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Thomas H. Adams

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TRANSCRIPTIONAL REGULATION OF NITROGEN FIXATION AND NITROGEN ASSIMILATION GENES IN *BRADYRHIZOBIUM JAPONICUM*

by

Thomas H. Adams

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ABSTRACT

TRANSCRIPTIONAL REGULATION OF
NITROGEN FIXATION AND NITROGEN ASSIMILATION
GENES IN BRADYRHIZOBIUM JAPONICUM

by Thomas H. Adams

I describe the isolation and characterization of three transcription units (nifH, nifDK, and nifB) that are required for nitrogen fixation by Bradyrhizobium japonicum. The promoters for transcription of each of these three B. japonicum gene units share a high degree of DNA sequence homology with each other and with nif gene promoters from K. pneumoniae and other diazotrophs. Transcription from these B. japonicum promoters is induced both during symbiotic association with soybean plants and during axenic free-living growth in microaerobic or anaerobic culture. I have isolated a regulatory gene, termed odcA, that is required for this nif transcription. The protein encoded by this regulatory locus (ODCA) has sequence homology to the product of the K. pneumoniae nifA gene. The odcA gene is also necessary for the microaerobic and symbiotic expression of the gene encoding glutamine synthetase II, glnII. The wildtype odcA allele somehow limits the final viable cell density achieved in microaerobicaly grown B. japonicum cultures. Finally, mutation of odcA results in defective nodulation of soybeans by B. japonicum. odcA mutant bacteria are released at a reduced efficiency into plant cells of the nodule cortex,

and infected plant cells rapidly enter a degenerative phase. Mutant bacteria continue to divide in the intercellular space of the nodule cortex. The abnormal developmental phenotype observed for this mutant strain cannot be attributed to nitrogen starvation due to the lack of nitrogen fixation, since nodules induced by a nitrogenase deficient strain (*nifDK*$^-$) differentiate normally. These results indicate that an important aspect of proper nodule development is the bacterial localization in the oxygen limited plant tissue resulting in the expression of a number of bacterial functions. One or more of these functions must be involved in signalling the plant of the symbiotic nature of the invading bacteria.

Three other *B. japonicum* *nifA*-like genes (*hna2*, *hna3*, and *hna5*) are described. None of these genes are required for transcriptional control of *nif* or *gln* gene expression under any condition tested. These genes are discussed in terms of their potential roles in regulating other aspects of cellular physiology in *B. japonicum*.

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CHAPTER 1

INTRODUCTION

The biological reduction of atmospheric dinitrogen into organic compounds accounts for more than two-thirds of global nitrogen fixation. The majority of the remaining ammonia production is by chemical means (Burris, 1980). Biological nitrogen fixation, as catalysed by the nitrogenase enzyme complex, is carried out by a number of diverse prokaryotes including obligate anaerobes (e.g. Clostridium pasteurianium), facultative anaerobes (e.g. Klebsiella pneumoniae), and obligate aerobes (e.g. Azotobacter vinlandii). Nitrogenases from all these different bacteria have similar properties (Eady et al., 1974). The nitrogenase complex consists of two oxygen-labile components. Component I (dinitrogenase) has two copies each of two unique subunits and contains the site for substrate binding and reduction. Component II (dinitrogenase reductase) functions in reducing component I and is a homodimer (Mortenson and Thornley, 1979).

The molecular genetics of nitrogen fixation is best understood in the free-living diazotroph K. pneumoniae. Here, a cluster of 17 genes, grouped in 7 operons encode the enzymes specifically involved in nitrogen fixation (nif genes; Roberts and Brill, 1981). Three of these genes, nifH, nifD, and nifK, encode dinitrogenase reductase and the two

subunit types of dinitrogenase, respectively. These three genes comprise a single operon that is transcribed in the order nifHDK (Merrick et al., 1978; Kaluza and Hennecke, 1979). The genes nifE, nifN, nifB, nifQ, nifC, and nifJ, all encode products involved in the synthesis of the FeMo cofactor of nitrogenase component I (Roberts and Brill, 1981). The products of the nifM, nifS, nifV, and nifU genes are required for modification or activation of nitrogenase component II (Roberts and Brill, 1981). Finally, nifA and nifL encode nif-specific regulatory molecules (Roberts and Brill, 1981).

The transcription of K. pneumoniae nif genes is modulated in response to oxygen and fixed nitrogen by two distinct regulatory systems each of which has positive and negative control elements. One links nitrogen fixation to the general nitrogen control pathway (Ntr) known for several enteric bacteria (Magasanik, 1982). The second is specific for nif gene control (Dixon, 1984).

The general nitrogen control (Ntr) system found in enteric bacteria has three regulatory components; ntrA (also termed glnF or rpoN), ntrB (also termed glnL), and ntrC (also termed glnG) (Leonardo and Goldberg, 1980; de Bruijn and Ausubel, 1981; Epsin et al., 1981; 1982; Ow and Ausubel, 1983; Merrick, 1983; Drummond et al., 1983). The products of these loci (NTRA, NTRB, NTRC; gene products for these and other regulatory genes will be referred to with capitals) interact to control the expression of genes for nitrogen assimilation, glnA (glutamine synthetase), amt (ammonia transport) (Jayakumar et al., 1986), and nif, as well as those genes involved in the utilization of secondary nitrogen sources including histidine (hut), proline (put), and arginine (aut). All of the loci that comprise the Ntr regulon have a similar promoter

structure characterized by the consensus sequence CTGGYAYR-N4-TTGCA (where C, T, G, and A represent the four standard nucleotides; Y=C or T, R=A or G, and N=C, T, G, or A) in the region -26 to -10 bp relative to the transcriptional initiation site (Beynon et al., 1983; Ow et al., 1983; Sundaresan et al., 1983). The identification of this novel consensus sequence for Ntr promoters led to the hypothesis that transcriptional initiation of these genes is mediated by a novel RNA polymerase holoenzyme (Ow et al., 1983). Subsequent experiments using in vitro transcription systems have demonstrated that NTRA is a sigma factor that is required for transcription of promoters in the Ntr regulon by bacterial core RNA polymerase (Hirschman et al., 1985; Hunt and Magasanik, 1985).

Transcription of ntrA is not controlled by nitrogen regulation (Costano and Bastacchea, 1984; de Bruijn and Ausubel, 1983). Instead, global Ntr control is mediated by modulation of the activity and expression of NTRC, a positive regulator of Ntr promoters. Ntr mediated activation of gene expression involves both the NTRA and NTRC proteins. NTRC functions as a dimer in binding double stranded DNA at the consensus sequence TGCACY-N5-GGTGCA (Ueno-Nishio et al., 1984; Hirschman et al. 1985; Reitzer and Magasanik, 1986; MacFarlane and Merrick, 1985; Ames and Nikaido, 1985). Deletion of NTRC binding sites upstream of the E. coli glnA promoter eliminates Ntr-mediated transcriptional activation of this gene (Reitzer and Magasanik, 1986). The function of these upstream binding sites in transcriptional activation is at least somewhat independent of their position and orientation with respect to the RNA initiation site (Reitzer and Magasanik, 1986). These properties

suggest that NTRC binding sites may function similarly to eukaryotic enhancer elements (Khoury and Gruss, 1983).

The activity of NTRC is modulated via a covalent modification mechanism involving NTRB (Ninfa and Magasanik, 1986). NTRB is a kinase/phosphatase and phosphorylates NTRC in response to nitrogen limitation. Dephosphorylation of NTRC occurs when nitrogen availability no longer limits growth. The activity of NTRB is controlled by an unknown mechanism requiring the glnB and glnD products. It has been proposed that these proteins sense the relative levels of glutamine and 2-ketoglutarate in the cell (Magasanik, 1982; Bueno et al., 1985). Thus, when nitrogen limits growth, the glutamine to 2-ketoglutarate ratio is low causing the conversion of NTRB to a kinase which then phosphorylates NTRC. This phosphorylated NTRC (NTRC-P) is then able to activate transcription of promoters under Ntr control. The manner in which NTRC phosphorylation affects its activity as a transcriptional regulator is not known. Nonphosphorylated NTRC is apparently able to bind DNA. In addition, ntrB deletion mutants do not abolish the ability of NTRC to activate transcription of Ntr promoters (Chen et al., 1982; Bueno et al., 1985). These ntrB mutants are slightly delayed in their response to nitrogen limitation. Perhaps phosphorylation affects the affinity of NTRC for DNA binding and/or interacting with NTRA.

General nitrogen regulation of nif gene expression in K. pneumoniae occurs through the action of NTRA, RNA polymerase, and NTRC on the expression of nifL and nifA (Leonardo and Goldberg, 1980; de Bruijn and Ausubel, 1981; 1983; Epsin et al., 1981; 1982; Ow and Ausubel, 1983; Drummond et al., 1983; Merrick, 1983). These genes comprise a single operon in K. pneumoniae and are transcribed from a Ntr promoter (Ow and

Ausubel, 1983). The nifL and nifA gene products (NIFL and NIFA) in turn control expression from other nif promoters (Buchanon-Wollaston et al., 1981a; 1981b; Dixon et al., 1980, Hill et al., 1981, Merrick et al., 1982). NIFA serves as a positive activator for transcription from nifLA and other nif operons. As with NTRC, NIFA mediated transcriptional activity requires a functional NTRA protein. When oxygen or intermediate levels of ammonia are present, NIFL prevents the activation of other nif gene transcription. The mechanism of NIFL mediated repression of nif gene transcription is not understood. It is likely that NIFL does not function as a repressor by binding DNA since it inhibits NIFA- but not NTRC-mediated activation of nifLA transcription (Cannon et al., 1985). In view of the NTRB-NTRC interaction described above, one might propose that NIFL exerts its effect through some covalent modification of NIFA.

In addition to their joint requirements for NTRA, NIFA and NTRC are structurally and functionally related. The amino acid sequences for both proteins are homologous throughout a large central region (Buikema et al., 1985; Drummond et al., 1986; Nixon et al., 1986; Gussin et al., In Press). Promoters for nif genes have the same -26 to -10 consensus sequence CTGGYAYR-N4-TTGCA as Ntr regulated promoters (Beynon et al., 1983; Ow et al., 1983). Finally, under certain conditions, some of these promoters can be activated by either regulatory system (Ow and Ausubel, 1983; Merrick, 1983; Buck et al., 1985).

The mechanism of NIFA mediated transcriptional activation is not as well understood as that for NTRC. However, genetic studies coupled with characterization of nif gene promoter sequences suggest certain analogies to NTRC dependent regulation. As with Ntr promoters, nif

promoters usually have a characteristic upstream consensus (TGT-N10-ACA) that is required but functions independantly of position or orientation with respect to transcription initiation (Buck et al., 1986). This sequence is similar to the proposed consensus binding sequence (TGTGT-N6-10-ACACA) for a variety of DNA binding protiens including LACI, CRP, ARAC, LEXA, and GALR (Buck et al., 1986; Giequel-Sanzey and Cossart, 1982). However, it has not yet been possible to detect binding of purified NIFA protein to this upstream region.

Studies of regulatory mechanisms for controlling nitrogen fixation by diazotrophs other than K. pneumoniae have relied on the models described above. This approach has proven to be particulaly useful in studying the regulation of nitrogen assimilation and nitrogen fixation by bacteria of the genera Rhizobium and Bradyrhizobium.

Rhizobia and bradyrhizobia reduce atmospheric dinitrogen primarily during symbiotic association with their leguminous host plants. The successful establishment of a nitrogen fixing symbiosis is complex, requiring the coordinate differentiation of both the plant and the bacterial cells. For the bacterium, this developmental process culminates upon differentiation into a morphologically and functionally distinct nitrogen fixing endosymbiont termed a "bacteroid". Nitrogen fixation by rhizobial bacteroids differs from nitrogen fixation by K. pneumoniae and most other free-living diazotrophs in that it is not coupled to nitrogen assimilation. Very little of the ammonia produced from dinitrogen reduction by bacteroids is used to support bacterial growth but instead, most of this ammonia is exported (Bergerson and Turner, 1967) into the plant cytoplasm where it is assimilated by plant enzymes (Miflin and Lea, 1976). Enzyme assays indicate the glutamine

synthetase activity is repressed in these nitrogen fixing cells (Brown and Dilworth, 1975; Stripf and Werner, 1978; Werner et al., 1980). In return for this fixed nitrogen, the plant supplies reduced carbon compounds to the bacteria to fuel the respiration needed to meet the high energy demands of nitrogen fixation.

The symbiotic mechanisms controlling plant and bacterial differentiation in root nodules are not well understood. One physiological factor that has been experimentally implicated in developmental control is oxygen limitation. Low oxygen levels presumably exist during the early stages of nodule development due to the presence of multiple diffusion barriers and the active respiration of both the plant and the bacterium. Later in nodule development, the plant encoded oxygen binding protein leghemoglobin maintains a very low concentration of free oxygen in the nodule, protecting the bacteroid nitrogenase from O_2 inactivation (Appleby, 1984). In response to these conditions rhizobia induce a terminal oxidase activity with a very high affinity for oxygen (Bergerson and Turner, 1980). In addition to its O_2 binding activity, leghemoglobin acts as an oxygen carrier protein in nodules, transporting oxygen to the bacteroids by facilitated diffusion. In this manner, bacteroids are provided a low oxygen environment to protect the oxygen labile nitrogenase complex from inactivation, coupled to a high oxygen flux for the vigorous respiration required to supply the energy necessary for nitrogen fixation.

Oxygen limitation has been shown to affect gene expression in both plants and bacteria. A small but detectable induction of uricase, a nodule specific enzyme, can occur in non-nodule soybean tissue when oxygen is limiting (Larsen and Jochimsen, 1986). In addition, many

Bradyrhizobium strains can be made to fix nitrogen in free-living culture under specialized conditions that include microaerobiosis (Keister, 1975; Pagan et al., 1975; Kurz and Larue, 1975; McComb et al., 1975; Tjepkema and Evans, 1975). Nitrogen metabolism during asymbiotic nitrogen fixation by bradyrhizobia is similar to that observed in bacteroids since most of the fixed nitrogen is not used to support growth but is instead exported into the medium (O'Gara and Shanmugan, 1976; Bergerson and Turner, 1978; Ludwig, 1980). For at least some Bradyrhizobium strains this failure to assimilate fixed nitrogen is partly due to a decrease in glutamine synthetase activity (Ludwig, 1980; Bergerson and Turner, 1978). There is also evidence that low oxygen induces an ammonia export system that could account for the observed accumulation of fixed nitrogen in the medium (Gober and Kashkett, 1983).

Ammonia export by bacteroids is "logical" in terms of their role as symbiotic nitrogen fixing "organelles". However, it is difficult to understand why such apparently altruistic behavior should have evolved. Two hypothesis have recently been put forward to explain the nitrogen fixation behavior of rhizobia. Ludwig (1984) proposed that nitrogen fixation coupled to ammonia export has evolved as a mechanism for syntrophic growth. This model implies that the differentiation of metabollically specialized nonviable cells occurs within a growing cell population that is limited for nitrogen. Thus, under conditions of low O_2 with a limiting supply of fixed nitrogen, some population of cells in a culture would switch their metabolic state to one in which nitrogen is fixed but growth is repressed. Nitrogen assimilation would be blocked in these cells and ammonia export activated (Ludwig, 1980; Gober and Kashkett, 1983). This exported ammonia could then be utilized by

non-nitrogen fixing members of the population (and by the plant) that are still active in ammonia assimilation. An alternative but perhaps related model for symbiotic nitrogen fixation was proposed by Kahn et al. (1985). According to this model the plant is able to induce the bacterial dinitrogen reduction by feeding amino acids. In this way, nitrogen might be used as a carbon carrier into the bacteroids. Nitrogen fixation would then occur to replace ammonia utilized by the plant. This would insure a continued flow of carbon to the bacteria. The mechanism for these models differ in that one requires bacterial differentiation while the other simply exploits known bacterial physiology. It is possible that both proposed models could play roles in controlling symbiotic nitrogen fixation.

Several laboratories have begun to characterize regulatory mechanisms for controlling the expression of nitrogen fixation and nitrogen assimilation genes in rhizobia and bradyrhizobia. These studies have been helped by the fact that DNA and amino acid sequences among nitrogenase genes from several organisms are highly conserved (Ruvkin and Ausubel, 1980; Chen et al., 1973; Emerich and Burris, 1978). Many genes required for nitrogenase activity have been identified in both Rhizobium and Bradyrhizobium strains using cloned K. pneumoniae nif genes as hybridization probes. The genes encoding the three nitrogenase polypeptides (nifH, D, and K) have been isolated from R. meliloti (Ruvkin and Ausubel, 1980; Ditta et al., 1980), R. trifolii (Scott et al., 1983a), R. leguminosarum (Ma et al., 1982; Schetgens et al., 1984; R. phaseoli (Quito et al., 1982), B. japonicum (Hennecke, 1981; Adams and Chelm, 1984), and several cowpea Bradyrhizobium strains (Scott et al., 1983b, Weinmann et al., 1984). In addition, genes homologous to

the K. pneumoniae nifA (Szeto et al., 1984; Adams et al., 1984; Fischer et al., 1986; Donald et al., 1986), nifB (Fuhrmann et al., 1985, Rossen et al., 1984; Noti et al., 1986) nifE (Hennecke et al., 1985; Norel et al., 1985; Donald et al., 1986) and nifS (Fischer et al., 1986b) genes have been identified in both Rhizobium and Bradyrhizobium strains. Other genes (fixA, B, and C) that are required for rhizobial nitrogen fixation but are not found in Klebsiella have been identified by transposon mutagenesis (Ruvkin et al., 1982; Corbin et al., 1983, Fuhrmann et al., 1985).

Despite the high degree of interspecies nucleotide sequence homology observed among nif genes, their location and organization is varied. All Rhizobium spp. studied contain symbiotic plasmids (Banfalvi et al., 1981). These plasmids carry clusters of genes that are required for nitrogen fixation and nodule development (nod genes; Long et al., 1982; Corbin et al., 1983; Downie et al., 1983; Schofield et al., 1983; Kondorosi et al., 1984). Although nif and nod genes are also clustered in Bradyrhizobium strains (Kaluza et al, 1983; Adams and Chelm, 1984; Fuhrmann et al., 1985; Lamb and Hennecke, 1986), there is no evidence for symbiotic plasmids in these bacteria (Haugland and Verma, 1981) indicating a probable chromosomal location.

