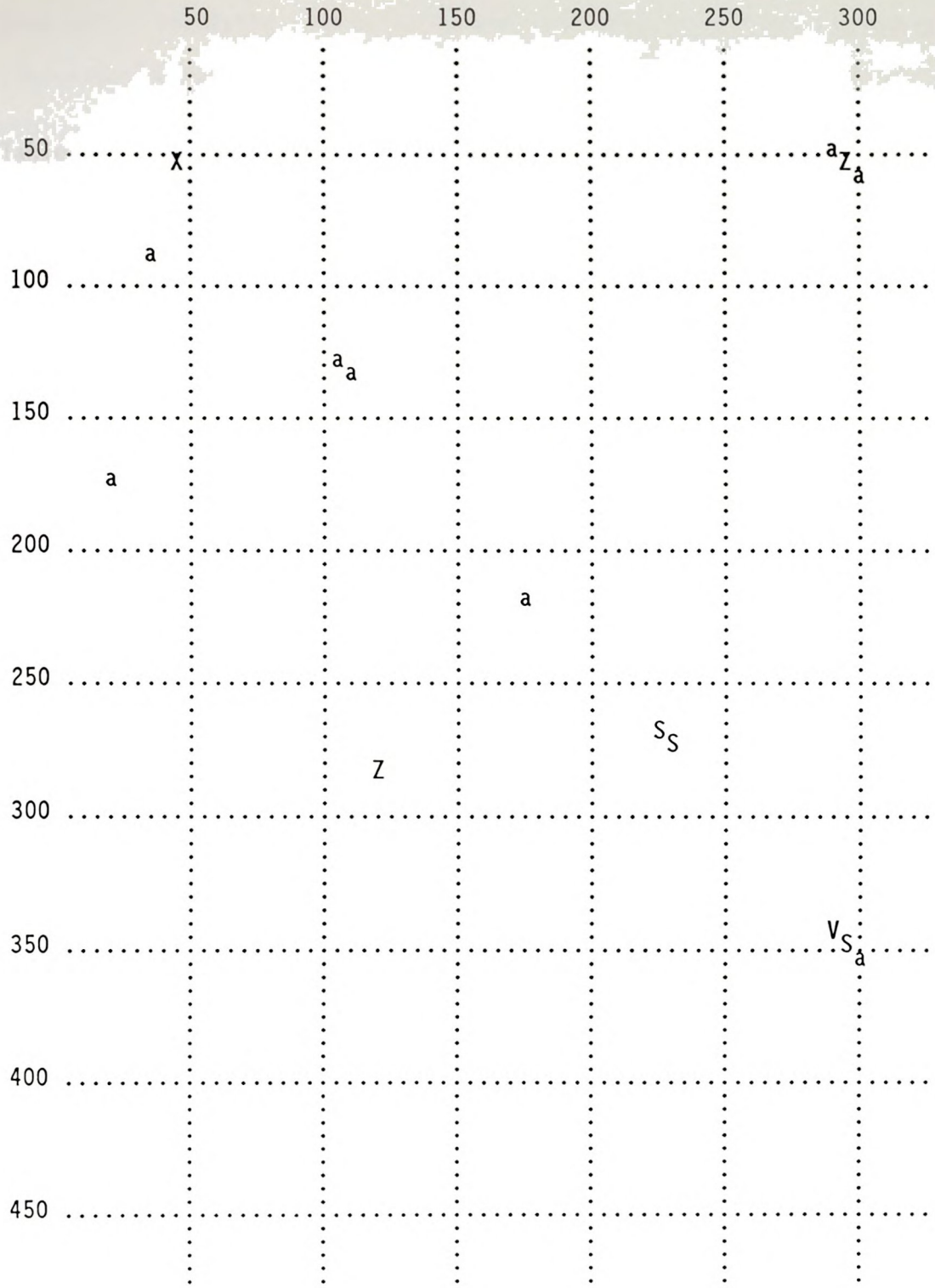


Figure 12. **Homology Matrix Comparing GSII With Anabaena GS.** The B. japonicum GSII (x axis) is compared to Anabaena GS (y axis). The homology matrix was plotted as described in Figure 11.

Table 3 **The Statistical Significance of Amino Acid Sequence Homologies Between B. japonicum GSII and other Glutamine Synthetases**[a]

Glutamine Synthetase Source	% Identity to GSII	Similarity Score	z Value[b]
Bradyrhizobium japonicum	100	1719	208.4
Phaseolus vulgaris[c]	43.8	663	77.3
Pisum sativum[d]	46.6	642	73.9
Medicago staiva[e]	43.8	674	78.9
Nicotiana plumbaginifola[d]	42.6	667	74.0
Anabaena 7120[f]	24.4	114	8.83

[a] All scores are from optimized alignments calculated with ktup = 1 and 1000 randomized sequences using the method of Lipman and Pearson (1985).

[b] The z value equals the similarity score minus the mean of the similarity scores of the randomized sequences divided by the standard deviation of the scores of the randomized sequences. A similarity score is considered significant if its z value is greater than 10. Z values less than 3 are not significant.

[c] Gebhardt et al., 1986

[d] Coruzzi et al., 1986

[e] Tischer et al., 1986

[f] Tumer et al., 1983

transfer, involving bacteriocuprein of Photobacterium leiognathi (Bannister & Parker, 1985), has recently come under dispute (Dunlap & Steinman, 1986).

Although sequence homology suggests the origin of the glnII gene, questions pertaining to the mechanism, frequency, or function of gene transfer between symbionts or pathogens and hosts cannot be directly addressed. Since plant GS genes are known to contain introns (Tischer et al., 1986), B. japonicum glnII had to evolve further by the loss of the introns, or the gene transfer must have occurred prior to the development of introns in the plant genes. In addition, there is no obvious reason why the aquisition of a eucaryotic gene would confer a selective advantage on a plant symbiotic bacterium. GSII is not known to serve an essential function, acting only to provide extra ammonia assimilatory capacity during growth under nitrogen limited conditions (Darrow et al., 1981). Nevertheless, it appears that the ability to acquire genetic information from eucaryotes can be a source of genetic diversity in the evolution of bacteria.