Much of what is known about the regulation of nif genes in rhizobia and bradyrhizobia comes from comparisons to and extrapolations from the detailed understanding of nif gene regulation in K. pneumoniae (see above). Promoters for nif and fix genes in both Rhizobium and Bradyrhizobium closely resemble those identified for nif operons in K. pneumoniae (Ow et al., 1983; Adams and Chelm, 1984; Alvarez-Morales and Hennecke, 1985; Alvarez-Morales et al., 1986). The similarities include

homology to both the -26 to -10 CTGGYAYR-N4-TTGCA Ntr promoter consensus sequence and the nif gene specific TGT-N10-ACA upstream activator consensus sequence. Sundaresan et al. (1983a,b) demonstrated that the NIFA and NTRC proteins from K. pneumoniae could each activate transcription from the R. meliloti nifH promoter in Escherichia coli and in R. meliloti. In E. coli, this transcriptional activation required a functional ntrA gene product (Sundaresan et al., 1983a). Such heterologous expression experiments have also been performed using promoters from the B. japonicum nifH and nifDK transcription units (Alvarez-Morales and Hennecke, 1985; Alvarez-Morales et al., 1986). Here, transcription could be activated by NIFA but not by NTRC. As with the R. meliloti nifH promoter, the transcriptional activation of B. japonicum nif promoters in a heterologous E. coli system requires a functional ntrA gene product. Finally, nifA-homologous genes have now been described in R. meliloti (Szeto et al., 1984), R. leguminosarum (Rossen et al., 1985), and B japonicum (Adams et al., 1984; Fischer et al., 1986). Mutations in at least some of these genes yield rhizobial strains that are unable to induce nif gene transcription. All of these data support the hypothesis that nif gene expression is controlled in rhizobia by a system that is somewhat analogous to the nif-specific regulatory pathway found in K. pneumoniae.

In contrast to Klebsiella, It seems unlikely that the expression of the rhizobial nifA-like regulatory genes are controlled by a central nitrogen regulatory system. As described above, nitrogen fixation by the rhizobia and bradyrhizobia differs from nitrogen fixation by K. pneumoniae in that it is not coupled to nitrogen assimilation. In addition, rhizobial nitrogenase activity is usually not repressed by the

presence of a fixed nitrogen source (Bergerson and Turner, 1967; Keister, 1975; Scott et al., 1979). Finally, a gene with ntrC-like properties has been described in R. meliloti (Ausubel et al., 1985). Mutations in this gene have no effect on the development of symbiotic nitrogen fixation. This differential control of nitrogen fixation and nitrogen assimilation must be central to effective symbiotic nitrogen fixation.

In free-living rhizobia, most ammonia assimilation occurs by the coordinate activity of glutamine synthetase (GS) and glutamate synthase (GOGAT) (Brown and Dilworth, 1975; Kondorosi et al., 1977; Vairinhos et al., 1983). This pathway is primarily controlled by regulating the levels of GS activity. GS regulation is complicated in rhizobia and bradyrhizobia by the fact that these bacteria contain at least two unique forms of the GS enzyme, GSI and GSII (Darrow and Knotts, 1977). These two distinct nitrogen assimilatory enzymes are differentially regulated in response to a number of environmental factors including nitrogen source, carbon source, oxygen concentration, and symbiotic development (Cullimore et al., 1983; Darrow et al.,1981; Fuchs and Keister, 1980; Ludwig, 1980b; Rao et al., 1978).

Rhizobial GSI is similar to the single GS enzyme found in most other Gram negative bacteria. It is a polymeric enzyme with 12 identical subunits of 59,000 daltons each. Like the GS in E. coli and other enterics (Magasanik, 1982), the activity of this enzyme is modulated by a reversible adenylylation cascade system (Darrow and Knotts, 1977; Darrow, 1980). The gene encoding GSI (glnA) has been isolated from both R. meliloti (Somerville and Kahn, 1983) and B. japonicum (Carlson et al., 1985). The transcriptional control of the B.

japonicum glnA gene is different from that for E. coli glnA. In E. coli, glnA transcription is regulated from two tandem promoters, glnAp1 and glnAp2 (Reitzer and Magasanik, 1985). glnAp1 is active under nitrogen excess conditions and provides the cell with basal levels of glutamine synthetase. glnAp2 is controlled by the Ntr system described above. When nitrogen availability limits growth, glnAp2 is activated to increase glutamine synthetase levels and thereby the cell's ability to assimilate nitrogen. In contrast, the B. japonicum glnA gene is transcribed from a single promoter and the amount of transcription is independent of nitrogen availability (Carlson et al., 1985). Thus, at least for B. japonicum, GSI activity would seem to be primarily controlled by adenylylation.

The gene encoding GSII in B. japonicum (glnII) has recently been described (Carlson and Chelm, 1986). This enzyme is unlike any known prokaryotic glutamine synthetases but is closely related to the analogous eukaryotic enzymes both in subunit structure (Darrow, 1980) and amino acid sequence (Carlson and Chelm, 1986). The level of GSII activity is regulated by nitrogen availability (Darrow et al., 1981; Ludwig, 1980b) and oxygen concentration (Rao et al.,1978). Since no post-translational control of GSII activity is known, it is likely that the activity of GSII is primarily modulated by transcriptional control. The promoter for glnII is similar in sequence to Ntr controlled promoters from enteric bacteria in the -26 to -10 region (Carlson, 1986). In addition, under aerobic growth conditions, glnII gene transcription is regulated by a mechanism that interprets the relative availabilities of carbon and nitrogen (Carlson, 1986). This Ntr-like control is similar to that observed for glnAp2 in E. coli.

The molecular mechanisms for transcriptional control of rhizobial nif and gln genes remain unclear. In this dissertation I describe the isolation and characterization of B. japonicum genes that are homologous to the K. pneumoniae nifH, nifD, and nifB genes (Chapters 2 and 3). I have examined the effects exerted by environmental factors (including both nitrogen and oxygen limitation) and symbiotic development on the expression of these nif genes (Chapter 5). In addition, I have isolated four separate DNA regions with homology to both the K. pneumoniae nifA and ntrC genes (Chapter 4). The effects of mutations in these genes on both nif and gln gene expression are described (Chapters 4 and 6). Finally, a model will be presented for the control of nitrogen fixation and assimilation in B. japonicum. The materials in Chapter 2 and most of the information in Chapters 3 and 4 has been presented elsewhere (Adams and Chelm, 1984; Adams et al., 1984). Chapter 6 has recently been submitted as a manuscript for publication (Journal of Bacteriology).

CHAPTER 2

The *nifH* and *nifDK* Promoter Regions from *Bradyrhizobium japonicum* Share Structural Homologies with Each Other and with Nitrogen-Regulated Promoters from Other Organisms

INTRODUCTION

The biological reduction of atmospheric dinitrogen, catalyzed by the nitrogenase enzyme complex, is carried out by a number of procaryotic organisms. The nitrogenase complex is composed of two enzymes, dinitrogenase (component I) and dinitrogenase reductase (component II). Component I itself is a multimeric enzyme composed of two α-subunits and two β-subunits encoded by the *nifD* and *nifK* genes. The reduction of component I is carried out by component II which is composed of two identical subunits encoded by the *nifH* gene.

The conservation of DNA and amino acid sequences among nitrogenase genes from a variety of nitrogen-fixing bacteria (including *Bradyrhizobium japonicum*) is well documented (Ruvkin and Ausubel, 1980; Chen *et al.*, 1973; Emerich and Burris, 1978). This interspecies conservation, however, does not extend to include a preservation of *nif* operon structure. Whereas *nifH*, *D*, and *K* genes are transcribed as a

single operon in Klebsiella pneumoniae (Reidel et al., 1979) and several Rhizobium spp., such as R. meliloti (Banfalvi et al., 1981), they occur as two separate transcription units nifHD and nifK in Anabaena 7120 (Rice et al., 1982) and as nifH and nifDK in B. japonicum and two cowpea Bradyrhizobium spp. (Kaluza et al., 1983; Scott et al., 1983).

The expression of nitrogenase activity is under complex and varied regulation. The control mechanisms are best studied in K. pneumoniae where transcriptional regulation of nitrogenase activity has been demonstrated to occur in response to concentrations of O_2, fixed nitrogen, and molybdenum (Roberts and Brill, 1981; Buchanan-Wollaston et al., 1981a; Dixon et al., 1980). In most cases, derepression of nif genes in Rhizobium species requires symbiotic association with a host legume leading to extensive differentiation of both organisms (Corbin et al., 1982; Paau and Brill, 1982). Symbiotic nitrogen fixation is affected by environmental factors; fixed nitrogen has been shown to inhibit this developmental process (Wong, 1980). Among Bradyrhizobium spp. some strains do not require symbiosis for expression of nitrogenase activity and can be induced to derepress nif genes in free-living culture (Keister, 1975; Scott et al., 1979). For these organisms, environmental control of nitrogenase expression appears to be somewhat similar to that seen for K. pneumoniae except that the presence of ammonia in the growth medium does not repress nif gene expression Scott et al., 1979)

Transcriptional control of nif genes in K. pneumoniae occurs in part by the general nitrogen regulatory system common to all enteric bacteria (Magasanak, 1982). The primary regulatory proteins involved in this process are encoded by ntrA (glnF), ntrB (glnL), and ntrC (glnG).

These proteins work together to control expression of genes involved in nitrogen assimilation, glnA (glutamine synthetase) and nif, as well as those involved in degradation of the amino acids histidine (hut), arginine (aut), and proline (put). The current model for regulation of the Klebsiella nif genes involves a concerted action of the ntrA, and ntrB, and ntrC gene products on the transcription of the nifLA operon (Leonardo et al., 1980; de Bruijn et al., 1981; Epsin et al., 1981; Epsin et al., 1982; Ow and Ausubel, 1983; Drummond et al., 1983; Merrick, 1983; de Bruijn and Ausubel, 1983). The nifL and nifA gene products in turn control expression from other nif operons (Buchanan-Wollaston et al., 1981a; Dixon et al., 1980; Roberts and Brill, 1980; Hill et al., 1981; Buchanan-Wollaston et al., 1981b; Merrick et al., 1982). Thus, under conditions when nitrogen limits growth, ntrA and ntrC combine to activate transcription of the nifLA genes. The nifA gene product then serves as a positive activator of transcription from other nif operons by a mechanism that also requires ntrA. In addition, it has recently been demonstrated that the nifA gene product can substitute for ntrC in activation of its own expression as well as expression from other nitrogen assimilatory promoters (Ow and Ausubel, 1983; Drummond et al., 1983). The function of the nifL gene product is to repress nif transcription as a consequence of rising concentrations of oxygen and ammonia. Finally, when fixed nitrogen is in excess, the ntrB and ntrC products combine to eliminate nif expression entirely through repression of the nifLA operon.

In order to begin to evaluate the molecular mechanisms involved in regulating the transcription of genes controlled by nifA, ntrC, and ntrA, recent work has centered on the determination of promoter

sequences for nitrogen assimilatory genes from Klebsiella. This has led to the establishment of a consensus sequence for promoters controlled by this pathway. Interestingly, the promoter for R. meliloti nifH shares these sequences with ntrC/nifA regulated promoters for Klebsiella (Ow et al., 1983; Beynon et al., 1983). In addition, transcription of the lacZ gene, when fused to the R. meliloti nifH promoter, is controlled in Escherichia coli via the general ntrC/nifA nitrogen regulatory pathway, suggesting similar control mechanisms may be involved (Sundaresan et al., 1983; Sundaresan et al., 1983).

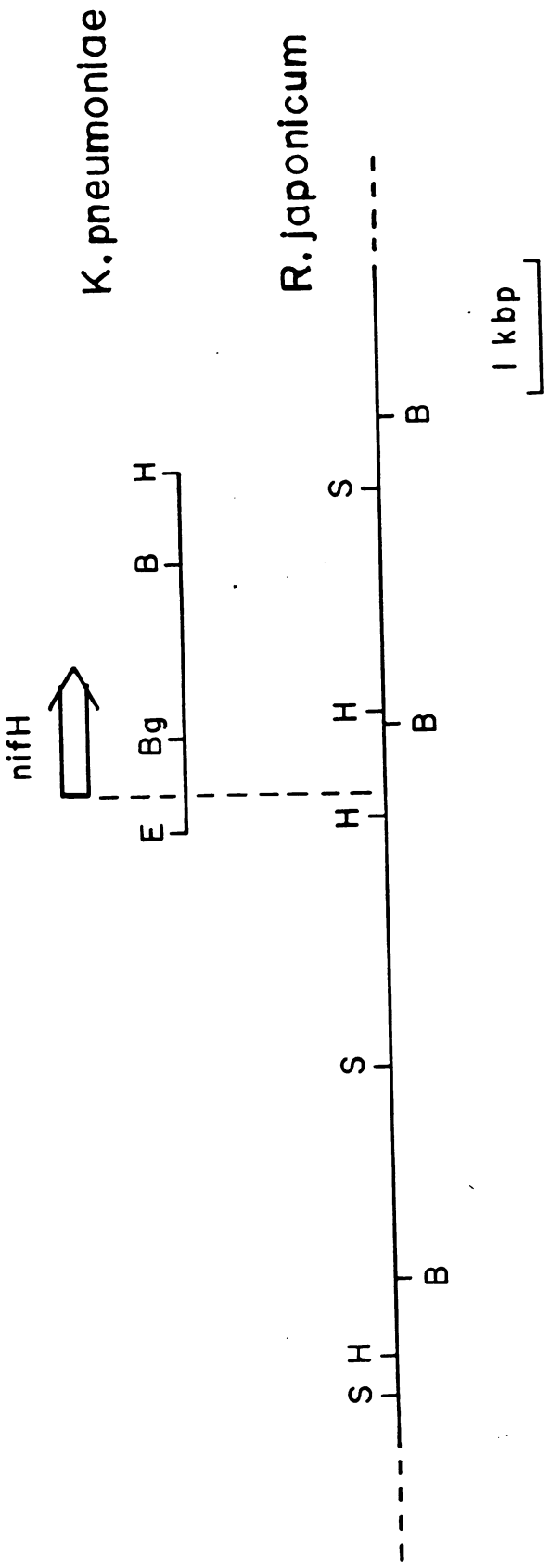
The genus Rhizobium has traditionally been divided into two groups based on growth rate (Vincent, 1982). The "slow-growing" bradyrhizobia are apparently only distantly related to the "fast-growing" rhizobia. In fact, when many slow growers were tested, all had less than 10% overall DNA homology with fast growers such as R. meliloti or R. trifolii. In addition the slow growers are themselves a very diverse group with overall DNA sequence homologies between different strains often being only in the 25% range (Hollis et al., 1981). In this paper we present the nucleotide sequences of the promoter regions for the nifH and nifDK operons from the slow-growing species B. japonicum strain 110. The comparison of these promoters with each other and with nif promoter regions from Rhizobium species shows striking structural homologies. In addition, conserved sequences are found with nif promoter sequences from Klebsiella.

RESULTS

Isolation of the Bradyrhizobium japonicum nifH Gene. In B. japonicum strain 110 the nitrogenase complex polypeptides are encoded by two separate operons, nifH and nifDK (Kaluza et al., 1983). The nifDK operon had previously been isolated as a cloned HindIII fragment by Hennecke (1981). We therefore isolated the nifH gene from a library of B. japonicum USDA strain 110 DNA constructed in the E. coli phage lambda vector BF101 as described previously (Carlson et al., 1983). The lambda library was screened by hybridization to the 730 bp EcoRI-BglII fragment isolated from the plasmid pSA30 (Cannon et al., 1979). This probe fragment contains a portion of the K. pneumoniae nifH gene and no nifD or nifK sequences (Fig. 1). This library screen resulted in the isolation of a single phage, λNH-1. A restriction map of the B. japonicum genomic DNA cloned in λNH-1 is illustrated in Fig. 1. The approximate location of the B. japonicum nifH gene was determined by Southern hybridization analysis (Southern, 1975) of NH-1 DNA using the K. pneumoniae nifH fragment described above as probe (data not shown) allowing the placement of the structural portion of the nifH gene within the region indicated in Fig. 1. Transcription of the nifH gene was determined to proceed in the direction indicated by S1 protection analysis of separated strands of the completely internal 180 bp BglII-HindIII fragment as described in Experimental Procedures (data not shown).

DNA Sequence of the nifH 5' Terminus. The nucleotide sequence of the 5' terminal region, identified by Southern hybridization and S1

Figure 1. *Klebsiella pneumoniae* and *B. japonicum* *nifH* genomic regions. The arrow indicates the position and transcriptional direction of the *nifH* gene within *K. pneumoniae*. The approximate location of the 5' end of the *nifH* coding region within *B. japonicum* is indicated by the dashed vertical line. Restriction endonuclease sites are *Eco*RI (E), *Bgl*II (Bg), *Bam* HI (B), *Hind*III (H), and *Sal*I (S).

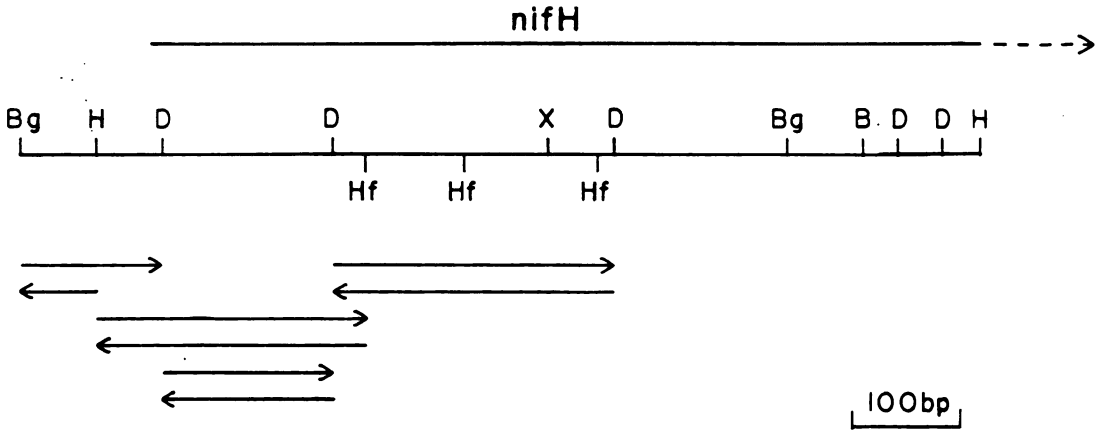

K. pneumoniae
R. japonicum
nifH
E
Bg
B
H
H
B
S
B
H
B
S
B
S H
1 kbp

protection analyses described above, was determined in order to verify the presence of nitrogenase reductase coding capacity (Fig. 2). Beginning at nucleotide 202 and proceeding to the end of the sequenced region is an open reading frame encoding 94 amino acids which have 74% homology to the amino terminus of K. pneumoniae nitrogenase reductase (Sundaresan and Ausubel, 1981; Scott et al., 1981). This open reading frame can actually be extended four amino acids in the 5' direction (to the ATG marked by ?). However, we favor the downstream start site based on homology with the N-terminal ends of nifH from other organisms. In addition, a purine-rich region precedes the ATG suggested to encode the N-terminal methionine. This sequence falls in a position analogous to that designated by Shine and Dalgarno (1975) as the ribosome binding site which precedes translataional initiation in enteric bacteria.

DNA Sequence of the nifDK 5' Terminus. The nifDK operon was isolated previously within a 7.6 kbp HindIII fragment by Hennecke (1981). Southern hybridization experiments with a DNA fragment containing the K. pneumoniae nifD gene as probe allowed the placement of the nifD amino terminus as illustrated in Fig. 3 (data not shown). The nucleotide sequence of this region was determined in order to confirm this placement (Fig. 3). An open reading frame encoding at least 115 amino acids begins at nucleotide 151 and proceeds to the end of the region sequenced. This region has 51% amino acid homology to the K. pneumoniae nifD amino terminal region (Scott et al., 1981).

As described for the amino terminus of the nifH gene, the initiation ATG codon of nifD is preceded by a purine-rich region in a position appropriate to be the Shine and Dalgarno ribosome binding site

Figure 2. DNA sequence of the 5' end of the B. japonicum nifH gene. The strategy used in sequencing the 5' end of the B. japonicum nifH gene is illustrated in the upper part of the figure. The approximate position and direction of transcriptional initiation are indicated by the arrow labelled "nifH". The sequenced DNA strands are illustrated by arrows below the restriction map. Restriction endonuclease sites are BglII (Bg), HindIII (H), DdeI (D), HinfI (Hf), XhoI (X), and BamHI (B). The nucleotide and amino acid sequence of this region are presented in the lower portion of the figure. The question mark indicates a second possible translational initiation codon as discussed in the text.



AGATCTTGTC AGATCCAAAA CAGCCTACGA TCGCGCGCCG GCTGGTTGCT TTTGGAAACG

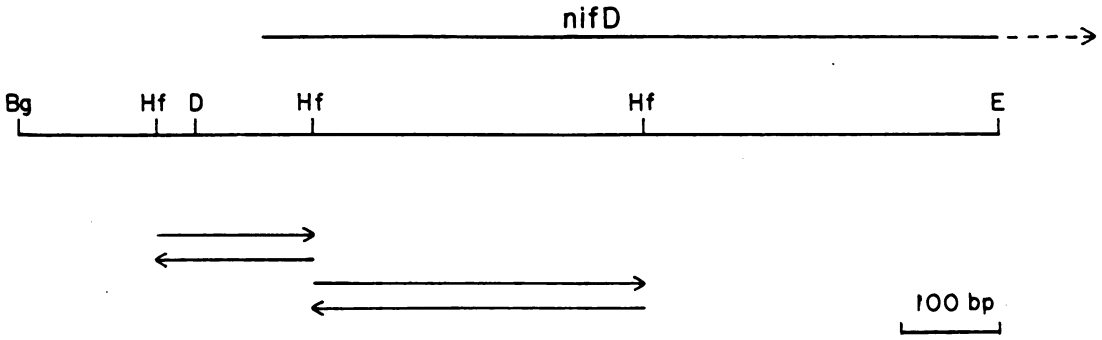
TAATCAGAAG CTTAAGGTGC CGGGTTAGAC CTTGGCACGG CTGTTGCTGA TAAGCGGCAG

CAACACTGAG TGAGGGCTGA GTGCACGCCG ACGTGTAAGG CGAGCGATGC GCTCCTTCCC

TTGAACCCGT GTGCCCCCGT TTCTGCGAGG GAAGCAAAGC TCGCAAAAGA AGCGCGCAAC

					?			MET	ALA	SER	LEU	ARG	GLN	ILE	ALA
GTTTGGCAAA	TCGGTTGATG	GAGAGCAGC	ATG	GCT	TCA	CTA	AGA	CAA	ATC	GCC
PHE	TYR	GLY	LYS	GLY	GLY	ILE	GLY	LYS	SER	THR	SER	SER	GLN	ASN	THR
TTC	TAC	GGG	AAG	GGC	GGA	ATC	GGC	AAG	TCC	ACC	ACT	TCG	CAG	AAC	ACG
LEU	ALA	ALA	LEU	ALA	GLU	MET	GLY	GLN	LYS	ILE	LEU	ILE	VAL	GLY	CYS
CTA	GCG	GCG	CTG	GCA	GAG	ATG	GGT	CAG	AAG	ATC	CTG	ATT	GTA	GGG	TGC
ASP	PRO	LYS	ALA	ASP	SER	THR	ARG	LEU	ILE	LEU	HIS	ALA	LYS	ALA	GLN
GAT	CCG	AAA	GCG	GAC	TCG	ACT	CGC	CTT	ATT	CTG	CAC	GCC	AAG	GCT	CAA
ASP	THR	ILE	LEU	SER	LEU	ALA	ALA	SER	ALA	GLY	SER	VAL	GLU	ASP	LEU
GAC	ACG	ATT	TTG	AGT	CTT	GCC	GCG	AGC	GCC	GGC	AGC	GTG	GAG	GAT	CTA
GLU	LEU	GLU	ASP	VAL	MET	LYS	VAL	GLY	TYR	ARG	ASP	ILE	ARG	CYS	VAL
GAG	CTC	GAG	GAC	GTA	ATG	AAG	GTT	GGC	TAC	CGA	GAC	ATT	CGC	TGC	GTT
GLU	SER	GLY	GLY	PRO	GLU										
GAG	TCC	GGT	GGC	CCT	GAG	.....									

Figure 3. DNA sequence of the 5' end of the B. japonicum nifD gene. The strategy used in sequencing the 5' end of the B. japonicum nifD gene is illustrated in the upper portion of the figure. The arrow labelled "nifD" indicates the approximate position of transcriptional initiation as well as the direction in which it proceeds. The sequenced DNA strands are illustrated by arrows below the restriction map. Restriction endonuclease sites are BglII (Bg), HinfI (Hf), DdeI (D), and EcoRI (E). The nucleotide and amino acid sequences for this region are shown in the lower part of the figure.



GATTCGCAAC AACAGCCCGT CACCGTACAA GTCGCGCTAA GAAACTGTTG TTGTTCTAGT

TTTAGTGCTC ATGAGACCCT GGCATGCCGG TTGCAAAGTC TTGGATCAAG AAGCCGCCCT

MET SER LEU ALA THR THR ASN SER
CCCAACAGCT AACCTTTTAA AGGACACCAG ATG AGT CTC GCC ACG ACC AAC AGC

VAL ALA GLU ILE ARG ALA ARG ASN LYS GLU LEU ILE GLU GLU VAL PRO
GTC GCA GAA ATC AGG GCT CGC AAC AAA GAG CTG ATC GAG GAG GTG CCG

LYS VAL TYR PRO GLU LYS THR ALA LYS ARG ARG ALA LYS HIS LEU ASN
AAG GTC TAT CCG GAG AAA ACC GCG AAA AGG CGT CGG AAG CAC CTC AAC

VAL HIS GLN ALA GLY LYS SER ASP CYS GLY VAL LYS SER ASN ILE LYS
GTG CAC CAA GCA GGT AAG TCG GAC TGC GGG GTT AAG TCC AAC ATC AAA

SER ARG PRO GLY VAL MET THR ILE ARG GLY CYS ALA TYR ALA GLY SER
TCC AGA CCG GGC GTG ATG ACG ATA AGA GGG TGC GCC TAT GCA GGG TCG

LYS GLY VAL VAL TRP GLY PRO ILE LYS ASP MET VAL HIS ILE SER HIS
AAG GGG GTG GTC TGG GGA CCA ATC AAG GAC ATG GTT CAT ATT AGC CAT

GLY PRO VAL GLY LYS GLY GLN TYR SER TRP GLY SER GLY ARG ASN TYR
GGC CCG GTT GGC TGC GGC CAA TAT TCA TGG GGC TCG GGG CGC AAC TAT

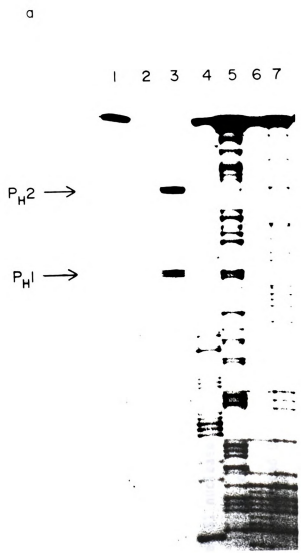
TYR VAL GLY THR THR GLY ILE ASP SER PHE VAL THR
TAC GTT GGC ACC ACG GGC ATC GAT AGC TTC GTG ACT C.....

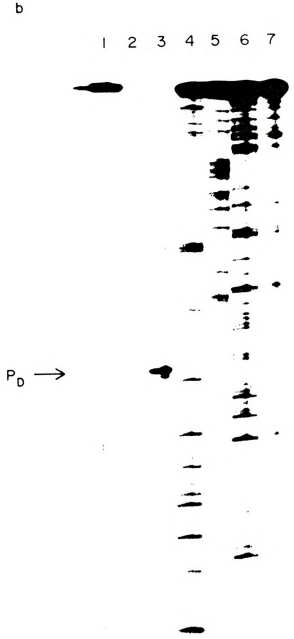
(1975). The comparison of these two genes would suggest that the B. japonicum USDA strain 110 ribosome binding site sequence can be approximated by the sequence 5'-ANGGANA-(4 or 5 nucleotides)-ATG. We do not detect any significant secondary structure possibilities around the ATG initiator codon of either of these genes.

Promoter Mapping. In order to determine the precise locations of the initiation sites for both nif operons, S1 nuclease protection analyses (Berk and Sharp, 1977) were initiated in the region 5' to the translational initiation sites of nifH and nifDK, respectively. All RNA used in these experiments was prepared from bacteria isolated from within soybean root nodules induced by infection with B. japonicum strain 110 (see Experimental Procedures).

A 245 bp HinfI fragment containing the region upstream from the nifH N-terminal methionine indicated in Fig. 2 as well as the capacity to code for the first 15 amino acids of nifH was 5'-end-labelled as described. The strands were separated electrophoretically and hybridized separately to nodule bacterial RNA. The partially S1 nuclease-resistant mRNA coding DNA strand was run opposite a DNA sequencing ladder of the same DNA strand (Fig. 4a). There are two major protected fragments separated by about 35 nucleotides and designated P_H1 and P_H2. The positions of these RNA 5' ends are indicated in Fig. 5 but must be considered to be $\pm$ 2 nucleotides due to the inherent ambiguity in the S1 nuclease digestion. These S1 protection products could correspond to either transcriptional initiation sites or sites of RNA processing or specific degradation. They have both been detected in several independent RNA preparations. However, the relative abundance

Figure 4. Determination of 5'ends of nifH and nifD mRNAs by S1 protection and DNA sequence analysis. Panel a shows S1 protection and DNA sequence analysis of the 245 bp HinfI-HindIII fragment containing the 5' end of the B. japonicum nifH gene (Fig. 2). In panel b the results of similar experiments using the 155 bp HinfI fragment (Fig. 3) containing the 5' end of the B. japonicum nifD gene are shown. Only the DNA strands which hybridize to bacterial RNA isolated from soybean nodules are presented. Lane designations for both panels are as follows: (1) S1 probe DNA marker; (2) DNA hybridized in the absence of RNA followed by S1 nuclease digestion; (3) protected DNA resulting from hybridization to RNA from N_2-fixing B. japonicum prior to digestion with S1 nuclease; (4-7) chemical DNA sequencing reactions, (4) G, (5) A>C, (6) T, and (7) C+T. The P_H and P_D transcripts are indicated.


a
1 2 3 4 5 6 7
P_H2
P_H1

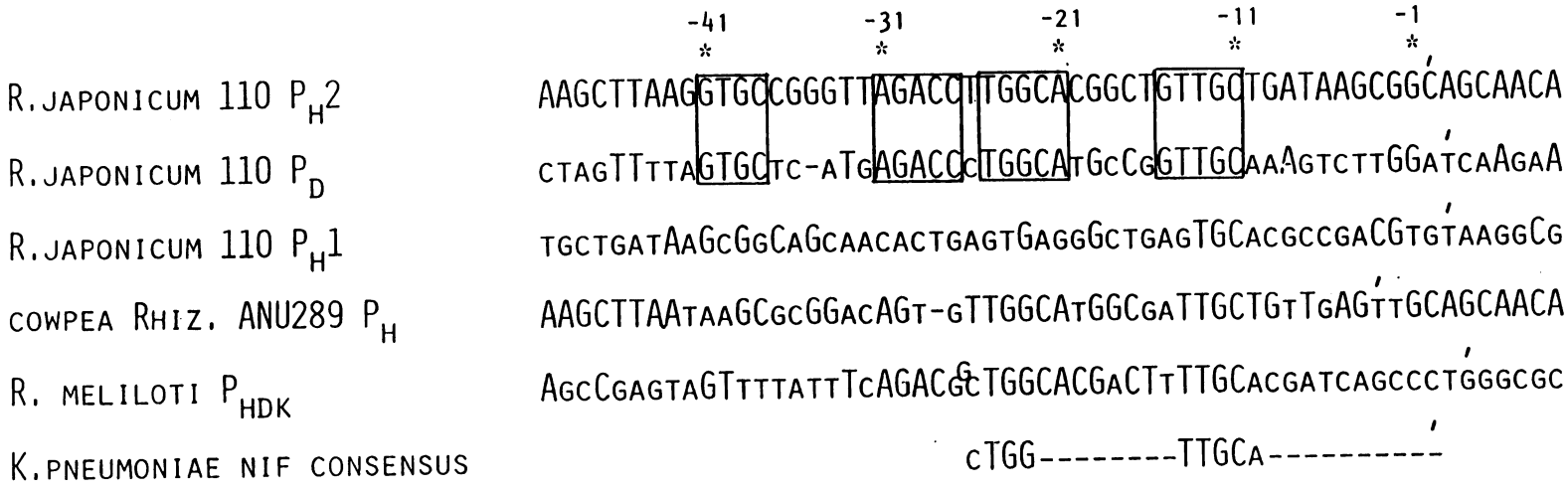

b
1 2 3 4 5 6 7
P_D

of the two transcripts varies, with the P_H2 5' end always predominating by a ratio of at least 2.5:1 (see Fig. 4a). No transcript initiated upstream of P_H2 has been observed in these RNA preparations. We therefore tentatively identify P_H2 as the major initiation site for nifH transcription in root nodules.

The nifD initiation site was determined by S1 nuclease protection of the separated strands of a 155 bp HinfI fragment which encodes the initiator methionine of nifD as well as 150 nucleotides of upstream sequence (see Fig. 3). Electrophoretic separation of the partially protected S1 nuclease digestion products is shown in Fig. 4b alongside DNA sequencing reactions of the same fragment. A single group of partially protected DNA fragments is observed and the position ($\pm$2 bp) of this RNA 5' end is designated as P_D in Fig. 5. Complete protection of the HinfI fragment has not been observed, indicating that no transcription initiates upstream of this fragment and reads through it. P_D can therefore be tentatively identified as the sole initiation site for nifD transcription in root nodules.

Promoter and Leader Region Sequence Comparisons. A comparison of the B. japonicum nifH and nifDK promoters is illustrated in Fig.5. Also shown for comparison are the sequences of the cowpea Bradyrhizobium sp. ANU289 nifH (Scott et al., 1983) and R. meliloti nifHDK (Sundaresan et al., 1983) promoters and the consensus sequences for K. pneumoniae nif proposed by Beynon et al. (1983) in agreement with that proposed by Ow et al. (1983).

Figure 5. Comparison of nucleotide sequences of nif promoters. Promoter sequences for nif genes from B. japonicum 110, cowpea Bradyrhizobium ANU289 (Scott et al., 1983), and B. meliloti (Sundaresan et al., 1983) are arranged to maximize their homology with the major B. japonicum nifH promoter P_H2. In addition, the K. pneumoniae nif consensus sequences discussed by Beynon et al. (1983) are included for comparison. Transcriptional initiation is indicated by the symbol '. Nucleotides which are homologous to the B. japonicum 110 promoter P_H2 are presented as enlarged letters. The boxes indicate highly conserved regions within the B. japonicum 110 P_H2 and P_D promoters, as discussed in the text. Dashes indicate gaps, and letters above the line indicate loopouts included to maximize the homologies.


-41
-31
-21
-11
-1
R.JAPONICUM 110 P_H2
AAGCTTAAGGTGCCGGGTTAGACCTTGGCACGGCTGTTGCTGATAAGCGGCAGCAACA
R.JAPONICUM 110 P_D
CTAGTTTTAGTGCTC-ATGAGACCCTGGCATGCCGGTTGCAAAGTCTTGGATCAAGAA
R.JAPONICUM 110 P_H1
TGCTGATAAGCGGCAGCAACACTGAGTGAGGGCTGAGTGCACGCCGACGTGTAAGGCG
COWPEA RHIZ. ANU289 P_H
AAGCTTAATAAGCGCGGACAGT-GTTGGCATGGCGATTGCTGTTGAGTTGCAGCAACA
R. MELILOTI P_{HDK}
AGCCGAGTAGTTTTATTTCAGACGCTGGCACGACTTTTGCACGATCAGCCCTGGGCGC
K.PNEUMONIAE NIF CONSENSUS
CTGG--------TTGCA----------

The two major B. japonicum nif promoters, P_H2 and P_D, show a very high degree of homology with the major blocks of conserved sequence occurring in the "-10 region" (-11 to -15), the -21 to -25 region, and in two blocks flanking the "-35 region" (-27 to -31 and -38 to -41) as shown in Fig. 5. Interestingly, these promoters also show a high degree of sequence conservation with the R. meliloti nifHDK promoter; this conservation is particularly strong in the -11 to -15 and -21 to -25 regions. The nifH promoter from the cowpea Bradyrhizobium sp. ANU289, which is more closely related to B. japonicum than is R. meliloti (Hollis et al., 1981), also has a high degree of sequence homology in the -11 to -15 and -21 to -25 regions. Neither the R. meliloti nifHDK nor cowpea Bradyrhizobium sp. ANU289 nifH promoter has sequence homologies with B. japonicum P_H2 or P_D in the -27 to -31 or -38 to -41 regions.