CHAPTER 4

Transcriptional Control of *glnII*, the Gene Encoding Glutamine Synthetase II of *Bradyrhizobium japonicum*

Intoduction

In bacteria, nitrogen is generally assimilated in the form of ammonia by the coordinated activity of the enzymes glutamine synthetase (GS) and glutamate synthase. When nitrogen becomes limiting, the cell's ammonia assimilatory capacity is enhanced by an increased synthesis and activity of GS. Nitrogen starvation also causes the induction of a number of other enzymes responsible for the production of ammonia from alternative nitrogen sources such as nitrate and amino acids. In the case of severe nitrogen starvation, diazotrophs have the ability to produce ammonia through the reduction of dinitrogen by the activity of the enzyme nitrogenase.

The large number of genes and enzymes involved in nitrogen metabolism are carefully regulated by a complex nitrogen regulatory (Ntr) system (Magasanik, 1982). Two genera of nitrogen fixing bacteria, *Rhizobium* and *Bradyrhizobium* are unique in that their ability to fix nitrogen is limited, for the most part, to the highly differentiated symbiotic form termed bacteroids which are found within the root nodules

of leguminous plants. These bacteria are also unusual because as much as 94% of the fixed nitrogen is exported from the bacteroid and is assimilated by the plant symbiont (O'Gara & Shanmugam, 1976). The purpose of this study is to investigate the mechanisms by which B. japonicum regulates the unusual physiology of symbiotic nitrogen fixation.

Nitrogen regulation has been best characterized in the enteric bacteria E. coli and K. pneumoniae (Magasanik, 1982; Alvarez-Morales et al., 1984). Genes that are regulated by the Ntr system have the unusual promoter sequence -27 CTGGCAC-N_5-TTGCA -10 (Dixon, 1984b; Ow et al., 1983). Transcriptional activation of Ntr promoters requires the RNA polymerase core enzyme, an Ntr-specific sigma factor (σ^{60}), and the regulatory protein NRI, encoded by the ntrC (glnG) gene (Hirschman et al., 1985; Hunt & Magasanik, 1985; Merrick & Gibbins, 1985). NRI also has DNA binding activity specific for the sequence 5'-TGCACC$\overset{AAAA}{TTTT}$TGGTGCA-3' (Ames & Nikaido, 1985). The DNA binding function of NRI bas been implicated in transcriptional activation (Reitzer & Magasanik, 1986), repression (Reitzer & Magasanik, 1983; Hawkes et al., 1985) and antitermination (Ames & Nidaido, 1985). The conversion of NRI from its DNA binding form to its Ntr promoter activating form seems to be central to the control of the Ntr system (Bueno et al., 1985).

One of the Ntr activated operons of K. pneumoniae is nifLA. The nifA gene product is a regulatory protein with homology to NRI (Buikema et al., 1985; Drummond et al., 1986) and is required for the activation of the genes encoding the nitrogenase enzyme complex (nif genes; Dixon, 1984a). nif promoters, like Ntr promoters, require the Ntr-specific sigma factor and have the σ^{60} consensus promoter sequence (Ow et al.,

1983). In addition, nif promoters have the upstream regulatory sequence 5'-TGT-N_{10}-ACA-3' which is required for efficient nifA dependent transcriptional initiation and is presumably responsible for the differential regulation of nif and Ntr promoters (Alvarez-Morales et al., 1986; Buck et al., 1986). The complex interactions among the Ntr promoters and regulatory proteins result in the effective regulation of bacterial nitrogen metabolism.

There is evidence that B. japonicum has a nitrogen regulatory system which is homologous to the Ntr system of the enteric bacteria. GSI of B. japonicum is regulated by an adenylylation cascade system homologous to the Ntr regulated adenylylation of GS in E. coli. The best evidence for the existence of an Ntr system in B. japonicum is from the characterization of the nif promoters. B. japonicum nif promoters are very similar in structure to the nif promoters of K. pneumoniae. Both have the σ^{60} recognition sequence (Adams & Chelm, 1984; Fuhrmann & Hennecke, 1984) and the upstream nif activation sequence (Alvarez-Morales et al., 1986; Buck et al., 1986). Furthermore, the nifA gene product of K. pnuemoniae is able to activate transcription from B. japonicum nif promoters (Alvarez-Morales & Hennecke, 1985).

The conservation of nif promoter sequences indicates extensive homology between the Ntr systems of B. japonicum and K. pnuemoniae. However, there may be differences in these systems which allow B. japonicum to express the unusual metabolism of a symbiotic nitrogen fixing bacterium. A number of studies report the characterization of mutations in Rhizobium and Bradyrhizobium that affect both the Ntr dependent GS regulation and the effectiveness of symbiotic nitrogen fixation (Donald & Ludwig, 1984; Konderosi et al., 1977; Ludwig, 1980).

The complex phenotypes of these strains suggests that they carry mutations in the Ntr system. Recently, several genes have been isolated from B. japonicum with homology to the K. pnuemoniae nifA gene (Adams & Chelm, 1986; Fischer et al., 1986). Mutations in some of these genes have similar pleiotrophic phenotypes.