On the other hand, the minor B. japonicum 5' end, P_H1, has only minimal sequence conservation with the other B. japonicum promoters, suggesting that this end arises either via a different regulatory pathway or by a post-transcriptional mechanism. The major B. japonicum nif promoters, P_H2 and P_D, also display limited conservation with the K. pneumoniae nifHDK promoter; however, the nif consensus sequences within the -11 to -15 and -21 to -25 regions are well conserved.

DISCUSSION

We have isolated the gene encoding dinitrogenase reductase, nifH, from Bradyrhizobium japonicum USDA strain 110 on the basis of hybridization to the K. pneumoniae nifH gene. DNA sequence of part of this gene indicated that the amino terminal 94 amino acids share 74% homology with the amino terminus of the K. pneumoniae dinitrogenase reductase. In addition, we have sequenced a portion of the B. japonicum nifD gene encoding the α-subunit of dinitrogenase which had been cloned previously by Hennecke (1981). The amino terminal 115 amino acids of the R. japonicum nifD gene product exhibit 51% homology with the K. pneumoniae α-subunit. Comparison of the two B. japonicum sequences indicates that translational initiation is directed by a Shine and Delgarno-like sequence (1975) having the structure 5'-ANGGANA-(4 or 5 nucleotides)-ATG. This purine-rich region is similar, both in position and composition, to the translational initiation signals found in other bacteria.

In order to investigate the mechanisms by which transcription from the nifH and nifDK operons is controlled, we have used S1 nuclease protection analysis (Berk and Sharp, 1977) to map the 5' ends of nif-specific RNAs prepared from B. japonicum isolated from soybean nodules. The RNA transcript encoding the and presumably subunits of B. japonicum nitrogenase component I has a single major 5' end (P_D) 46 nucleotides upstream from the site of nifD translational initiation. We have not directly determined whether this RNA arises through initiation from this site or through processing or degradation of an RNA initiated upstream. However, as no protection is seen 5' to this site, we

tentatively consider P_D to be the major transcriptional initiation site for nifD and presumably nifK in soybean nodules. S1 nuclease protection analysis of the region upstream from the nifH initiation codon yields two major protection products (P_H1 and P_H2) whose 5' ends are 117 and 152 nucleotides from the initiation codon, respectively. The relative abundance of RNA species with each 5' end varies considerably among RNA preparations. P_H2 is always dominant, with P_H1 transcript ranging from approximately 10 to 30% of the level of P_H2 transcript. These results, together with the sequence analyses described below, suggest that the P_H1 ends arise by a different mechanism from that for P_H2. Since soybean nodules contain at least three morphologically and physiologically distinct bacterial cell types (Ching et al., 1977) all of which express nif, P_H1- and P_H2-initiated transcripts might reflect different expression mechanisms in specific cell populations. Alternatively, P_H1 ends may result from variable levels of RNA degradation during preparation.

Despite their physical separation, expression of the nifH and nifDK operons is controlled in a developmentally coordinate manner (our unpublished results). Thus, the two major nif promoters, P_D and P_H2, are expected to share sequences involved in the action of positive and/or negative regulators of nif gene expression as well as in RNA polymerase recognition. There are indeed several blocks of highly conserved sequence shared by P_H2 and P_D (see Fig. 5). These include sequences from -11 to -15 and -21 to -25 which are also shared with R. meliloti and K. pneumoniae nif promoter sequences. nifA-mediated recognition of nif promoters by RNA polymerase has been discussed in detail and these conserved regions have been implicated in this control

mechanism (Ow et al., 1983; Beynon et al., 1983). Their conservation in similar position, with respect to transcriptional initiation sites for B. japonicum nif genes, suggests that a similar regulatory mechanism may control transcription from P_D and P_H2. The minor B. japonicum nifH 5' end, P_H1, shares only minimal sequence homology with P_H2 and P_D. This further supports the model presented above in which the appearance of transcripts with 5' ends at P_H1 is controlled by a separate mechanism from that controlling P_D and P_H2 transcription.

In addition to the regions discussed above, P_H2 and P_D share a pair of sequence homologies which are located from -27 to -37 and -38 to-41. These conserved regions flank the "-35 region," which has been shown to play a role in transcriptional initiation in enteric bacteria, and they are also separated by precisely one helical turn. A structurally analogous situation has recently been presented by Ho et al. (1983) concerning the activation of expression of lysogenic functions of the E. coli phage lambda. In this case, a positive regulatory protein, cII, contacts a 4 bp sequence which is repeated on each side of the "-35 region" and is separated by one helical turn. This interaction of cII with the repeated sequence allows RNA polymerase to interact with a previously unrecognizable -35 sequence, apparently resulting in transcriptional initiation. One could invoke a similar model for B. japonicum in which the conserved sequences flanking the -35 region interact with a positive activator, possibly the product of a nifA-like gene, to allow RNA polymerase to bind and initiate transcription.

Further comparison of nif promoter sequences can be made between the B. japonicum sequences presented here and the sequence of the nifH promoter from the cowpea Bradyrhizobium miscellany. Cowpea

Bradyrhizobium are much more closely related to B. japonicum in terms of physiological parameters (Vincent, 1982) as well as overall DNA sequence homology (Hollis et al., 1981) than are R. meliloti and K. pneumoniae. Scott et al. (1983) have used S1 protection analysis to map and sequence a transcriptional initiation site for cowpea Bradyrhizobium ANU289 nifH which is in a position analogous to B. japonicum P_H2 (Fig. 5). From -1 through -26, 19 nucleotides are conserved between P_H2 and the cowpea P_D. The -26 to -42 region has diverged significantly but from -43 to -60 there is almost complete homology (Fig. 6A). Interestingly, despite the high degree of conservation in this region, the two blocks of conserved sequence between P_H2 and P_D which flank the -35 region are not present in the cowpea nifH promoter. Since the sequence of the cowpea Bradyrhizobium strain ANU289 nifD promoter has not yet been reported, we cannot presently determine if intrastrain conservation in the regions flanking -35 as found in B. japonicum strain 110 is a generalization for the "slow-growing" rhizobia. It would be curious that the -35 region conservations could have diverged even in close relatives while the sequences in the -23 and -11 regions have been conserved over longer evolutionary time.

Beyond the promoter region discussed above, an extremely high degree of conservation is found throughout the approximately 154 nucleotide leader region of the nifH transcript (see Fig. 6A). The overall homology in the leader region is 87%, and when two small blocks of divergent sequence (19 nucleotides; see Fig. 6A) are discounted, the remaining leader sequences are 93% homologous. Surprisingly, this degree of conservation is higher than that found within the nifH coding regions of which 84% of the first 282 bp are homologous. As expected,

the vast majority of mismatches in the coding region are in the third position of codons.

The functional significance of this leader region in the nifH transcript is not yet clear; however, this high degree of conservation along with the potential presence of a secondary 5' end (P_H1) found in B. japonicum led us to investigate possible secondary structures for B. japonicum nif transcripts. No significant secondary structure can be formed by the 45 bp nifD leader region. In contrast, the nifH leader can form several possible stable secondary structures. Two of these structures are illustrated in Fig. 6B with emphasis on the structures which are P_H2 vs. P_H1 specific. The P_H2 transcript can form two stem and loop structures with ΔG's (Tinoco et al., 1973; Borer et al., 1974) of -15.8 kcal (regions 1 and 2) and -12.3 kcal (regions 3 and 4). Since the region 1 is not present in the P_H1 transcript, the most stable structure has only one stem and loop with a ΔG = -15.9 kcal (regions 2 and 5). The postulated P_H1 stem and loop formed by hybridization of regions 2 and 5 would therefore be both thermodynamically and kinetically disfavored in the P_H2 transcript. Although the implications of these alternative structures are unclear, there are precedents for leader region structures affecting transcriptional attenuation (Yanofsky and Kolter, 1982), mRNA stability (Friedman et al.,1983) and translational efficiency (Walz et al., 1976; Ptashne et al., 1976; Queen and Rosenberg, 1981). No evidence has yet been found for attenuation in this system. If each of these transcripts were to predominate in physiologically different cell types, it would therefore be reasonable to presume that the amount of nifH product made per message would differ as well.

Figure 6. Analyses of nifH mRNA leader region. A: Comparison of the B. japonicum 110 (upper) and cowpea Bradyrhizobium ANU289 (Scott et al., 1983; lower) nifH leader region sequences is presented with the regions of relatively high degree mismatching boxed. The positions of the major B. japonicum promoter P_H2 and the cowpea Bradyrhizobium promoter P_H (Scott et al., 1983) are indicated by arrows. The translational initiation codons are underlined. B: Potential secondary structures for the B. japonicum 110 mRNA leader regions as discussed in the text are presented. G-C and A-U base pairs are indicated by solid lines while G-U base pairs are indicated by dashed lines. Regions 1 through 5 which are capable of base pairing (see text) are indicated. The translational initialtion codons are underlined.

A

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AGATCTTGTCAGATCCAAAACAGCCTACGATCGCGCGCCGGCTGGTT-GC-TTTTGGAA
AGATCCTGTCCGGTGCAAACCTCCCTGGGGGTAGCTCAGCGGCTCGTTGGCTTTTTAGAG

ACGTAATC-AGAAGCTTAAGGTGCCGGGTTAGACGTTGGCACGGCTGTTGCTGATAAGC
-CGTAATCAAGAAGCTTAATAAGCGCGGACAGT-GTTGGCATGGCGATTGCTGTTGAGT

      PH2
GGCAGCAACACTGAGTGAGGGCTGAGTGCACGCCGACGTGTAAGGCGAGCGATGCGCTC
TGCAGCAACACTGAGTGAGGGCTGGGTGCACGCCGACGCGTAAGACGAGCGATGCGCTC
      PH
CTTCCCTTGAACCCGTGTGCCCCCGTTTCTGCGAGGGAAGCAAAGCTCGCA-A-AAGAA
CTTCCCTTGAACCCGTGTGCCCC-GTTTCTGAGAGAGAAACAA-GCTCGCGTGTCGGAA

GCGCGCAACGTTTGGCAAATCGGTTGATGGAGAGCAGCATG·····················
GCACGCAACTTTTGGCAAATCGGTTGATGGAGAACAACATG·····················
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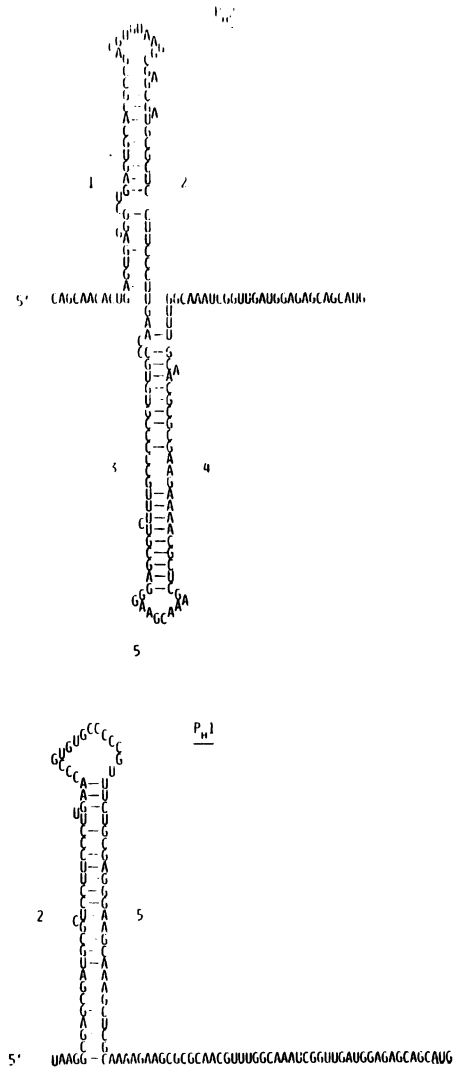
B



The qualitative S1 protection analyses presented here suggested that the abundances of nifH and nifDK 5' ends differ dramatically (see Fig. 4). In order to quantitate this difference more precisely we have employed an S1 protection scheme in which both nifH and nifDK 5' ends are probed in the same reaction (unpublished data). This analysis confirms that there is an excess of P_H over P_D transcripts; a result which is puzzling in light of data indicating that component I and component II are present in equimolar amounts in fully developed pea and cowpea nodules (Bisseling et al., 1979). On the other hand, [^{35}S] methionine pulse-labeling experiments with lupin bacteroids (Shaw, 1983) or B. japonicum cultures which had been derepressed for nitrogenase synthesis (Scott et al., 1979) have shown higher levels of incorporation into component II than component I. Given these conflicting results, the relative levels of expression of the nifH and nifDK operons in Bradyrhizobium under different developmental and physiological conditions and the functional significance of modified stoichiometries for the components of nitrogenase still require clarification.

In conclusion, we find that the major promoters for the nifH and nifDK operons of B. japonicum USDA strain 110 have a high degree of homology with each other as well as with nif promoters from cowpea Bradyrhizobium. In addition, a lesser but significant degree of homology is seen to nif promoters from R. meliloti and K. pneumoniae. These sequence conservations suggest sites at which nif regulatory functions may act. The actual importance and mechanism by which these sites are involved in the regulation of nitrogen fixation will only be uncovered by direct biochemical and genetic analyses.

EXPERIMENTAL PROCEDURES

Bacterial strains. The E. coli K-12 strain ED8654 (gal met $hsdR_k$ supE supF) was used for general plasmid cloning and maintenance. Escherichia coli K802 (lacY met $hsdR_k$ galK supE) was used as a host for lytic growth of the phage lambda recombinant library described previously (Carlson et al., 1983). Bradyrhizobium japonicum strain USDA 110 was used for inoculation of surface sterilized soybean (Amasoy variety) seeds.

Soybean Growth and Nodule Bacteria Isolation. Inoculated soybeans were planted six to a pot in a sterilized mixture of vermiculite and perlite (1:2) containing 1.2 g of gypsum per quart, and watered regularly with N-free medium (Johnson et al., 1966). Following 5-6 weeks of greenhouse growth, nodules were harvested, immediately frozen in liquid nitrogen, and stored at -70°C for future use.

To isolate B. japonicum from soybean nodules, 30 g frozen nodules were placed in 200 ml of grinding buffer (Ching et al., 1977) at 4°C to thaw. All subsequent steps were caried out at 4°C. After thawing, nodules were rinsed twice with 200 ml cold gringing buffer, transferred to a mortar and pestle with 30 ml of buffer, and ground to a homogeneous paste. The nodule paste was filtered through four layers of Miracloth (Chicopee Mills, Inc., Milltown, NJ). This material was centrifuged at 250 g for 10 min and the pellets were discarded. This low-speed centrifugation was then repeated. Bacterial cells were pelleted by centrifugation for 10 min at 8000 g. This material was used immediately

to isolate RNA as described below; it contains a mixture of cells at varying stages of differentiation to bacteroids (Ching et al., 1977).

RNA Isolation. Soybean nodule bacteria were suspended in 4-9 vol of 4% sarkosyl, 0.1 M Tris-HCl, pH 8, and passed three times through a french pressure cell (Aminco; Silver Spring, MD) at 12,000 psi. For each milliliter of broken cell solution, 1 g of CsCl was added. RNA was isolated by discontinuous CsCl gradient centrifugation as described previously (Chelm and Hallick, 1976). The RNA pellet was drained thoroughly, redissolved in 0.15 M sodium chloride, 0.015 M sodium citrate, and then extracted twice with phenol and four times with diethyl ether. Following ethanol precipitation, the RNA was redissolved in H_2O and stored in aliquots at $-70^{o}C$.

DNA Techniques. Recombinant lambda phage were prepared using cesium chloride block density gradient centrifugation (Davis et al., 1980). Phage DNA was extracted by treatment with formamide (Davis et al., 1974). Plasmid DNA was isolated from E. coli by CsCl-ethidium bromide equilibrium centrifugation (Clewell and Helinski, 1972). Strand separation of DNA fragments was by electrophoresis on 8% polyacrylamide gels (Szalay et al., 1977; Maxam and Gilbert, 1977; Maxam and Gilbert, 1980). Isolation of both double and single stranded DNA fragments from polyacrylamide gels was carried out as described (Maxam and Gilbert, 1977; Maxam and Gilbert, 1980) using a modified elution buffer [0.3 M LiCl, 0.1 mM EDTA, 0.05% sodium dodecyl sulfate (SDS), 10 mM Tris-HCl, pH 7.5].

Hybridization methods. Lambda library plaques were prepared as escribed (Davis et al., 1980) using E. coli K802 grown in tryptone roth (Miller, 1972) plus 10 mM Mg SO_4 and 0.2% maltose as the host. nage DNA was transferred to cellulose nitrate sheets by the methods of enton and Davis (1977). Prior to hybridization, filters were incubated or 1 h at 65°C in 5 x Denhardt's solution (Denhardt, 1966), 5 x SSPE [1 SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, pH 7.7, 1mM thylenediaminetetraacetate (EDTA)] and 200 ug/ml sheared and denatured almon sperm DNA. Hybridizations to nick-translated (Maniatis et al., 975) DNA probes were caried out overnight (12-24 h) at 65°C in 5 x SPE, 1.5 x Denhardt's solution, and 100 ug/ml salmon sperm DNA. ollowing hybridization, filters were washed twice for 15 min each at oom temperature in 2 x SSPE, 0.1% SDS followed by two washes in 0.1 x SPE, 0.1% SDS.

S1 Nuclease Protection Analysis. Transcription unit mapping by S1 uclease protection techniques was adapted from the methods of Berk and harp (1977).

Since Southern hybridization analysis using the K. pneumoniae nifH ene as probe (see Results) indicated the 180 bp BglII-HindIII fragment hown in Fig. 2 was totally internal to the gene, this fragment was hosen for S1 protection analysis in order to determine the direction of . japonicum nifH transcription. The fragment was 5'-end-labelled with olynucleotide kinase (Maxam and Gilbert, 1977; Maxam and Gilbert, 1980) id the DNA strands denatured and separated as described above. To fferentiate the two strands, the same fragment was labelled only on he HindIII 5' end by first labelling a larger HindIII fragment,

followed by excision of the 180 bp BglII-HindIII segment This was followed by strand separation alongside the doubly labelled fragment. The 5'-end-labelled single-stranded DNAs were then hybridized to 40 ug of RNA in 10 ul of 80% formamide, 0.4 M NaCl, and 40 mM PIPES [piperazine-N,N'-bis(2-ethanesulfonic acid)], pH 6.4, for 3 h at 52°C. Following hybridization, samples were transferred to 0.3 ml of ice-cold S1 digestion buffer (280 mM NaCl, 30 mM NaOAc, pH4.4, 4.5 mM $ZnSO_4$, and 20 ug/ml sheared and denatured salmon sperm DNA) containing 25 units of S1 nuclease (BRL) and incubated at 37°C for 30 min. The S1 digestion was stopped by adding 75 ul of 2.5 M NaOAc, 50 mM EDTA, followed by ethanol precipitation. Samples were resuspended in 80% formamide containing 0.1% xylene cyanol and 0.1% bromophenol blue, heat denatured, and separated electrophoretically on polyacrylamide gels containing 8.3 M urea (Maxam and Gilbert, 1980). Full protection of the 5' HindIII-labelled fragment was seen while the BglII-labelled 5' end showed no protection. Thus, transcription of nifH proceeds as shown in Figs. 1 and 2. In order to determine 5' ends of nifH and nifDK transcripts precisely, the fragments shown in Fig. 2 and 3 were 5'-end-labelled and strand separated. Hybridization to total nodule bacteria RNA was at 56-60°C. All other conditions were as described above. DNA sequence ladders were used as markers to determine the exact location of transcription initiation. DNA sequence analysis was performed as described by Maxam and Gilbert (1980).

CHAPTER 3

Physical Organization of the *Bradyrhizobium japonicum* Nitrogenase Gene Region

INTRODUCTION

The biological reduction of dinitrogen is carried out by a number of procaryotic organisms via the nitrogenase enzyme complex. The conservation of DNA and amino acid sequences among nitrogenase genes (*nifH*, -*D*, and -*K*) from a variety of nitrogen-fixing organisms (including *Bradyrhizobium japonicum*) is well documented (Chen *et al.*, 1973; Emerich and Burris, 1978; Ruvkin and Ausubel, 1980). This interspecies conservation, however, does not extend to include a preservation of *nif* operon structure. Whereas the *nifH*, -*D*, and -*K* genes are transcribed as a single operon (in the order *nifHDK*) in *Klebsiella pneumoniae* (Reidel *et al.*, 1979) and in several fast-growing *Rhizobium* spp. (including *Rhizobium meliloti* [Banfalvi *et al.*, 1981] and *R. leguminosarum* [Krol *et al.*, 1982]), they occur as two separate transcription units, *nifH* and *nifDK*, in at least some bradyrhizobial strains (Kaluza, *et al.*, 1983; Scott *et al.*, 1983).

In addition to the genes encoding the three constituent polypeptides of the nitrogenase complex, the synthesis of an active

nitrogenase in the free-living nitrogen-fixing bacterium K. pneumoniae requires the expression of at least 14 other genes (Reidel et al., 1979; Roberts and Brill, 1980; Roberts and Brill, 1981). These nitrogen fixation (nif) genes are arranged in seven or eight operons clustered within a 24-kilobase-pair (kbp) section of the chromosome. The expression of these nif genes is coordinately controlled via the products of the nifLA operon (Buchanon-Wollaston et al., 1981; Filser et al., 1983; Hill et al., 1981; MacNeil and Brill, 1980; Merrick et al., 1982; Ow and Ausubel, 1983; Roberts and Brill, 1980), which is in turn controlled by the general nitrogen regulatory system (Ntr) common to all enteric bacteria studied (Magasanak, 1982).

I have begun to characterize the organization and expression of the genes encoding the nitrogen fixation process in the soybean symbiont B. japonicum USDA 110. In this chapter I report the determination of the physical arrangement of the nifH and nifDK transcription units on the B. japonicum genome. In addition, I have localized a region that is homologous to another K. pneumoniae nif gene, nifB. This B. japonicum nifB gene is transcribed from its own promoter and, as with nifH and nifDK, there is considerable sequence homology between this promoter and the consensus for Klebsiella nif promoters (Beynon et al., 1983).

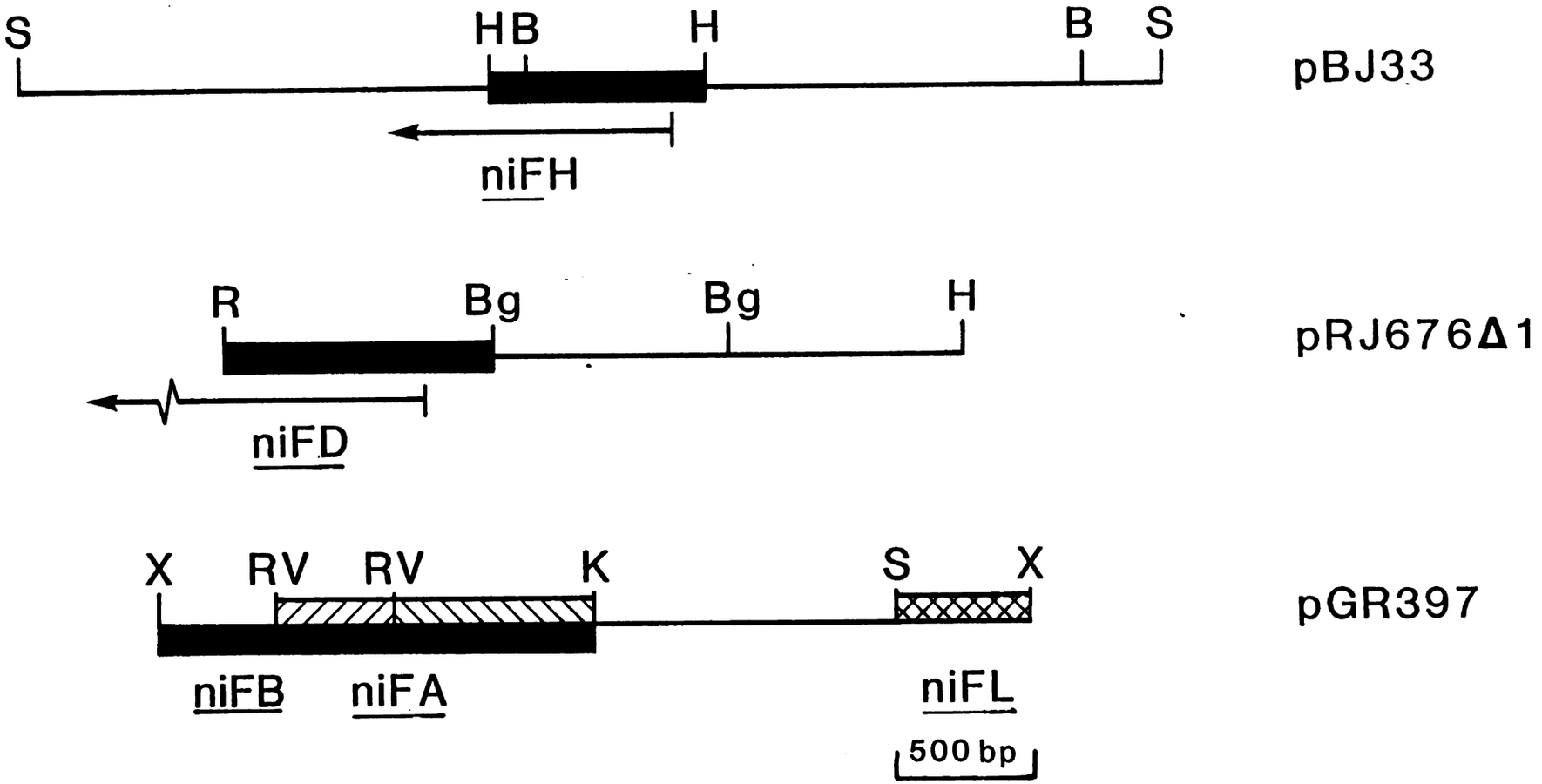
RESULTS

Identification and characterization of nif-specific cosmid clones.

determine whether B. japonicum USDA 110 nif genes are clustered in a nner similar to that seen for other nitrogen-fixing organisms, we have ed DNA sequences from the nifH and nifD genes of B. japonicum (see g. 7) as hybridization probes to screen a library of B. japonicum enomic DNA cloned into the broad-host-range cosmid vector pLAFR1 riedman et al., 1982). This library is maintained as an ordered array f 1,426 individual transformants. The average insert size per ecombinant cosmid is 24 kbp, therefore, with a genome size of 10,000 bp (T. Caspar and B. K. Chelm, unpublished), any genomic sequence has a 7% probability of being represented at least once. In this manner, we ave identified two cosmids which hybridize to the nifH probe pRJcos1-62 and pRJcos14-58) and one cosmid which hybridizes to nifD pRJcos2-63).

Ethidium bromide staining patterns for restriction endonuclease igests of DNA from pRJcos1-62 (nifH) and pRJcos2-63 (nifD) suggested hat these cosmids may represent overlapping regions of the B. japonicum enome. To verify this observation, radioactively-labelled pRJcos2-63 nifD) was used to probe Southern transfers of various restriction igests of the nifH cosmid pRJcos1-62 (Fig. 8). Since these recombinant osmids were constructed as EcoRI partial digestion products (see bove), the full extent of the overlapping region can most easily be een in the EcoRI digest of pRJcos1-62 (Fig. 8a and b, lanes 4). There re four EcoRI fragments totalling 13 kbp in length in pRJcos1-62 that ybridize to pRJcos2-63.

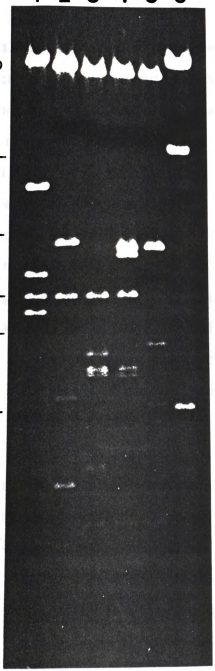
Figure 7. Summary of hybridization probes. Boxed regions indicate restriction endonuclease fragments isolated for use as hybridization probes. The positions of transcriptional initiation sites for the B. japonicum nifH and nifD genes within the probe fragments isolated from pBJ33 and pRJ676-1 (Hennecke, 1981), are indicated by the arrows. The plasmid pBJ33 is a subclone of the SalI fragment containing the nifH gene from NH1. The 1.6 kbp KpnI-XmaI fragment from pGR397 (Reidel et al., 1983) was used as a general nifA-nifB hybridization probe.


S
HB
H
B
S
pBJ33
niFH
R
Bg
Bg
H
pRJ676Δ1
niFD
X
RV
RV
K
S
X
pGR397
niFB
niFA
niFL
500 bp

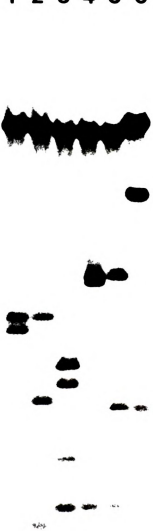
50

Figure 8. Hybridization of nick-translated pRJcos2-63 to restriction endonuclease digests of pRJcos1-62. (a) Ethidium bromide-stained agarose gel of restriction endonuclease-digested pRJcos 1-62. Lanes: 1, HindIII; 2, HindIII plus BamHI; 3, HindIII plus EcoRI; 4, EcoRI; 5, EcoRI plus BamHI; 6, BamHI. (b) Hybridization of nick-translated pRJcos2-63 to cellulose nitrate transfer of the gel in a. Lane designations are the same as in a. The top band in each lane contains the vector DNA, pLAFRI. All other bands represent B. japonicum DNA.

a



b



The overlapping cosmids pRJcos1-62 and pRJcos2-63 were used to construct a restriction endonuclease map representing approximately 33 kbp of B. japonicum DNA (Fig. 9). The second nifH cosmid clone (pRJcos14-58) exhibits an EcoRI restriction pattern that is nearly identical to pRJcos1-62 and thus has not been further analyzed. The positions and directions of nifD and nifK genes and the small gene region of unknown function marked (?) were defined previously within the 9.5 kbp HindIII fragment by using the plasmid clone pRJ676 (Fuhrmann and Hennecke, 1982; Hennecke, 1981). The nifH gene was previously isolated as a recombinant lambda phage, λNH1 (Adams and Chelm, 1984) and its position within this region is shown. The approximate position of a B. japonicum nifB gene is also illustrated on the map in Fig. 3. The identification and localization of this region is discussed below.

To verify that these cosmid clones accurately represent the true genomic arrangement of the nif gene cluster in B. japonicum, total genomic DNA was digested with the restriction enzymes used to map this region, separated on agarose gels, and blotted to cellulose nitrate. The resulting blots were hybridized to either the 1.2 kbp XhoI fragment in pBJ86 (Fig. 10a) or to pBJ87 (Fig. 10b), each of which had been nick-labelled by nick translation (see Fig. 9 for map positions of probes). In each case the hybridization pattern predicted from the map shown in Fig. 3 was in agreement with the results. This, coupled with the fact that B. japonicum DNA regions that were independently cloned in cosmids and pRJ676 are unaltered in these cosmid clones, indicates that pRJcos1-62 and pRJcos2-63 accurately reflect the physical structure of the B. japonicum genome.

Figure 9. Restriction endonuclease map of the B japonicum nif gene cluster. The positions and transcriptional directions of nif and fix genes described previously (Adams and Chelm, 1984; Fuhrmann and Hennecke, 1982; Hennecke, 1981; Fuhrmann et al., 1985) are indicated by the hatched arrows. The position and transcriptional direction of the nifB gene described here is indicated by the hatched arrow labelled nifB. Recombinant DNA clones of this region that were used in this work and are discussed in the text are illustrated at the bottom. Restriction endonuclease sites are EcoRI (E), HindIII (H), XhoI (X), and BamHI (B).

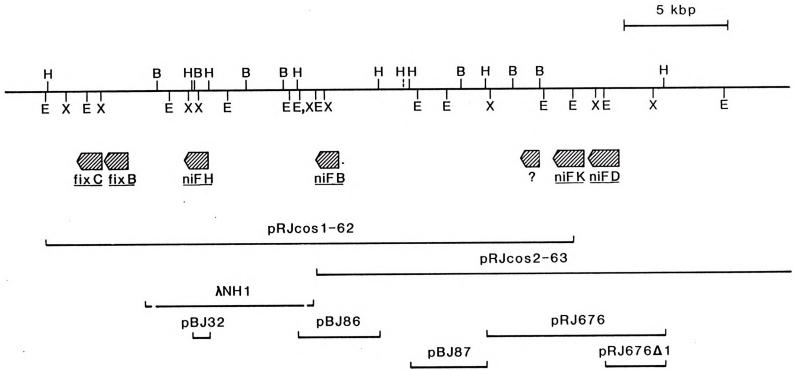
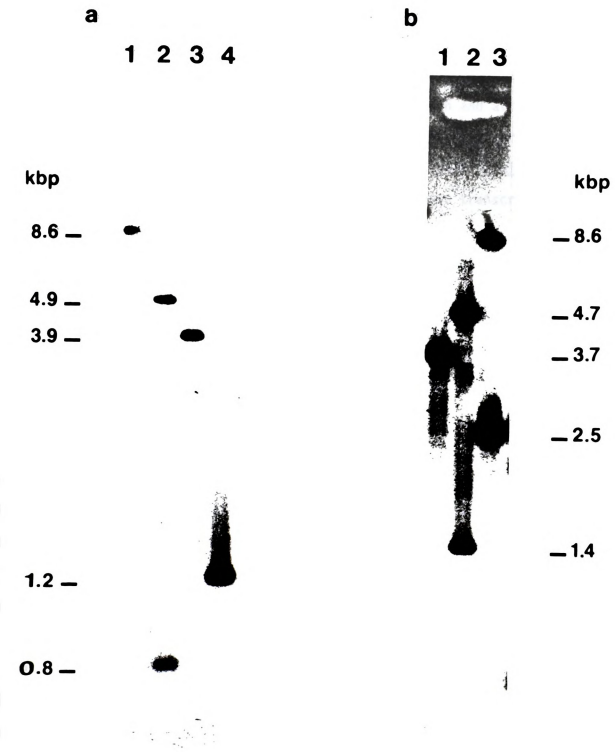

5 kbp
H B HBH B B H H HH B H B B H
E X E X E XX E EE,XEX E E X E E XE X E
fixC fixB niFH niFB ? niFK niFD
pRJcos1-62
pRJcos2-63
ΔNH1
pBJ32
pBJ86
pRJ676
pBJ87
pRJ676Δ1

Figure 10. Verification of the B. japonicum nif genomic structure by Southern hybridization to restriction endonuclease digests of total genomic DNA. Genomic DNA from B. japonicum was digested with restriction endonucleases and separated electrophoretically on 1% agarose gels. Cellulose nitrate transfers of these gels were then hybridized to radioactively labelled (a) pBJ87 or (b) the 1.2 kbp XhoI fragment from pBJ86 (Fig. 3). (a) Lanes: 1, BamHI; 2, EcoRI; 3, HindIII. (b) Lanes: 1, XhoI; 2, HindIII; 3, EcoRI; 4, BamHI.


a
1 2 3 4
kbp
8.6
4.9
3.9
1.2
0.8
b
1 2 3
kbp
8.6
4.7
3.7
2.5
1.4

Localization of a B. japonicum nifB gene. In all species of Rhizobium examined thus far, at least some of the genes responsible for nitrogen fixation (including the nifHDK operon) and nodulation of host legumes are closely linked on large plasmids (Banfalvi et al., 1981; Hirsch et al., 1980). In R. meliloti, the nifHDK operon and at least two other transcription units essential for nitrogen fixation are found within 15 kbp of one another (Buikema et al., 1983; Corbin et al., 1982; Corbin et al., 1983; Ruvkin et al., 1982). One of these transcription units encodes a positive regulator of symbiotic nitrogen fixation that is similar in both structure and function to the K. pneumoniae nifA gene product (Zimmerman et al., 1983; Buikema et al., 1986). We were interested in determining whether a similar nifA-like gene is closely linked to the B. japonicum nifH, D, and K gene region. With this in mind, a 1.8 kbp SmaI-KpnI fragment from pGR397 (see Fig. 7, Chen et al., 1982) which carries most of the K. pneumoniae nifA gene and a portion of the amino-terminus of nifB was used to probe Southern transfers of EcoRI digested pRJcos1-62 and pRJcos2-63. This probe hybridizes predominantly to the 4.9 kbp EcoRI fragment common to both pRJcos1-62 and pRJcos2-63 that is located approximately 11 kbp downstream of the nifDK promoter (Fig. 11). Subsequent analysis (not shown) of this nifAB hybridization has localized the region of homology to the 1.2 kbp XhoI-SalI fragment shown in Fig. 9.