Bacteria of the Rhizobiaceae family are distinct from all other bacteria by having a second form of GS, designated GSII (Darrow & Knotts, 1977), which seems to have been acquired from plants by a eucaryote to procaryote gene transfer event (Carlson & Chelm, 1986). The level of GSII activity is regulated in response to nitrogen and carbon source (Darrow et al., 1981; Ludwig, 1980b) and oxygen concentration (Rao et al., 1981). Because GSII is not known to be post translationally regulated, the gene encoding GSII, glnII, is presumably regulated by an Ntr promoter. One possible role of the B. japonicum Ntr system is the repression of GS activity in nitrogen fixing bacteroids (Brown & Dilworth, 1975; Cullimore et al., 1983; DeVries et al., 1983). This repression is presumably responsible for the observed export of ammonia from bacteroids during symbiotic nitrogen fixation (O'Gara & Shanmugam, 1976). To date, no Ntr promoters have been characterized from B. japonicum. In Chapter 3 I described the isolation of the gene encoding GSII (glnII) of B. japonicum. In this chapter, I describe the characterization of the structure and expression of the Ntr regulated glnII promoter and discuss its potential role in the regulation of nitrogen metabolism in B. japonicum.

Promoter Mapping. Transcriptional start sites were mapped by the S1 nuclease protection method of Berk and Sharp (1977). Single stranded 5' end-labeled probes were generated by the primer extension method (Adams & Chelm, 1986). 80 ng of oligonucleotide were labeled with 10 units of T4-polynucleotide kinase (Bethesda Research Laboratory) at 37°C for 1 h in 100 mM tris(hydroxymethyl)aminomethane (Tris) -HCl [pH 7.5], 10 mM $MgCl_2$, 6 mM dithioerythritol and 100 uCi of [γ-^{32}P]ATP (3000 Ci per mmole). In experiments with mixed probes, the specific activities of the 5' end-labeled oligomers were adjusted to 250 cpm per pg with unlabeled oligonucleotides prior to the elongation reaction. Labeled oligonucleotides were combined with 20 ug of single stranded M13 recombinant phage DNA, ethanol precipitated, suspended in 50 ul of Klenow buffer (10 mM Tris-HCl [pH 8.5], 10 mM $MgCl_2$), heated to 90°C for 10 min and allowed to hybridize for 1 h at 37°C. The oligonucleotide primer was extended with 3 units of the large fragment of E. coli DNA polymerase (Klenow fragment, Bethesda Research Laboratory) for 1 h at 37°C in 100 ul of Klenow buffer with 0.6 mM each of ATP, CTP, GTP and TTP. The partially double stranded DNA was ethanol precipitated, suspended in SalI reaction buffer (Bethesda Research Laboratory), digested with 10 units of SalI (Bethesda Research Laboratory) for 3 h at 37°C, ethanol precipated, and suspended in formamide dye buffer. The elongated primer was purified by denaturing polyacrylamide gel electrophoresis (Maxam & Gilbert, 1980), detected by autoradiography, and eluted by the crush and soak method (Maxam and Gilbert, 1980).

S1 nuclease protection experiments were carried out as described in Chapter 2, using 9000 cpm of probe hybridized with 10 ug of total cell RNA. Protected fragments were separated by denaturing polyacrylamide gel electrophoresis. Gels were dried onto filter paper by aspiration under vacuum prior to autoradiography. Bands of radioactivity from dried gels were cut out and the amount of radioactivity was determined by scintillation counting.

Bacterial Cultures. Batch cultures of B. japonicum USDA 110 were grown at 28°C in 14 l fermenters (New Brunswick) with an agitation rate of 200 RPM and an aeration rate of 500 ml per min. The culture medium consisted of 50 mM 3-(N-morpholino)-propanesulphonate (MOPS), 0.03% (w/v) KH_2PO_4, 0.03% Na_2HPO_4, 0.012% $MgSO_4 \cdot 7\ H_2O$, 0.45 mM $CaCl_2$ and trace minerals and vitamins to the following concentrations (per liter): 10 mg H_3BO_4, 1.0 mg $ZnSO_4 \cdot 7\ H_2O$, 0.5 mg $CuSO_4 \cdot 5\ H_2O$, 0.5 mg $MnCl_2 \cdot 4\ H_2O$, 0.1 mg $Na_2MoO_4 \cdot 2\ H_2O$, 1.0 mg $FeCl_3$, 0.2 mg riboflavin, 0.12 mg biotin, 0.8 mg thiamine HCl, 0.48 mg inositol, 0.08 mg p-aminobenzoic acid, 0.5 mg nicotinic acid, 0.8 mg pantothenic acid, and 0.001 mg cyanocobalamine. The pH of the medium was adjusted to 6.8 by the addition of NaOH prior to autoclaving. The $CaCl_2$, vitamins, carbon source and nitrogen source were filter sterilized and added after autoclaving.

Cultures were inoculated with cells grown to stationary phase in YEX medium (Chapter 2) with 5 mM NH_4Cl. Samples from the batch cultures were taken every 12 h. Culture density was measured by optical density at 420 nm. Protein concentration was measured on whole cell lysates by the method of Lowry et al. (1951) with the following modifications. Protein samples were prepared by mixing 0.5 ml of the bacterial culture

with an equal volume of trichloroacetic acid. The protein was precipitated in a microcentrifuge and suspended in 0.1 ml of 0.3 M NaOH.

RNA Purification. Cells for RNA purifications were harvested from 1 l samples by centrifugation, washed in 20 ml of H_2O, recentrifuged, suspended in 8.6 ml of H_2O and stored at -70°C. To 8.6 mls of cell suspension was added 0.4 mls of 10% (w/v) N-lauroyl sarkosine and 1 ml of 1 M Tris-HCl [pH8.0]. The cells were lysed by two passes through a French pressure cell at 12,000 psi and immediately added to 10 gm of CsCl. The resulting mixture was layered over a 7 ml cushion of 5.6 M CsCl. The tube was filled with 0.1 M Tris-HCl [pH8.0], 0.4% (w/V) N-lauroyl sarkosine and centrifuged in a Beckman SW28 rotor at 22,500 RPM at 20°C for 36 h. RNA pellets were suspended in autoclaved H_2O, extracted twice with H_2O-saturated phenol, extracted four times with diethylether, precipitated with ethanol, suspended in autoclaved H_2O and stored at -70°C.

Results

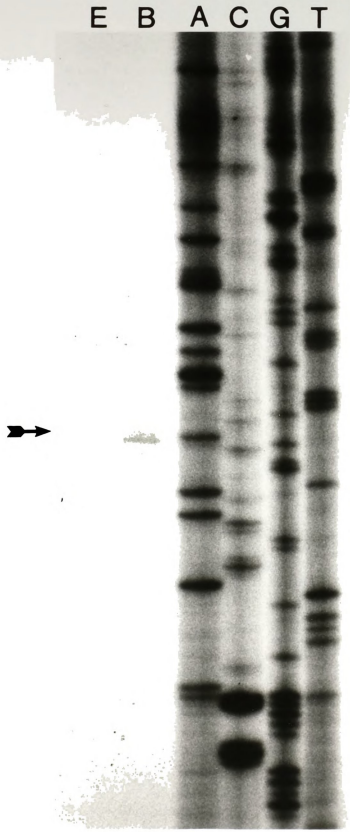
Promoter Mapping. The site of transcriptional initiation upstream of glnII was mapped by the S1 nuclease protection method. Single stranded 5' end-labeled probe was generated using the recombinant phage M13glnII, which has the 2.1 kbp SalI fragment of pBJ196 (Chapter 3) cloned in M13mp18 (Yanisch-Perron et al., 1985) oriented so that the non-coding strand is produced in the single stranded phage. Second strand synthesis was primed with the oligonucleotide TCII

(5'-CGACGCGAATTCCTTGA-3') which hybridizes to the DNA encoding amino acids 26 through 31 of GSII (Figure 10). The resulting probe, which includes about 700 bases upstream of the initiation codon of glnII, was hybridized to RNA prepared from B. japonicum USDA 110 and treated with S1 nuclease. A single protected fragment of about 170 bases in length was observed. The precise location of transcriptional initiation upstream of glnII can be determined by comparing the length of the protected fragment to a sequencing ladder prepared with M13glnII and TCII. The sequence of the glnII promoter can be read directly from the gel (Figure 13).

Sequence Analysis. The DNA sequence upstream of glnII was determined from pBJ196 as described in Chapter 3 and has some interesting features (Figure 14). 40 bp upstream of the glnII initiation codon is the center of an imperfect inverted repeat. This sequence should produce a "hairpin" secondary structure in the 5' untranslated region of the glnII mRNA. Upstream of the promoter is an unusual run of 13 or 14 consecutive G-C base pairs. The exact length of this structure is uncertain due to difficulties in sequencing regions of high G-C content.

The promoter region of glnII is shown in Figure 15. As expected, there is little homology between the promoters of glnII and glnA (Figure 6). This lack of promoter homology is presumably responsible for the differential regulation of the two glutamine synthetases of B. japonicum. However, Figure 15 shows that there is extensive homology between the glnII promoter and several B. japonicum nif promoters. This suggests that glnII and nif genes are regulated, at least in part, by a common mechanism. However, the differential expression of glnII and nif

Figure 13. **S1 Nuclease Protection Mapping of the glnII Promoter.** Lanes ACGT show DNA sequence reactions (Sanger et al., 1977) for the region upstream of glnII. S1 nuclease protection reactions used the following RNA types: E, E. coli ET8051; B, B. japonicum USDA 110 grown to mid-log phase in MOPS buffered medium (Methods) plus 0.2% xylose and 10 mM glutamate.


E B A C G T

```
-280
CATCGGCAAC CTCCGGCACC GGCCGGCCTG CTCCAGAATC AGGCGTTTTC CGCGGGATTT

-220                        [- - - -NRI- - - -]
TTGCAGTGCA GCTTCACGCA AAGGTGCGCC GCTATGACGC ACGCCGCCGG GGACGACCGG

-160
CCGCTCGCGG GGGGGGGGGG GGCGGCCGCG GTGGAAAACC TCCCGCAATG CGGCCTTTTG

-100
GCACGCTAAA TGCTTGTAAA CGGTCGGCCG ATGGTGGCCG GGTACAAACG TGGGGGGCCC
                                                  M   T   K   Y   K   L
CCGGGCCCCC AACCTTTCGC ATCATCTACA GAGAGGCTCA ATG ACC AAG TAT AAG CTC
```

Figure 14. **DNA Sequence Upstream of glnII.** The DNA sequence was determined as described in Chapter 3. The asterisk marks the point of transcriptional initiation as indicated in Figure 13. The lines over the sequence from -55 to -24 mark the inverted repeat in the 5' untranslated region of the glnII mRNA. The region with a potential NRI binding site is marked in brackets.

```
B. j. nifH          taaggtgCcgggttaGaCCtTGGCACGgctGTTGCTgATaagCGGca*
B. j. nifDK         ttTtagtgctcatGaGaCCcTGGCAtGCcgGTTGCaaAgtcttGGat*
B. j. nifB          caTtcgcgtcatcctcgCCacGGCAtGCaAGTTGCTaATcctCctgaa*
B. j. fixA          gcggtccCaagcgGcGgggaTGGtACaagAcTTGCTgtTctcttccc*
B. j. glnII         ccTcccgCaatgcGgcctttTGGCACGCtAaaTGCTtgTaaaCGGtc*

B. j. consensus     --T----C-----G-G-CC-TGGCACGC-AGTTGCT-AT---CGG***
Ntr consensus       -------------------CTGGCAC-----TTGCA----------*
```

Figure 15. **B. japonicum Promoter Sequence Comparison.** The B. japonicum glnII promoter and a variety of nif promoters are aligned to maximize homology. The B. japonicum consensus sequence was determined by matches in 3 out of 5 sequences. Homologies between the B. japonicum promoters and the B. japonicum consensus are shown as uppercase letters. Transcriptional initiation sites are shown with asterisks. The nif promoters are from Adams and Chelm (1984) and Adams et al. (1986). The fixA promoter is from Fuhrmann et al. (1985). The consensus sequence for Ntr promoters from enteric bacteria is from Dixon (1984b).

genes suggests that other sequences in these promoters are involved in transcriptional regulation. In E. coli, the transcription of glnA is partially controlled by NRI through binding at a number of NRI binding sites (see Introduction). A search of the 600 bp of DNA sequence upstream of the B. japonicum glnII promoter reveals only one region with better than 65% homology to the consensus NRI binding sequence of enteric bacteria (Figure 14).

glnII Promoter Characterization. The control of the glnII promoter was characterized in a series of experiments which measure changes in the amount of cellular glnII mRNA during the transition of B. japonicum cultures from log to stationary phase. In each culture, either the carbon or the nitrogen source is added at a low concentration so that the transition to stationary phase is concurrent with the depletion of a known nutrient. Therefore, any changes in glnII transcription at this point in the growth curve can be attributed to specific physiological changes.