The nucleotide sequence of the B. japonicum DNA that hybridizes to the K. pneumoniae nifAB probe was determined in order to confirm the presence of nifA- or nifB genes in this region. Although no homology to the K. pneumoniae nifA gene was observed, an open reading frame extending for at least 193 amino acids and with 49% amino acid homology

Figure 11. Hybridization of the K. pneumoniae nifAB genes to EcoRI digested recombinant cosmids from the nif gene cluster. (a) Ethidium bromide-stained agarose gel of EcoRI-digested cosmid clones. Lanes: 1, pRJcos1-62; 2, pRJcos2-63. (b) The KpnI-SmaI fragment of pGR397 (Reidel et al., 1983), that contains portions of the K. pneumoniae nifA and nifB genes, was nick-translated and hybridized to a cellulose nitrate transfer of the gel shown in a. Lane designations are the same as in a.

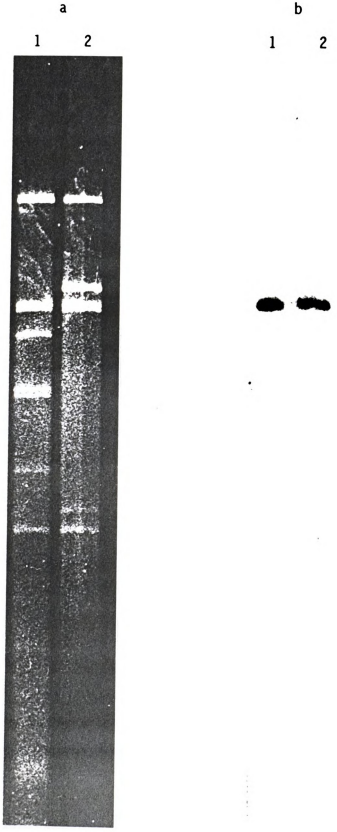

a
1
2
b
1
2

Figure 12. Nucleotide sequence of the 5' end of the B. japonicum nifB gene. A DNA and amino acid sequence for the N-terminal portion of a nifB open reading frame from B. japonicum is presented. Amino acid homologies to the R. leguminosarum nifB gene are indicated by *. The approximate transcriptional inititiation site as determined by S1 nuclease analyses (Fig. 7) is indicated by the arrow. Nucleotide sequences in this region with homology to the B. japonicum nifH promoter (Chapter 2) are underlined.

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GATCGGGCCA ATTGAGAGCG GCAGCGCGCG AAAGCCCGTT CAGATGAGCT GGATCGGGGC

GAGCCCCGCG AGAGTGAAAA GCACACGAGA TGGAGCCCCG AGTGAGAATG GCGACGCATC

TGTCGCTTTG CCAACAAGCT CATCATTGCT GTCTTGCAAG TGCCAACGCT ATCGCTGCGC

CTACTCGGTG TACTGTGGTT GCATTCGCGI CATCCTCGCC ACGGCATGCA AGTTGCTAAI
   PB
------>
CTTCCTGAAG CGCGCTCTAG GATGATCTGT GGGCGTCGAT GCTTGCAGGG GAGTGATCTC

                                                          M   Q
GGTCGTGGAG CGCGGAAAAT ATATCAAAGC AGCGGTCAAT AGCGGGAAGA TC ATG CAG

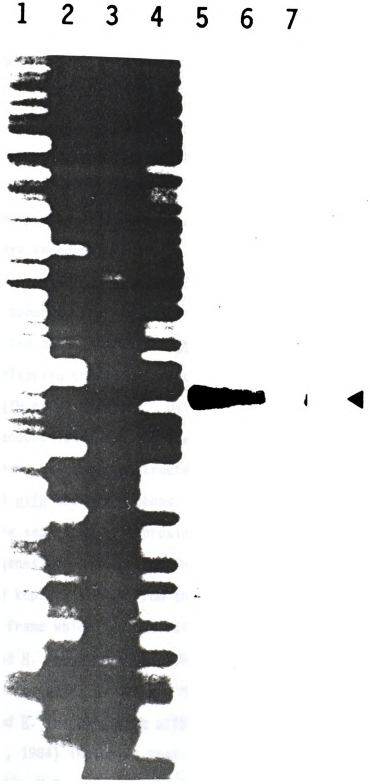
 S   I   T   E   H   K   G   C   R   A   S   A   K   T   G   R   A
TCC ATA ACC GAG CAT AAG GGC TGC CGC GCT TCG GCG AAG ACC GGG CGG GCG

 S   C   G   S   Q   A   G   R   G   D   L   P   V   E   I   R   E
AGC TGC GGC TCG CAG GCC GGC CGA GGC GAT CTG CCG GTC GAA ATC AGG GAA
 *
 R   V   K   N   H   P   C   Y   S   E   D   A   H   H   H   Y   A
AGG GTG AAA AAC CAT CCC TGT TAC AGC GAG GAT GCG CAC CAT CAT TAC GCT
 *       *           *   *   *   *   *   *   *   *   *   *   *   *
 R   M   H   V   A   V   A   P   A   C   N   I   Q   C   N   Y   C
CGC ATG CAT GTC GCG GTC GCA CCT GCC TGC AAT ATC CAG TGC AAC TAC TGC
 *   *   *   *   *   *       *   *   *       *   *           *
 N   R   K   Y   D   C   A   N   E   S   R   P   G   V   V   S   E
AAC CGA AAA TAC GAC TGC GCC AAT GAA TCG CGT CCG GGT GTG GTG AGC GAG
 *
 K   L   T   P   E   Q   A   V   R   K   V   I   A   V   A   T   T
AAG CTC ACC CCT GAG CAG GCA GTG AGA AAA GTG ATC GCG GTC GCG ACG ACC
                                    *   *   *   *   *   *
 I   P   Q   M   T   V   L   G   I   A   G   P   G   D   A   L   A
ATT CCG CAG ATG ACG GTA CTT GGC ATC GCT GGT CCC GGC GAT GCC CTG GCC
            *   *           *               *                   *
 N   P   A   K   T   F   K   T   L   A   L   V   T   E   A   A   P
AAT CCA GCA AAG ACG TTC AAA ACG CTC GCG TTG GTC ACC GAG GCT GCT CCT
 *       *   *   *       *   *   *   *   *   *   *   *   *   *       *
 D   I   K   L   C   L   S   T   N   G   L   A   L   P   D   Y   V
GAC ATC AAG CTG TGT CTG TCA ACC AAC GGA CTA GCG CTG CCA GAC TAT GTC
 *                           *   *   *   *   *   *   *   *   *   *
 D   T   I   V   R   A   K   V   D   H   V   T   I   T   I   N   M
GAT ACC ATC GTG AGG GCC AAA GTT GAC CAC GTC ACC ATC ACC ATC AAC ATG
 *   *   *   *   *   *   *   *   *   *   *   *   *                   *
 V   D   P   E   I   G   A   K   I   Y   P   W   I   F   F   N   H
GTC GAT CCT GAA ATC GGA GCC AAG ATT TAT CCA TGG ATC TTC TTC AAC CAC
      *   *
 K   R   Y   T
AAG CGA TAC AGG
```

to the R. leguminosarum nifB gene (Rossen et al., 1984) was identified (Fig. 12). As described for the amino termini of the B. japonicum nifH and nifD genes (Chap. 2), the proposed nifB initiation ATG codon is preceded by a purine rich region in a position appropriate to be the Shine and Dalgarno ribosome binding site (Shine and Dalgarno, 1975) suggesting that this is indeed the translational start for this gene.

nifB Promoter Mapping. In order to determine the precise transcriptional initiation site for the nifB gene, S1 nuclease protection analyses (Berk and Sharp, 1977) were initiated in the region 5' to the proposed translational start for the nifB open reading frame. A 302 bp MspI-AvaI fragment that contains the first 16 amino acids of nifB coding sequence as well as about 250 bp upstream of the N-terminal methionine was 5' end-labelled using T4 polynucleotide kinase. The DNA strands were separated electrophoretically and hybridized separately to bacterial RNA from soybean root nodules. As expected from the DNA sequence, only the MspI 5' end-labelled strand showed partial S1 nuclease-resistance confirming that this is the mRNA coding strand. To precisely define the nifB transcriptional initiation site, the protected DNA strand was separated electrophoretically next to a DNA sequencing ladder of the same DNA strand (Fig. 13). A single group of partially protected fragments was observed approximately 105 (± 2) bp upstream from the putative nifB translational initiation site indicating that a nifB RNA 5'-end is located at the position shown in Fig. 6. We tentatively consider this to be the sole initiation site for nifB transcription in root nodules. As with nifH and nifDK (Chap. 2), the promoter region for this transcription unit shares extensive DNA

Figure 13. Identification of the B. japonicum nifB promoter sequence. S1 protection and DNA sequence analysis was carried out using a 302 bp MspI-AvaI fragment containing the 5' end of the B. japonicum nifB gene. Only the DNA strand which hybridizes to bacterial RNA isolated from soybean root nodules is shown. Lane designations are as follows: (1) C+T, (2) T, (3) A>C, (4) G, (5-7) S1 protection analysis using either 2 ug (5), 1 ug (6), or 0.5 ug (7) of RNA isolated from soybean nodule bacteria.


1 2 3 4 5 6 7

sequence homology in the -10 to -27 region with other B. japonicum nif promoters (Adams and Chelm, 1984; Alvarez-Morales et al., 1985) and with nif promoters from other nitrogen fixing species (Beynon et al., 1983; Ow et al., 1983).

DISCUSSION

Unlike many other diazotrophs where the constituent polypeptides of nitrogenase are encoded by a single operon in the order nifHDK (Reidel et al., 1979; Banfalvi et al., 1981; Krol et al., 1982), these genes occur as two separate transcription units, nifH and nifDK, in B. japonicum strain USDA 110 (Kaluza et al., 1983; Chap. 2). We have isolated overlapping cosmid clones of B. japonicum genomic DNA carrying either the nifH or nifDK genes. Using these clones, a partial restriction endonuclease map representing approximately 33 kbp of the B. japonicum genome has been constructed. The transcriptional initiation sites for the nifH and nifDK genes, which have been mapped previously (Chap. 2), are separated by approximately 20 kbp, and all three nitrogenase genes are transcribed in the same direction (Fig. 9).

About 13 kbp downstream from the nifDK operon we have identified an open reading frame which has extensive homology to the nifB genes of K. pneumoniae and R. leguminosarum. This gene is also transcribed in the same direction as nifH, D, and K. Mutational analyses of the K. pneumoniae and R. leguminosarum nifB genes (Roberts and Brill, 1981; Rossen et al., 1984) indicates that their products are required for synthesis of the MoFe cofactor of nitrogenase component I. Although the

exact function of the nifB product is unknown, analysis of the predicted amino acid sequence of the R. leguminosarum nifB gene (Rossen et al., 1984) indicated the presence of clustered cysteine residues in the N-terminal region of the protein. This data has been used to suggest that the nifB gene product functions in the binding of $[Fe_x:S_x]$ clusters (Rossen et al., 1984). We have observed similar groupings of cysteine residues in the N-terminus of the B. japonicum nifB gene (Fig. 12).

Using S1 protection analyses, we have mapped a putative transcriptional inititiation site for the B. japonicum nifB gene. As expected, the sequences 5' to this initiation site share several blocks of highly conseved sequence with the major nifH and nifD promoters described in chapter 2. A genetic analysis of the role these sequences play in the coordinate control of this gene set will be useful in understanding the symbiotic control of nif gene expression.

EXPERIMENTAL PROCEDURES

Bacterial strains and media. The Escherichia coli K-12 strain ED8654 (gal met $hsdR_K$ supE supF) was used for general plasmid cloning and maintenance as well as for maintenance of the B. japonicum genomic DNA library cloned into the broad-host-range cosmid cloning vehicle pLAFR1 (Friedman et al., 1982). B. japonicum USDA 110 was grown in YEX medium (0.04% yeast extract, 0.3% xylose, 3 mM K_2HPO_4, 0.8 mM $MgSO_4$, 1.1 mM NaCl).

DNA techniques. Genomic DNA from B. japonicum was purified by phenol extraction (Marmur and Doty, 1962). Relaxed replicon plasmid DNA was isolated by the method of Clewell and Helinski (1972). Isolation of plasmid DNA from the low-copy-number cosmid clone band was modified as described by Friedman et al. (Friedman et al., 1982). Isolation of DNA restriction endonuclease fragments for use as probes was as described previously (Adams and Chelm, 1984). All other restriction endonuclease mapping and enzymatic cloning techniques were standard (Maniatis et al., 1982).

S1 nuclease protection analysis was done as described (Adams and Chelm, 1984). DNA sequencing was by the methods of Maxam and Gilbert (1981). All sequences were determined using both DNA strands and all restriction sites were overlapped.

Construction and maintenance of a B. japonicum cosmid gene bank. Total DNA from B. japonicum 110 was partially digested with EcoRI and subjected to centrifugation for 8 hr at $4^{o}C$ and 155,000 x g_{av} through a 12-ml 5 to 20% (wt/vol) sucrose gradient dissolved in 1 M sodium acetate, 10 mM Tris-hydrochloride, 1 mM EDTA, 0.01% sodium lauryl sarcosinate (pH 8). Fractions from this gradient that contained DNA molecules greater than 15 kbp in size were pooled and used for construction of a gene bank in the broad-host-range cosmid vector pLAFR1 (Friedman et al., 1982). EcoRI-cleaved, phosphatase-treated vector DNA was ligated to the partially digested B. japonicum DNA at a molar ratio of 5:1 (vector/insert) as described by Maniatis et al. (1982). After ligation, DNA was packaged as described previously (Maniatis et al., 1982) and used to transduce E. coli K-12 ED8654 to tetracycline

resistance. Transductants were replicated in an ordered array to both agar stabs and liquid medium in polycarbonate microtiter dishes. For screening, the cosmid-containing isolates were transferred with the aid of a steel prong replicator to sheets of cellulose nitrate lying on a dish of agar-solidified medium and then allowed to grow to colonies.

Hybridization procedures. E.coli ED8654 colonies harboring the ordered cosmid library were lysed, and their DNA was bound to nitrocellulose filters as described by Grunstein and Hogness (1975). Before hybridization, filters were incubated for 1 h at 65°C in 5 x Denhardt solution (Denhardt, 1966), 5 x SSPE (1 x SSPE is 0.18 M NaCl, 10 mM $NaPO_4$ [pH 7.7], 1 mM EDTA), and 200 ug of sheared and denatured salmon sperm DNA per ml. γ^{32}P-labelled hybridization probes were prepared by nick translation (Maniatis et al., 1975). Hybridization reactions were carried out for 12 to 24 h at 65°C in 5 x SSPE, 1.5 x Denhardt solution, and 100 ug of salmon sperm DNA per ml. After hybridization, filters were washed twice for 15 min each at room temperature in 2 x SSPE, 1% sodium dodecyl sulfate followed by two washes in 0.1 x SSPE, 0.1% sodium dodecyl sulfate. Hybridization signals were detected by autoradiography.

CHAPTER 4

Bradyrhizobium japonicum Genes With Sequence Homology to the *Klebsiella pneumoniae nifA* Gene

INTRODUCTION

The free-living nitrogen-fixing bacterium *Klebsiella pneumoniae* contains a cluster of at least 17 genes arranged in 7 or 8 operons which are required for nitrogen fixation (*nif* genes; Reidel *et al.*, 1979; Roberts and Brill, 1981). This complex regulon is controlled by two separate systems. One links nitrogen fixation to the general nitrogen control pathway (Ntr) known for several enteric bacteria (Magasanik, 1982). The second is specific for *nif* gene control (Dixon, 1984).

The *nif*-specific aspect of this regulation requires the *nifA* gene product (NIFA). NIFA works in conjunction with the *ntrA* gene product (NTRA), a putative sigma factor for recognition of *ntr*-activated promoters (Hirschman *et al.*, 1985; Hunt and Magasanik, 1985), to turn on transcription from other *nif* operons (Dixon *et al*, 1980; Merrick, 1983; Ow and Ausubel, 1983). The *nifA* gene itself is the site at which the more general Ntr system acts to control *nif* expression. Under nitrogen limiting conditions, transcription of the *nifA* gene is activated through the concerted action of NTRA and the product of another gene, *ntrC*

(NTRC; Ow and Ausubel, 1983; Dixon, 1984). Under these conditions NTRC and NTRA are also necessary for the activation of genes involved in nitrogen assimilation including those for glutamine synthetase, histidine utilization, proline utilization, and arginine utilization (Magasanik, 1982).

In addition to their mutual requirement of NTRA for activity, NIFA and NTRC are structurally and functionally related. The amino acid sequences for both proteins are homologous throughout a large central region (see Fig. 19; Buikema et al., 1985; Drummond et al., 1986; Nixon et al., 1986; Gussin et al., In Press). Also, the promoters for genes under NIFA or NTRC control share a common consensus sequence (Beynon et al., 1983; Ow et al., 1983). Under certain conditions, some of these promoters can be activated by either regulatory system (Ow and Ausubel, 1983; Merrick, 1983; Buck et al., 1985).

For Bradyrhizobium japonicum, the soybean symbiont, several genes required for nitrogen fixation have been localized in two distinct clusters (Adams et al., 1984; Fuhrmann et al., 1985). Promoter sequences for these genes share a high degree of homology with those identified for NIFA/NTRA and NTRC/NTRA regulated promoters in K. pneumoniae (Adams and Chelm, 1984; Fuhrmann et al., 1985; Chap. 2). In addition, when overexpressed in a heterologous E. coli system, the K. pneumoniae nifA gene product is able to activate transcription from the B. japonicum nifH and nifDK promoters (Alvarez-Morales and Hennecke, 1985; Alvarez-Morales et al., 1986), supporting the hypothesis that B. japonicum nif genes may be controlled by similar regulatory mechanisms. With this in mind, we have used the K. pneumoniae nifA gene to screen libraries of B. japonicum genomic DNA. In this chapter, I report on the

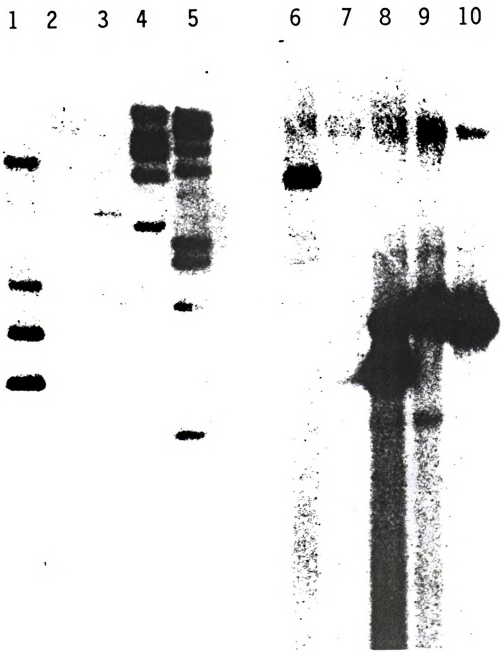
isolation and structural characterization of four separate genes from B. japonicum with sequence homology to K pneumoniae nifA.

RESULTS

Identification and cloning of B. japonicum nifA-like regions. A 950 bp PstI-EcoRV DNA fragment which is entirely internal to the K. pneumoniae nifA gene (see Chap. 3, Fig. 7; Buikema et al., 1985) was radioactively labelled and hybridized to Southern transfers of EcoRI digested B. japonicum genomic DNA. As shown in Figure 14, B. japonicum contains several EcoRI fragments with homology to the nifA gene of K. pneumoniae. Rhizobium meliloti (Fig. 14, lanes 2 and 3) and Escherichia coli (Fig. 14, lanes 4 and 5) also contain multiple restriction fragments with homology to nifA.

In order to isolate the B. japonicum nifA-homologous regions a phage library of B. japonicum genomic DNA was screened with the nifA-specific hybridization probe described. To reduce the background observed from hybridization to the host chromosome, phage were plated on an E. coli deletion strain which lacks the single known nifA-like E. coli gene, ntrC (Fisher et al., 1981). Several recombinant phage with nifA-like B. japonicum sequences were isolated. These phage were then used to isolate larger genomic DNA regions by directly screening the ordered cosmid library described previously (Adams et al., 1984). Cosmids identified in this manner were isolated and grouped into four distinct classes based on restriction site patterns. nifA-specific hybridization to Southern transfers of EcoRI digested DNA from cosmids

Figure 14. nifA-homologous sequences in B. japonicum, R. meliloti, and K. pneumoniae. Southern transfers of total cellular B. japonicum DNA digested with EcoRI (lane 1); R. meliloti DNA digested with HindIII (lane 2) or EcoRI (lane 3); E. coli DNA digested with HindIII (lane 4) or EcoRI (lane 5); and cosmid clones of B. japonicum DNA, pRJcos2-43 (lane 6), pRJcos1-62 (lane 7), pRJcos7-36 (lane 8), pRJcos14-28 (lane 9), or pRJcos3-44 (lane 10) were hybridized with a probe specific for internal sequences from the nifA gene of K. pneumoniae as described in the Experimental Procedures.

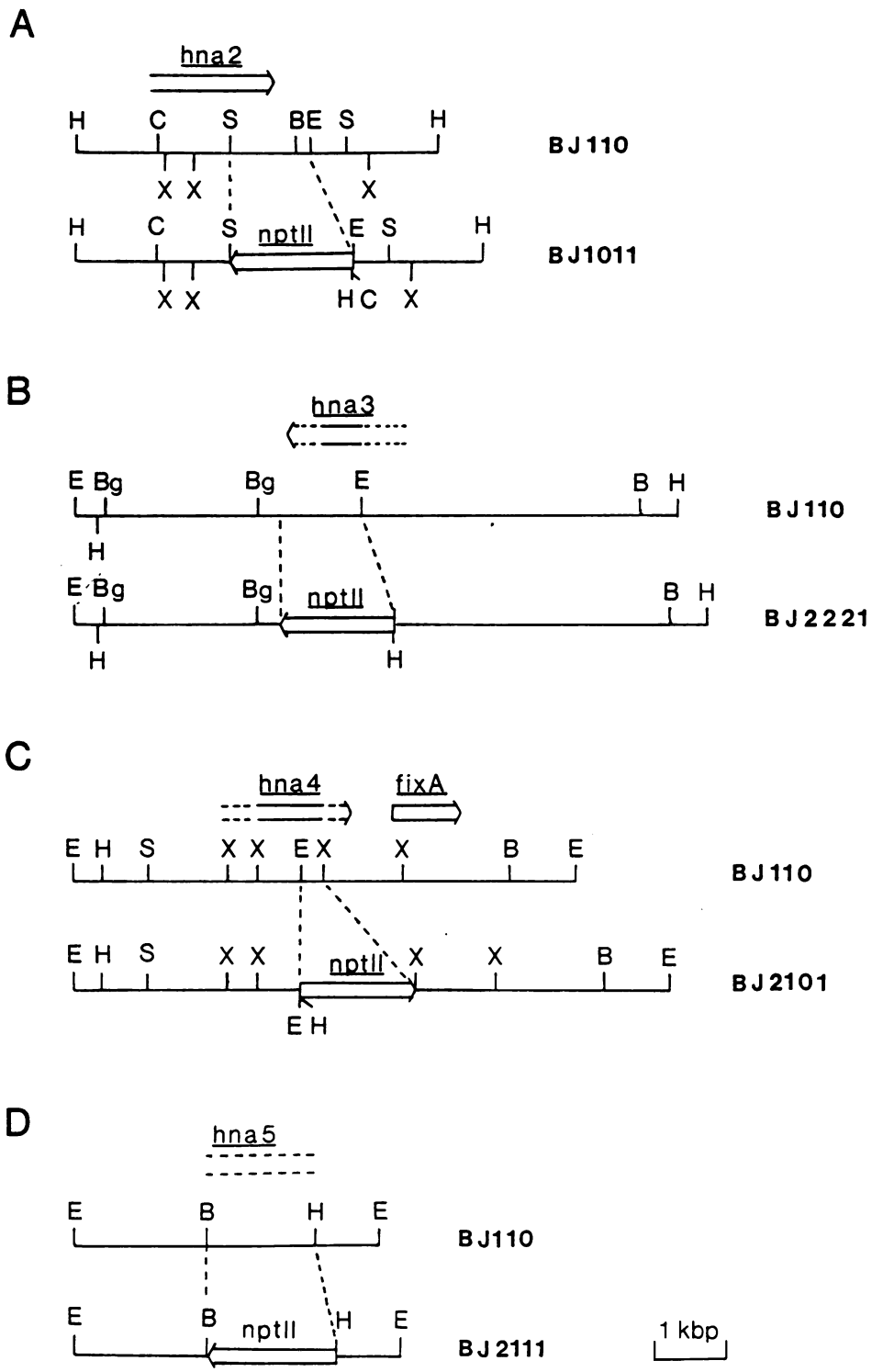

1 2 3 4 5 6 7 8 9 10

representing each of these classes is shown in Fig. 14 (lanes 6, 8, 9, and 10). Restriction endonuclease maps for each of the nifA-homologous regions (designated hna2-hna5 for homology to nifA) are shown in Fig. 15. By inspection of the restriction map for the region labelled hna4, this gene was found to be linked with another gene required for nitrogen fixation termed fixA (Fig. 15; Fuhrmann et al., 1985). This localization of fixA was confirmed by DNA sequence analyses (see below). This DNA region also contains several genes required by B. japonicum for nodulation (nod genes) of soybean plants (Lamb et al., 1986). None of the other nifA-like regions are closely linked to any B. japonicum genes that have been described.

DNA sequence analyses. To determine whether the B. japonicum regions descussed above have the capacity to encode NIFA-like proteins, partial DNA sequences for three of the four nifA-like regions have been determined (Figs. 16-18). In each case, open reading frames with extensive amino acid sequence homology to the K. pneumoniae nifA and ntrC (Buikema et al., 1985) genes were found (Fig. 19). This homology is most apparent through the central domain of the Klebsiella genes. No sequence data is available for the nifA-like cloned region (hna5). However, this region does hybridize to each of the other three B. japonicum regions as well as to nifA (not shown).

Construction and characterization of B. japonicum hna deletion strains. To determine whether any of the NIFA-like regions described above are involved in regulating nitrogen fixation or nitrogen assimilation genes, we have constructed four B. japonicum mutant strains

Figure 15. Genomic restriction endonuclease maps for B. japonicum strain BJ110 nifA-homologous genes hna2 (A), hna3 (B), hna4 (C), and hna5 (D). Also shown are genomic maps for B. japonicum hna2, hna3, hna4, and hna5 deletion strains BJ1011, BJ2221, BJ2101, and BJ2111 respectively. In each case, a portion of the nifA-like gene region was deleted and replaced with the nptII gene. Arrows indicate the orientation and approximate position of each nifA-like gene region. The solid line portion of each arrow indicates the gene region that has been sequenced (Figures 16-18). Dashed lines indicate approximate dimensions for each hna gene assuming that their size and structure are like that of the K. pneumoniae nifA gene. For hna5 (D), the dashed lines represent the DNA region that hybridizes to the K. pneumoniae nifA gene as well as to other B. japonicum hna regions. The position and orientation of fixA within the hna4 genomic region (C) has been described previously (Fuhrmann et al., 1985). Restriction sites indicated are H, HindIII; C, ClaI; S, SalI; B, BamHI; E, EcoRI; X, XhoI; and Bg, BglII.


A
hna2
H C S BE S H
BJ110
X X X
H C S nptII E S H
BJ1011
X X H C X
B
hna3
E Bg Bg E B H
BJ110
H
E Bg Bg nptII B H
BJ2221
H H
C
hna4
fixA
E H S X X E X X B E
BJ110
E H S X X nptII X X B E
BJ2101
E H
D
hna5
E B H E
BJ110
E B nptII H E
BJ2111
1 kbp