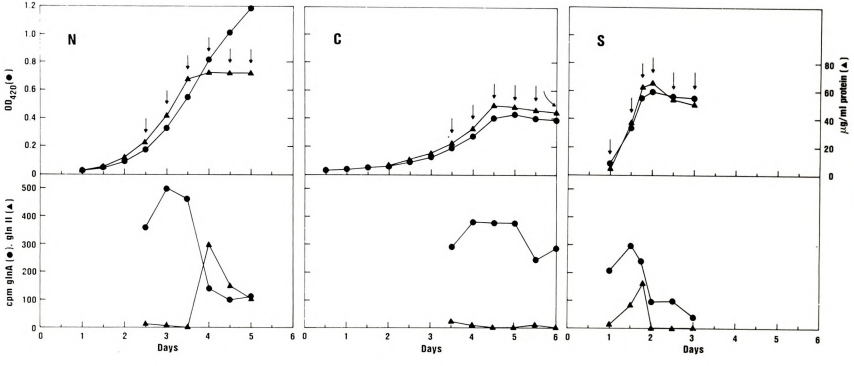
The activity of the glnII promoter was determined using quantitative S1 nuclease protection analysis so that the amount of protected fragment is proportional to the amount of glnII mRNA (Chapter 2). RNA for these experiments was purified from B. japonicum culture samples taken at six points in the growth curve, three before and three after the transition to stationary phase. The transcription of glnA was also monitored for comparison. The glnA S1 nuclease probe was prepared using the primer extension method described above with the oligonucleotide TCA (5'-CCCCTTTTGGCTCTGCG-3') and the template M13glnA, which has the 391 bp SalI fragment of pBJ93 cloned in M13mp18. This gives a 5' end-labeled

single stranded probe 331 bases long and produces a 125 base S1 nuclease protected fragment.

The first culture was grown on 0.2% xylose and 1 mM NH_4Cl. These conditions were chosen so that the culture would deplete the nitrogen source before growing to an OD_{420} of 1.0. The growth curve of the low ammonia xylose culture is shown in Figure 16N. The results of the quantitative S1 protection assay (Figure 17N) show that there is no detectable glnII transcription in B. japonicum when growing on xylose and ammonia, but when the nitrogen source is depleted, the glnII promoter is activated. In contrast, glnA transcription is relatively constant throughout the growth curve. There is about a 3-fold decrease in glnA transcription during stationary phase. This type of differential regulation between the glnA promoter and GSII was observed in Chapter 2. The continued increase in the optical density of the culture following the cessation of protein accumulation (Figure 16N) is due to the production of extracellular polysaccharide (EPS) after the depletion of nitrogen in stationary phase.

Both the activation of glnII and the production of EPS seem to be the result of the nitrogen limited and carbon excess condition following the depletion of ammonia. This conclusion is supported by the results of an analysis using a similar culture grown with a low concentration of xylose (0.02%) and excess nitrogen (5 mM NH_4Cl; Figure 16C). Just as with the first culture, there is no detectable glnII transcription during growth on xylose and ammonia. However, when stationary phase is brought on by carbon limitation, the culture does not induce glnII transcription or EPS production (Figure 17C).

Figure 16. B. **japonicum Minimal Media Growth Curves.** Ten liter cultures of B. japonicum USDA 110 were grown and sampled as described in Materials and Methods. The upper portion of each panel shows the growth curve for a particular culture as measured by the OD at 420 nm and total protein concentration. The arrows show the time points when cells were harvested for RNA preparation. The lower half of each panel summarizes the S1 nuclease protection data by showing the amount of radioactivity protected by glnA and glnII mRNA in the six samples described above. Panel N is the growth curve for the low ammonia xylose culture (0.2% xylose and 1.0 mM NH_4Cl). Panel C is the growth curve for the low xylose ammonia culture (0.02% xylose and 5.0 mM NH_4Cl). Panel S is the growth curve for the low succinate ammonia culture (0.4% succinate and 5.0 mM NH_4Cl).


N
C
S
OD_{420} (●)
μg/ml protein (▲)
cpm glnA (●), gln II (▲)
Days
Days
Days

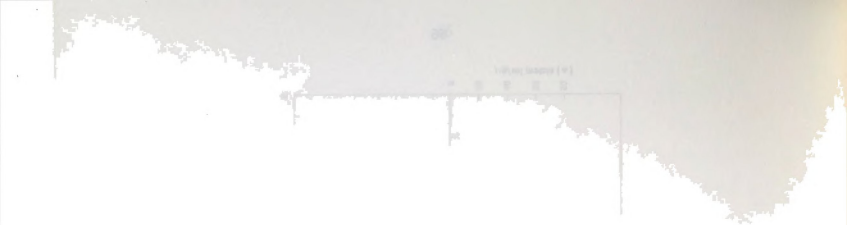
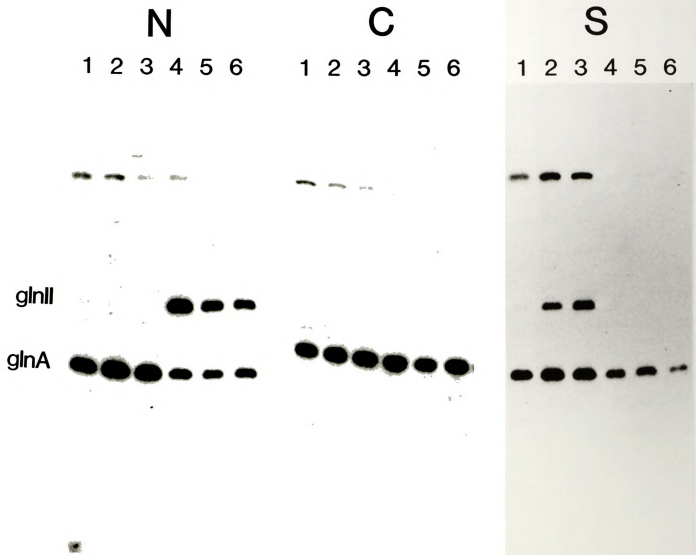