```
   1  TGACTAGGCT CCAGCGCCCA GATTTCCGCC GCGTTGCGGA AGTACTTGGC GATGCGGCCA
  61  CAATGCTGGT CATCACCGAC AGGAATGGTG TCGTGCTTGG TGTCGGGGGC GATCTGAGGA
 121  GTGCCGGGCA CGGTGTTCGC CTCGAAGTGC GCCGCCAGGG AGAGACGGTG ACCGGCACCA
 181  ACGGCATTGG CACGGCGCTC GTCACAGGAA AGCCGGTTCA TGTCCACGCC GCGGAGCATT
 241  TTTCCGAGGG CATCAAGGCC TGGACCTGCG TCGGTAGCCC CATTCGTAGC CCGATCGACG
 301  GCAGCATCAT AGGCATCATC GATTTTTCCG GCCCCCAGGC GATCTTTCAT CGTCACAATG
 361  TCGCGCTGGC GGTGATCGCG GCCAATCATA TTGAGCTTGC GCTCTCGGAG AAGATTCGGC
 421  TCGAAAGAAT ACGCCTGCTC GAGGCAAGCA TTTGTCGAAT GCCAGGGATG GGCAGTGCCG
 481  ACGGCGTGGT GATCCTGGAT CGGTTTGGTC GGGTCGTCCA TCACAACGAT ATGGCGTCGG
 541  CTCGCTGGCG GCGGATCACG CAGTCTCGTG AGTTGAGCAT TGGGTCACAG ATCCTTCCAA
 601  GTCGCGAAGG CCTGTTGGGC GAAGACTTGG CCGAGGGGTT GCCCGAGGAG CTGCGGGAGC
 661  AGCGGATCGA GCCACTGATC GTTGACGGGG TCGTGAAGGG CGCAATGCTC GTGCTTGCTT
 721  CGAAGCCGCG AACCCATCCT GCTGCATCCG AGATGTCGCC GACAGCCCGC ACTGCCCTGA
 781  TGTCGGCAAA GGAAGCGATC GTCGGATGCA GCGAGGCACT GCTGGACGTG GCGCAGAAAG
 841  TTGAGCGGCG GGCCCTCGGC CGGACCGCGG TCCTGCTCGA GGGCGAGACG GGCGTCGGCA
 901  AGGAACTGTT CGCCCGCCTG GTTCATGCCG CCAGTCTGAA GACGGGCAAG GAGCCGTTCG
 961  TTGCCTTCAA TTGCGGTGCG GTGTCGAAAG AGTTGCTCGG CGGCGAATTG TTCGGGCATG
1021  CGCCGGGCGC TTGTACACCG GCTACGCGCG AGGGAAGGCC CGGGCGGTTC GAATTTGCCA
1081  ATGGCGGCGT GCTCAGCTTA GACGAGATCG GCGAGATGCC GATGGACCTG CAACCCTACC
1141  TGCTCCGTGT GCTGGAAGAG CGCGCGGTGT ACAGACTGGG TGACAGCAAG GCGCGCCCGG
1201  TGGACGTTCG GCTGGTGGCG TCGACCAATC GGAATTTGAA GCAGGAAGTG GCCGAGGGAC
1261  GTTTCCGCAA GGATCTGTAC TTCCGGATCG GCGTCGTCAA GTTCACCATC CCGCCGCTCC
1321  GCGATCGGCT GGGCGATGTC GAGATCCTGA TCGATCACTT CAACAGGCAA TTCGCCACGA
1381  TCTACAGCAC CCAGCCGCTG CGGTTCGAGG CGGCCACTAT GGAGTTGCTG CGCCGCTATT
1441  CCTGGCCGGG AAACGTTCGC GAGCTGCGTA ATCTGATTGA GAACCTCGTG CTGATGACGA
1501  TCACCGGTAT CGTCACTCCC AGGGACCTGC CAGAGGATTT CGTGGAGGTG GCGCCGGCTC
1561  AACCTCCCTC AATCTCCGTG CAGTCGGAGA ATAGCGCGAC GGGATCGGAG AGTGACGAGA
1621  TCGCGCGCTT TGACGAGATG GAACGTCGTG CCATCGAGAG AGCCGTCGCA AACGCCGGCG
1681  GCAATATTGC CGCTGCCGCG GAAAAGCTGG GGCTTTCGCG CGCTACGCTG TACCGCAAGT
1741  TGCACCAATA CCGTTCCCGG ACCTAGCGAT CGGGGGCTGG CATGGCGGCT GTCTGGGATG
1801  AACAAAAGGG CGGGGAAGGT CCGGCCCCCG CGGCGACGCT GGCCTCGCAG ATCCTTGCGC
1861  GCGCCGACCG CGACGTCACT TTCGTGACGC GCCTGGTTGG CGAAAGCAAG CTCGCTGGTG
1921  CGACATTTCA TCATGTTCAT CGAGACCGAG CTCGCAAAGC GCGGCATGAG CTACAGCGTA
1981  CATCCGCTAC TGCGCCCTTT CGTCGAACGC ATGCACGCGA GCTGTCGGAT TTCGTATTGC
2041  AGGGTATCGG CTTGCGGCAT CAGTTCGGCC TCCAGACCAT CGAGACGATG GCGGGCGATC
2101  CTGCGCGGTT GCTGAGGGTC GATTTGAGGG ACTCGCTGCA ATCTCACGTT AATGACGCGA
2161  GCAACACTTC GTATCCGATG CCCGGGGCCT CCAGCGGATC CTGGATCAAA TCGAATCGAG
2221  CGCATGAGTG TCGATCCGGA AGTCCTTGTC GAAATGCTGA AGGAGCGGTT GTTCGTCGTT
2281  CAGCAGATAT CGGCCGCTCA ATCGTGGAAC CTTCTGAACC GTCTGCTTGC TGGT
```

Figure 16. Nucleotide sequence of the B. japonicum nifA-like region hna2.

```
  A1  GTCGACGTCA CGATCTATGG TGAGACCGGA ACCGGCAAGG ACGTCGTCGC GCGCTGCCTG
 A61  CACAATCACA GCGGCCGCCG CGGCGAGCAT TATGTCGCGG TGAATTGCGG CGGTCTGCCG
A121  GAATCGCTGG TCGAGAGCGA GCTGTTCGGC CACGAGGTGG GCGCGTTCAC CGGCGCCACC
A181  CTGTTCGGCC ACGAGGTGGG CGCGTTCACC GGCGCCACCC GTCAGCGGAT CGGAAAGATC
A241  GAATACGCCA GTGGCGGAAC GCTATTCCTC GACGAGATCG AGAGCGTGCC GCTGA
```

```
  B1  CTGAGCGAAA AGCGTCTGTT TCGTGCCGAT CTGTACTATC GCCTCGGCGT CGCTTTCATC
 B61  GAGCTCCCGC CGTTGCGCGA ACGGCGGGAG AATATCCCGC TGCTGTTCGA GCACTTCACC
B121  CTGGATGCGG CGAGGCGGTT CGAGCGTGAC GCCCCGATTC TGGACGAACA GACCATGTCT
B181  CACCTGCTGG CCTATTCGTG GCCGGGCAAC GTACGCGAGC TTCGCAACGT CGCCGACCGT
B241  TTCGTGCTCG GTGTCCTCGA CAGCAAGGCG GTCAATAAAT CCGGTTCGGT ACGTCGGATG
B301  TGTCATTGCC GCGGCAACTC GAGCGATCAT CGAGGACCGC TCCGGCGCAA GCAGGGCGAC
B361  GTCCAGGCAA CGAGCCGCGC ATCTCA
```

Figure 17. Nucleotide sequence of the B. japonicum nifA-like region hna3. The two blocks (A and B) of sequence shown are from separate regions of this nifA-like gene.

```
  1  CTCGAGTACG ATGCGCGGCT GCTCGCCATG GTCGCGAACG TGATAGGACA AACGATCAAG
 61  TTACATCGCT TGTTCGCCGG CGATCGCGAA CAATCGTTGG TGGACAAGGA CCGGCTAGAG
121  AAACAGACAG TTGATCGTGG GCCGCCCGCT CGCGAGCGCA AGCAGCTTCA GGCACACGGG
181  ATCATTGGCG ACAGCCCGGC GCTGAGCGCA CTGCTTGAGA AGATTGTCGT TGTAGCCAGA
241  TCAAACAGCA CGGTTCTGCT GCGTGGCGAA TCCGGTACCG GGAAGGAGCT GGTAGCCAAG
301  GCCATTCACG AGTCGTCCGT TCGTGTAAGC GGCCGTTCCG TTAAGCTGAA TTGCGCGGCG
361  CTCCCCGAGA CGGTCCTGGA ATCGGAATTG TTTGGCCATG AGAAAGGAGC CTTTACCGGT
421  GCTGTCAGGC GCCCGCAAGG GCGCTTCGAG CTTGCTGACA AAGGGACGCT ATTTCTTGAC
481  GAGATCGGAG AGATCTCACC TCCGTTCCAG GCGAAGTTGC TGCGAGTTCT GCAAGAGCAG
541  GAGTTCGAGC GCGTCGGCAG CAATCACACG ACAAAGTCGA TGTTCGGGTG ATAGCTGCGA
601  CCAACAGGAA CCTTGAAGAG GCTGTGGCAA GGAGCGAATT CCGCGCGGAC CTCTACTATC
661  GTATTAGCGT AGTTCCCTTG TTGTTGCCGC CGCTTCGCGA AAGACGCAGT GATATTCCGC
721  TGCTCGCAAG AGAGTTCCTC AGAAAGTTTA ACAGCGAGAA CGGCCGCTCT CTTACTCTGG
781  AGGCGAGTGC GATCGATGTA CTGATGAGCT GTAAATTTCC GGGAAATGTC CGCG
```

Figure 18. Partial nucleotide sequence of the B. japonicum nifA-homologous region hna4.

Figure 19. Comparison of derived amino acid sequences from the K. pneumoniae (Kp) nifA and ntrC (Buikema et al., 1985; Drummond et al., 1986) genes with amino acid sequences for hna2, hna3, and hna4 from B. japonicum (Bj). Sequences are aligned to maximize homology with the K. pneumoniae nifA gene. Amino acids homologies to this gene are indicated by underlining.