Figure 17. **Comparison of glnA and glnII Transcription.** The relative abundance of glnA and glnII mRNA was measured by quantitative S1 nuclease protection analysis. RNA was prepared from six samples taken from each of the batch cultures N, C, and S as indicated in Figure 16. The positions of the partially protected glnA and glnII probes are indicated. The uppermost band is full length glnA probe. No full length glnII probe was detected.


N
1 2 3 4 5 6
C
1 2 3 4 5 6
S
1 2 3 4 5 6
glnII
glnA

A third culture was designed to study the effect of a preferred carbon source on GSII production. This culture is similar to the low xylose ammonia culture except that the xylose was replaced with succinate (0.04%). Succinate proved to be a very effective carbon source, supporting a rapid growth rate, even at this low concentration (Figure 16S). Succinate also had a dramatic effect on glnII transcription. Figure 17S shows that the glnII promoter is active during growth on succinate and ammonia but is repressed after carbon source depletion. In the succinate culture, as in the low ammonia xylose culture, EPS production parallels glnII expression. EPS accumulates during the growth phase and disappears after carbon source depletion. The pattern of glnA expression in the succinate culture is very similar to the pattern observed in the first culture (compare Figure 17N and 17S). These data are in agreement with earlier observations (Chapter 2) that the glnA promoter is not under general nitrogen control. The glnII promoter, on the other hand, seems to be induced specifically during conditions of nitrogen limitation.

In all conditions that I have tested, glnII is transcribed from a single promoter. There is no other partial or full length protection of the glnII probe. However, several RNA samples gave full length protection of the glnA probe (Figure 17) suggesting the presence of an upstream promoter. Earlier S1 nuclease protection studies using strand separated probes did not detect any transcription upstream of the primary glnA promoter (Chapter 2). This discrepancy may be due to differences in the methods of probe synthesis although the possibility of a second glnA promoter cannot be excluded.

DISCUSSION

I have previously demonstrated that the glnA gene of B. japonicum is transcribed from a single promoter which is not regulated by nitrogen source (Chapter 2). In contrast, GSII levels fluctuate greatly in response to the cell's nitrogen nutrition (Ludwig, 1980b; Darrow et al. 1981). These data suggested that glnII may be transcribed from a nitrogen regulated promoter similar to the nitrogen regulated promoter of the glnA genes in other bacteria (Dixon, 1984b, Reitzer & Magasanik, 1985; Tumer et al., 1983). In this chapter I have shown that glnII is transcriptionally regulated in response to changes in nitrogen metabolism and has a promoter distinct from the glnA promoter but very similar to B. japonicum nif promoters. These data indicate that B. japonicum has a nitrogen regulation (Ntr) system homologous to the well characterized Ntr systems of E. coli and K. pnuemoniae. glnII is the first Ntr regulated gene to be characterized from B. japonicum.

The Ntr regulation of glnII is best illustrated by the results of the nutrient limited batch culture experiments, which show that glnII is induced in conditions of nitrogen limitation. This response occurs in the low ammonia xylose culture (Figures 16N and 17N) when the cells go from a carbon limited to a nitrogen limited condition. B. japonicum growing on xylose and ammonia is presumably carbon limited. Xylose in not a very good carbon source since substituting even a low concentration of succinate results in a much more rapid growth rate (compare Figures 16N and 16S). Furthermore, ammonia is an excellent nitrogen source since even at low concentrations it will repress GS activity in both E. coli (Magasanik, 1982) and B. japonicum (Ludwig, 1978).

However, when ammonia is depleted and the cells become limited for nitrogen, B. japonicum responds by inducing glnII transcription. This response is not due to general starvation since a similar low xylose ammonia culture does not induce glnII in stationary phase (Figure 17C).

The amount of glnII transcription during exponential growth is also controlled by nitrogen limitation. However, the extent of nitrogen limitation is not controlled by the nitrogen source but rather by the relative qualities of the carbon and nitrogen sources. For example, growth on xylose and ammonia is carbon limited as previously explained. However, by substituting the relatively poor carbon source xylose with a good carbon source such as succinate, the cells become nitrogen limited and glnII is transcribed during the growth phase (Figure 17S). When the succinate is depleted, the cells are no longer nitrogen limited and glnII transcription stops. This interpretation is supported by the observation that EPS accumulates during growth on succinate and ammonia. In most bacteria, EPS is produced during nitrogen limited growth (Harder & Dijkhuizen, 1983). The disappearance of EPS in stationary phase indicates that, unlike most bacteria, B. japonicum is able to utilize its own EPS as a carbon source (Dudman, 1977; Patel & Gerson, 1974). Apparently succinate is a sufficiently good carbon source to cause growth to be nitrogen limited even with ammonia as the nitrogen source. Similarly, carbon limited growth on xylose and ammonia can be made carbon rich by substituting ammonia with a relatively poor nitrogen source such as glutamate. Thus xylose glutamate grown cells have high levels of glnII transcription (Figure 13).

Because glnII is subject to nitrogen regulation, a comparison of the DNA sequence of the glnII promoter with well characterized Ntr

promoters of other bacteria may show that homologous mechanisms of gene regulation are functioning in B. japonicum. A number of different DNA sequences have been shown to be important in Ntr promoters, including the consensus Ntr promoter, the NRI binding sequence and the upstream nif activating sequence. An analysis of the DNA sequence upstream of glnII reveals some similarities and some differences to these Ntr promoter features. A comparison of Figure 6 and Figure 15 shows that there is little homology between the B. japonicum glnA and glnII promoters but a large block of homology among glnII and B. japonicum nif promoters. This region of homology is similar in structure to the sequence of other bacterial Ntr promoters responsible for transcriptional initiation by RNA polymerase and the Ntr-specific sigma factor, encoded by the gene rpoN. It is therefore reasonable to conclude that the conserved sequence upstream of glnII is the RNA polymerase binding site and that B. japonicum has a gene analogous to rpoN which encodes an Ntr-specific sigma factor.

Although Ntr and nif genes share an Ntr specific promoter sequence, only nif genes have the upstream regulatory sequence. This sequence has been found upstream of nif promoters in many nitrogen fixing bacteria (Buck et al., 1986) and may account for the differential regulation of nif genes and other Ntr activated promoters. In accordance with this model, I find that the nif activating sequence does not occur upstream of the glnII promoter.

Another feature of Ntr promoters is the NRI binding site. NRI is a DNA binding protein encoded by the gene ntrC (glnF) and is required for Ntr promoter activation. NRI may also bind to DNA to function as a transcriptional activator, repressor, or antiterminator. I find only

one potential NRI binding site upstream of glnII (Figure 14). It is not known if this sequence is involved in glnII regulation by NRI binding, or even if B. japonicum has a regulatory protein analogous to NRI. In contrast, the Ntr regulated glnA promoter of E. coli has five NRI binding sites involved in glnA transcriptional regulation (Reitzer & Magasanik, 1986). More experiments are necessary in order to determine if a similar mechanism of regulation functions in B. japonicum.

Despite extensive differences between the structures of E. coli and B. japonicum GS genes, the overall regulation of GS seems quite similar. In E. coli a single GS gene, glnA, is transcribed from two differentially regulated promoters (Reitzer and Magasanik, 1985). One promoter is homologous to the consensus E. coli promoter and provides a constant low level of GS during growth on carbon limited media. This function is served in B. japonicum by the glnA gene which is transcribed in all conditions from a promoter independent of the cell's nitrogen nutrition. The other E. coli glnA promoter has multiple NRI binding sites and is activated by the Ntr system to provide high levels of GS during growth on nitrogen limited media. In B. japonicum, nitrogen starvation causes the production of high levels of GSII by means of a single inducible nitrogen regulated promoter.

Although the glnII promoter is responding to nitrogen limitation, there is evidence that other factors are involved in glnII regulation. Adams and Chelm (1986) have shown that glnII is transcribed at significant levels in bacteroids and in free living cultures grown at low oxygen concentrations, and that this transcription originates from the same promoter as the aerobic expression. glnII transcription in these conditions requires the presence of a gene, odcR (also termed nifA in

Fischer et al., 1986), which is homologous to the regulatory genes nifA and ntrC of K. pneumoniae. odcR, however, is not responsible for aerobic expression of glnII since odcR deletion strains show normal aerobic regulation of glnII. The microaerobic specific activation of glnII is unknown in the well characterized Ntr systems of the enteric bacteria and contrasts with previous studies which reported the repression of B. japonicum GSII in bacteroids (Cullimore et al., 1983) and microaerobic cultures (Rao et al., 1978). It is not yet known if the odcR dependent transcription of glnII results in the production of functional GSII, although it is possible that GSII is more abundant in bacteroids than previously thought. odcR mutants do not fully differentiate to form nitrogen fixing bacteroids. It will be of interest to characterize the role of odcR and other nitrogen regulatory factors in the regulation of nitrogen metabolism in symbiotic nitrogen fixing bacteria. The glnII promoter, along with nif promoters, will serve as important tools in the characterization of the B. japonicum Ntr system.

CHAPTER 5

Summary and Conclusions

Glutamine synthetase (GS) catalyzes the first step in the primary pathway of bacterial ammonia assimilation. Thus the regulation of GS is an important element in the control of overall nitrogen metabolism. The enteric bacteria regulate GS via a complex nitrogen regulatory system (Ntr) which has been the subject of extensive genetic and biochemical characterization (Magasanik, 1982). In contrast, the mechanisms controlling rhizobial nitrogen metabolism are not well understood. Rhizobial genes encoding the enzyme nitrogenase have been isolated and characterized. The concurrent activation of nitrogenase and repression of GS is apparently responsible for the export of fixed nitrogen from symbiotic bacteroids. In this dissertation, I have demonstrated the isolation and characterization of the genes encoding GSI and GSII of Bradyrhizobium japonicum, the soybean symbiont. This work will ultimately lead to a more complete understanding of the specific adaptations necessary for rhizobium to function as a symbiotic nitrogen fixing bacteria.

In Chapter 1, I discussed the isolation and characterization of the gene encoding GSI. Unlike GSII, GSI is homologous in structure and sequence to the single GS of E. coli. Furthermore, GSI and E. coli GS

are both regulated by an adenylylation cascade system. Therefore, I chose the E. coli nomenclature glnA for the gene encoding B. japonicum GSI. The most significant finding of Chapter 1 is that glnA is constitutively transcribed from a single promoter. This is in contrast to other bacteria (E. coli, K. pnuemoniae, and Anabaena) where GS is transcribed from multiple promoters. Therefore, GSI is primarily regulated post-translationally by adenylylation. The constitutive production of a reversibly modified GS presumably allows for a very rapid adjustment of total GS activity as determined by the changing needs of the cell. The glnA promoter is the first "normal" promoter to be characterized from B. japonicum. Although the glnA promoter has some homology to the E. coli consensus promoter, it appears to have little or no activity in E. coli. A similar result was observed with the Rhizobium meliloti glnA promoter. Because it is always active, the B. japonicum glnA promoter may be useful for the expression of foreign genes in rhizobia.

GSII is not known to be regulated by a post-translational modification. Therefore, the gene encoding GSII is probably transcriptionally regulated from an Ntr-like promoter. For this reason, the isolation and characterization of the GSII gene (glnII) was of particular interest. Numerous screenings of B. japonicum genomic libraries using heterologous hybridization probes resulted in the identification of only the glnA gene. glnII was not detected because of the limited homology between the eucaryote-like glnII sequence and the procaryotic probes. Attempts to isolate glnII by complementation of an E. coli glutamine auxotroph were also unsuccessful. Ultimately, glnII was identified in a cosmid clone bank using a mixed oligonucleotide hybridization probe based on

a partial amino acid sequence determined from purified GSII (Chapter 3). This is the first report of the isolation of a glnII gene.

Amino acid sequence comparisons revealed extensive homology between GSII and eucaryotic glutamine synthetases but limited homology between GSII and procaryotic glutamine synthetases. Based on these data, I suggest that GSII evolved as the result of a eucaryote to procaryote gene transfer event. This conclusion is supported by the observation that the GSII isozyme is found in a single family of bacteria, the Rhizobiaceae. Because bacteria of this family are characterized by their ability to form cortical hypertrophies on plants, I suggest that a plant provided the progenitor glnII gene. Little is known of the role of horizontal gene transfer in evolution. I know of no selective pressure for the maintenance of a plant GS gene in plant pathogenic bacteria. GSII is not known to serve any special role in the development of rhizobial symbioses. It will now be possible to construct a glnII$^-$ rhizobium using site directed gene replacement mutagenesis. It will be of interest to analyze the symbiotic phenotype of a glnII mutant rhizobium. Glutamine auxotrophs have been utilized in the study of rhizobial physiology. Because there are two GS genes in rhizobia, glutamine auxotrophs generated by traditional mutagenesis techniques will generally carry mutations in regulatory genes, resulting in complex, pleiotropic phenotypes. I have shown that GSII is transcriptionally regulated to provide high levels of total GS activity during nitrogen limited growth. I predict that a glnII$^-$ rhizobia would not be a glutamine auxotroph (due to the presence of a functional glnA gene) and would not grow well on poor nitrogen sources (due to the lack of a transcriptionally inducible GS).

The site of transcriptional initiation upstream of the glnII coding region was mapped by S1 nuclease protection analysis. Upstream of the RNA start site is a region of homology to B. japonicum nif promoters. This conserved region is the same sequence responsible for σ^{60} dependent RNA polymerase binding in K. pneumoniae Ntr promoters. Furthermore, the glnII promoter has no significant homology to the glnA promoter. Based on these observations, I suggest that glnII and nif genes of B. japonicum require an alternate sigma factor for transcriptional initiation, analogous to the Ntr system of enteric bacteria. This model, however, does not account for the differential expression of glnII and nif genes. Presumably, sequences other than the RNA polymerase binding sites are involved in glnII and nif regulation. In K. pneumoniae, upstream activation sequences and the protein NRI are involved in Ntr promoter regulation. A similar system may function in B. japonicum. The glnII promoter will serve as a useful tool in the characterization of possible NRI-like proteins. Successive deletions upstream of glnII can be used to identify regions necessary for promoter activity. Promoter activity can be monitored in vivo GS assays or in vitro with a reconstituted RNA polymerase transcription system. Similar experiments have identified possible upstream activation sequences in B. japonicum nif promoters (Alvarez-Morales, et al., 1986).

I have shown that, in free-living aerobic cultures, the glnII promoter is activated by either nitrogen starvation or nitrogen limited growth. Adams and Chelm (personal communication) have identified a possible ntrC-like gene which is required for induction of glnII in nitrogen limited cultures. Although these data are still preliminary, it appears that the B. japonicum "ntrC" gene is not required for nif

gene activation as it is in K. pneumoniae. Therefore, nif regulation is not linked to general nitrogen regulation. It is not clear what factors affect the expression of the glnII promoter in microaerobic cultures and bacteroids. Rao, et al. (1978) have shown that GSII activity (and presumably glnII transcription) in a nitrogen limited culture decreases dramatically when the O_2 concentration drops below 0.40%. In contrast, Adams and Chelm (1986) have demonstrated significant glnII transcription in nitrogen rich microaerobic cultures. The microaerobic induction of glnII is dependent on another regulatory gene, odcR, which is required for bacteroid development and nif activation. These data can be explained as follows. glnII transcription in nitrogen limited aerobic cultures is "ntrC" dependent. When the O_2 concentration drops below a threshold level, the cells become oxygen (or energy) rather than nitrogen limited, and "ntrC" dependent glnII transcription stops. These same conditions (but not "ntrC") cause the induction of odcR dependent promoters, including nif genes. Because of the homology between the odcR and "ntrC" gene products, odcR stimulates transcription of glnII at a lowered level. Thus, glnII regulation is "normal," responding primarily to nitrogen metabolism. Alternately, it is possible that microaerobic cultures are still nitrogen limited and that the glnII promoter is inactivated by a microaerobic-specific regulatory system, possibly mediated by odcR. It would be of interest to characterize the microaerobic response of glnII in odcR$^-$ mutants grown on a poor nitrogen source. The preliminary results of Adams and Chelm, which suggest that nif transcription is not activated by general nitrogen regulation, are highly significant since this model implies that the regulation of nif, and not glnII, has been modified so that B. japonicum can export fixed

nitrogen to its host plant. However, the induction of *nif* gene transcription *ex planta* does not necessarily result in the assembly of a functional nitrogenase enzyme. Further investigations are needed to determine what additional regulatory factors are involved in the development of the rhizobial-legume symbiosis.

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