```
Kp nifA   MIHKSDSDTTVRRFDLSQQFTAMQRISVVLSRATEASKTLQEVLSVLHNDAFMQHGMICLYD
Bj hna2...TVTGTNGIGTALVTGKPVHVHAAEHFSEGIKAWICVGSPIRSPIDGSIIGIIDFSGPQAIFH

Kp nifA   SQQEILSIEALQQTEDQTLPGSTQIRYRPGEGLVGTVLAQGQSLVLPRVADDQRFLDRLSL
Kp ntrC             MQRGIAWIVDDDSSIRWVLERALTGAGLSCTTFESGNEVLDALTTKTPDVL
Bj hna2   RHNVALAVIAANHIELALSEKIRLERIRLLEASICRMPGMGSADGVVILDRFGRVVHHNDM

Kp nifA   YDYDLPFIAVPLMGPHSRPIGVLAAHAMARQEERLPACTRFLETVANLIAQTIRLMILPTS
Kp ntrC   LSDIRMPGMDGLALLKQIKQRHPMLPVIIMTAHSDLDAAVSAYQQGAFDYLPKPFDIDEAV
Bj hna2   ASARWRRITQSRELSIGSQILPSREGLLGEDLAEGLPEELREQRIEPLIVDGVVKGAMLVL
Bj hna4   ............................LEYDVRLLAMVANVIGQTIKLHRLFAGDREQ

Kp nifA   AAQAPQQSPRIERPRACTPSRGFGLENMVGKSPAMRQIMDIIRQVSRWDTTVLVRGESGTG
Kp ntrC   ALVDRAISHYQEQQQPRNAPINSPTADIIGERPAMQDVFRIIGRLSRSSISVLINGESGTG
Bj hna2   ASKPRTHPAASEMSPTARTALMSAKEAIVGCSEALLDVAQKVERRALGRTAVLLEGETGVG
Bj hna3   ..................................................VDVTIYGETGTG
Bj hna4   SLVDKDRLETVDRGPPARERKQLQAHGIIGDSPALSALLEKIVVVARSNSTVLLRGESGTG

Kp nifA   KELIANAIHHNSPRA-AAAFVKFNCAALPDNLLESELFGHEKGAFTGAVRQRK-GRFELADG
Kp ntrC   KELVAHALHRHSPRA-KAPFIALNMAAIPKDLIESELFGHEKGAFTGANTVRQ-GRFEQADG
Bj hna2   KELFARLVHAASLKTGKEPFVAFNCGAVSKELLGGELFGHAPGACTPATREGRPGRFEFANG
Bj hna3   KDVVARLLHNHSGRR-GEHYVAVNCGGLPESLVESELFGHEVGAFTGATRQRI-GKIEYASG
Bj hna4   KELVAKAIHESSVRA-KRPFVKFNCAALPETVLESELFGHEKGAFTGAVRRPQ-GRFELADK

Kp nifA   GTLFLDEIGESSASFQAKLLRILQEGEMERVGGDETLRV-NVRIIAATNRHLEEEVRLGHFRE
Kp ntrC   GTLFLDEIGDMPLDVQTRLLRVLADGQFYRVGGYAPVKV-DVRIIAATHQNLELRVQEGKFRE
Bj hna2   GVLSLDEIGEMPMDLQPYLLRVLEERAVYRLGDSKARPV-DVRLVASTNRNLKQEVAEGRFRK
Bj hna3   GTLFLDEIAESVPL.....................................LSEKRLFRA
Bj hna4   GTLFLDEIGEISPPFQAKLLRVLQEQEFERVGSNHHTSKVDVRIIAATNRNLEEAVARSEFRA

Kp nifA   DLYYRLNVMPIALPPLRERQEDIAELA-HFLVRKIAHSQGRTLRISDGAIRLLMEYSWPGNV
Kp ntrC   DLFHRLNVIRVHLPPLRERREDIPRLARHFLQIAARELGVEAKQLHPETEMALTRLAWPGNV
Bj hna2   DLYFRIGVVKFTIPPLRDRLGDVEILIDHFNRQFATIYSTQPLRFEAATMELLRRYSWPGNV
Bj hna3   DLYYRLGVAFIELPPLRERRENIPLLFEHFTLDAARRFERDAPILDEQTMSHLLAYSWPGNV
Bj hna4   DLYYRISVVPLLLPPLRERRSDIPLLAREFL-RKFNSENGRSLTLEASAIDVLMSCKFPGNV

Kp nifA   RELENCLERSAVLSESGLIDRDVILFNHRDNPPKALASSGPAEDGWLDNSLDERQRLIAALE
Kp ntrC   RQLENTCRWLTVMAAGQEVLTQDLPSELFETAIPDNPTQMLPDSWATLLGQWADRALRSGHQ
Bj hna2   RELRNLIENLVLMTITGIVTPRDLPEDFVEVAPAQPPSISVQSENSATGSESDEIARFDEME
Bj hna3   RELRN.....................................................
Bj hna4   REL.....................................................

Kp nifA   KAGWVQAKAARLLGMTPRQVAYRIQIMDITMPRL
Kp ntrC   NLLSEAQPEMERTLLTTALRHTQGHKQEAARLLGWGRNTLTRKLKELGME
Bj hna2   RRAIERAVANAGGNIAAAAEKLGLSRATLYRKLHQYRSRT
```

each of which contains a deletion in a single nifA-like open reading frame. For purposes of manipulation, each deleted region has been replaced by a DNA fragment carrying the nptII gene from the transposon Tn5 which confers kanamycin resistance to B. japonicum. Restriction endonuclease maps for the B. japonicum genomic regions carrying these deletions are shown in Fig. 15.

Each deletion strain was assayed for the expression of nif genes, the glnII gene, and nitrogen fixation activity in B. japonicum grown under a variety of conditions. These results are summarized in Table I. Gene expression data was obtained using the quantitative S1 protection assay described in Chapter 6. In this way, we have assayed for aspects of nifA-like (nifH and nifDK transcription) and ntrC-like (glnII transcription; Carlson,1986) control.

A detailed analysis of the hna4 deletion strain, BJ2101, is reported in Chapter 6. This mutation affects the microaerobic and symbiotic expression of a number of genes, including nifH, nifDK, and glnII. In addition, a wildtype copy of this gene is required for the normal development of soybean root nodules. Aerobic, Ntr-like control of glnII is normal in this mutant strain. We believe that this gene is part of a regulatory system (termed Odc) that is required for oxygen and developmentally mediated control of gene expression. The gene encoded by the hna4 region has been termed odcA as the first gene in this system to be described.

No changes in the expression of nifH, nifDK or glnII have been observed for the hna2, hna3, or hna5 mutant strains (BJ1011, BJ2221 and BJ2111 respectively). The gene encoded by the hna2 region does, however, appear to have an effect on development of the symbiosis

Table 1. Summary of *nif* and *gln* Gene Expression[a] data for *B. japonicum* *hna* Deletion Strains.

Mutant Strain	*nif* gene Expression		Nitrogen Fixation[b] in Root Nodules	*glnII* Expression			
						aerobic (Ntr)	
	microaerobic	symbiotic		microaerobic	symbiotic	N rich	N poor
BJ110 (wt)	+	+	+	+	+	–	+
BJ1011 (Δ*hna2*)	nd[c]	+	delayed	+	+	–	+
BJ2222 (Δ*hna3*)	+	+	+	+	+	–	+
BJ2101 (Δ*hna4*)	–	–	–	–	–	–	+
BJ2111 (Δ*hna5*)	+	+	+	+	+	–	+

[a] A (+) symbol indicates mRNA for the genes monitored was present under the conditions described (see Experimental Procedures) while (–) indicates that no transcript was observed.

[b] Nitrogen fixation activity was measured by acetylene reduction (Hardy *et al.*, 1968).

[c] not done.

Table 2. Acetylene Reduction by Soybean Root Nodules 2 and 4 weeks after Inoculation with BJ110 or BJ1011.

Strain	Time After Inoculation	Plant Number	Nitrogenase Activity umoles C_2H_2 Reduced/hr	Nodule Number/Plt	Nodule Fresh Weight(g/Plt)
BJ110	2 weeks	10	1.74	19	0.106
BJ1011	2 weeks	10	0.071	18	0.0525
BJ110	4 weeks	3	12	55	0.80
BJ1011	4 weeks	3	10	41	0.56

Data presented for nitrogenase activity, nodule number, and nodule fresh weight are averages determined using the number of plants indicated.

(Table 1). Soybean root nodules harvested two weeks after infectio with BJ1011 reduced approximately twenty-five-fold less acetylene p hour than did wildtype infected nodules of the same age (Table 2). four weeks after inoculation and sowing, nodules induced by both th wildtype and mutant strains reduced acetylene at nearly equal rates addition to this symbiotic phenotype, BJ1011 typically grows more s than wildtype under all media conditions that have been tested. Th slow growth phenotype is very unstable in liquid culture in that on growth is initiated, it proceeds at a rate similar to wildtype. Th return to normal growth rate is retained when cells are transferred fresh media. Since the mutation in this strain is a deletion of th hna2 region, the high frequency of phenotypic reversion observed mu arise through genetic changes at a second site. This tendency towa pseudoreversion has complicated efforts to determine the actual rol this gene in B. japonicum.

DISCUSSION

Bradyrhizobium japonicum, Rhizobium meliloti, and Escherichia genomic DNAs all contain multiple restriction endonuclease fragment which hybridize to a Klebsiella pneumoniae nifA DNA probe. At pres only one nifA-like gene has been described in E. coli (Buikema et a 1985) and two in R. meliloti (Szeto et al., 1984; Buikema et al., 1 Gussin et al., In Press). It will be interesting to see whether th different nifA-homologous regions encode a family of NIFA-like regulatory proteins responsible for controlling different gene syst

Recently, it has been pointed out that the E. coli nifA homo gene ntrC is related to a number of other regulatory genes in its N-terminal domain (Drummond et al., 1986; Nixon et al., In Press) of these genes, with the exception of the R. leguminosarum dctD g (Gussin et al., In Press), share any homology with the central do ntrC. This middle portion is the region of ntrC that is closely to the K. pneumoniae nifA gene. It has been suggested that this is involved in the interaction of these proteins with NTRA and/or polymerase (Drummond et al., 1986; Nixon et al., In Press). Sinc was this internal region of nifA which we used as the hybridizati probe in these experiments (see Chap. 3, Fig. 7), the multiple homologies described here may represent a group of as yet unident regulatory genes which function in tandem with NTRA.

We have isolated 4 and determined partial DNA sequence for 3 B. japonicum nifA-like regions. In each case, we have found that nifA hybridizing DNA from B. japonicum potentially encodes an ope reading frame with extensive amino acid sequence homology to the pneumoniae nifA and ntrC genes. Four separate mutant strains of japonicum, each containing a deletion in a single hna region, hav constructed and are described here. These mutant strains have be examined for alterations in the Nif-like (transcription of nifH a nifDK) and Ntr-like (transcription of glnII; Carlson, 1986) contr systems.

A detailed discussion of the hna4 mutant is presented in Cha The gene encoded by this region has some properties that are sim the K. pneumoniae nifA gene. In addition, this B. japonicum gene more general role in controlling gene expression in response to

limitation and symbiotic development than expected for nifA. We that this gene is part of a general regulatory system that is re for oxygen availability and developmentally mediated control. T regulatory system has been termed Odc. We call the nifA-like gen in the hna4 region odcA as the first gene identified in this reg pathway.

The hna2 deletion strain, BJ1011, has been characterized as a delayed fixation phenotype in soybean root nodules. The mecha which this delay occurs is not yet clear. Since at least some n (nifH and nifDK) are expressed normally during this lag in the o nitrogen fixation, it is unlikely that the delay in nitrogen fix due to a general alteration in nif gene control. One characteri the hna2 deletion strain in free-living culture is a tendency fo reversion from a very slow growing phenotype to a more normal gr rate. It is possible that the delay observed in nitrogen fixati activity results from a similar requirement for phenotypic rever

Ausubel et al. (1985) described a R. meliloti mutant strain which a transposon, In5, had been inserted into a nifA-homologou reading frame. Like the hna2 deletion, this R. meliloti mutatio results in a delayed nitrogen fixation phenotype. In addition, meliloti mutation results in an altered response to nitrogen lim such that a nifH-lacZ fusion is no longer activated when R. meli starved for ammonia. No such nitrogen limitation response has b observed in the B. japonicum mutant strain, BJ1011. A better understanding of the relationship between these two mutants may helpful in defining the factors necessary for early nodule devel

No phenotypic differences from wildtype have yet been observed for the hna3 or hna5 mutant strains, BJ2221 and BJ2111 respectively. Expression of genes in response to microaerobic or nitrogen limited growth is unperturbed in these strains. In addition, BJ2111 and BJ2221 grow like wildtype on a variety of carbon sources, including the dicarboxylic acid succinate, indicating that neither hna3 nor hna5 are likely to encode a B. japonicum analog of the R. leguminosarum nifA homologous dctD gene which regulates the uptake of dicarboxylic acids (Gussin et al., In Press). It is possible that these nifA homologous regions are simply genetically silent copies of nifA or related genes. Pseudogene copies of nif genes in other bacteria have been described previously (Scolnik and Haselkorn, 1984). In this case, Nif$^-$ mutations in the active gene copy were found to revert at high frequency, presumably due to an activation of one of the previously silent genes. Such an event might explain the high reversion frequency observed with the hna2 deletion strain. In addition, the lack of any obvious phenotype for the hna3 and hna5 deletion strains might be explained in such a way. Alternatively, hna3 and hna5 may represent two gene copies both of which are always active for regulating the same system. A B. japonicum strain in which both hna3 and hna5 have been deleted would address this possibility.

Finally, one must consider that hna3 and hna5 could encode regulatory genes for some as yet uncharacterized gene systems. A number of ntrC- and/or nifA-homologous genes have been described. In many cases, these genes regulate expression of other genes in response to environmental stimuli such as altered osmolarity (E. coli ompR; Ramakrishnan et al., 1986), phosphate limitation (E. coli phoB; Makino

et al., 1986), the presence of dicarboxylic acids (R. leguminosarum dctD; Gussin et al., In Press), the presence of detergents (E. coli sfrA; Drury and Buxton, 1985), or the presence of plant exudates (A. tumefaciens virG; Gussin et al., In Press) in the growth media (Drummond et al., 1986; Gussin et al., In Press; Nixon et al., In Press). An examination of the B. japonicum mutants described here for altered responses to environmental stimuli other than oxygen or nitrogen limitation would be useful.

B. japonicum contains other nifA-homologous regions which have not been described in this paper. Recently, two of these regions have been isolated (G. Martin, unpublished) in our laboratory. These regions are homologous to a gene isolated from B. parasponia (Nixon et al., In Press). No mutation in this gene has yet been described but the B. parasponia gene has a very similar sequence to the K. pneumoniae ntrC gene and is linked to a gene which is homologous to ntrB, another gene in the general nitrogen regulatory pathway (Magasanik, 1982). It will be interesting to see the effects of mutations in these genes and other nifA-like sequences on bradyrhizobial gene expression.

EXPERIMENTAL PROCEDURES

Bacterial strains. The wildtype B. japonicum strain used in all experiments is a small-colony forming derivative of B. japonicum USDA 33I1b110 (BJ110) isolated as described (Kuykendall and Elkan, 1976; Meyer and Pueppke, 1980). BJ1011, BJ2111, BJ2101, and BJ2222 are B. japonicum strains in which a nifA-like region has been deleted and

replaced by the nptII gene. The E. coli strain ED8654 (galK, galT, trpR, hsdR, supE, supF, lacY) was used for construction and maintenance of plasmids and cosmids (Adams et al., 1984), as well as for preparation of E. coli genomic DNA. The B. japonicum genomic DNA library maintained in lambda phage BF101 was screened for nifA homologous sequences using the E. coli host ET8051 ((rha-glnA), $hutC_k$, rbs, nal_r) which contains a deletion of the entire glnAntrBC operon (Fisher et al., 1981).

Bacterial media, growth conditions, and strain construction. All growth conditions, media, and gene replacement techniques used are described in Chapter 6.

Nucleic acid techniques. Preparation of genomic DNAs, library screening, Southern hybridization, and DNA sequencing were all as described (Adams and Chelm, 1984; Adams et al., 1984). The quantitative S1 protection assay used for evaluating gene expression levels is described in Chapter 6.

CHAPTER 5

Microaerobic Induction of *nif* and *gln* Gene Expression in *Bradyrhizobium japonicum*

INTRODUCTION

The expression of both nitrogen fixation (*nif*) and nitrogen assimilation (e.g. *glnA*, the gene encoding glutamine synthetase) genes in the free-living diazotroph *K. pneumoniae* is mediated by the same general nitrogen regulatory (Ntr) system (Magasanik, 1982; Dixon, 1984). Under nitrogen-limiting anaerobic conditions, the transcription of genes encoding both the nitrogen fixation and nitrogen assimilation processes is activated. In this way, *K. pneumoniae* is able to utilize atmospheric dinitrogen to support growth.

Rhizobia and bradyrhizobia reduce atmospheric dinitrogen primarily during symbiotic association with their leguminous host plants. The development of symbiotic nitrogen fixation is a complex process requiring the coordinate differentiation of both the plant and the bacterial cells. For the bacterium, this developmental process culminates upon differentiation into a morphologically and functionally distinct nitrogen fixing endosymbiont termed a "bacteroid". Very little

of the ammonia produced from dinitrogen reduction by bacteroids to support bacterial growth since glutamine synthetase activity repressed in these cells (Brown and Dilworth, 1975; Stripf and W 1978; Werner et al., 1980). Instead, most of this ammonia is ex (Bergerson and Turner, 1967) into the plant cytoplasm where it i assimilated by plant enzymes (Miflin and Lea, 1976). In return, plant feeds reduced carbon coumpounds to the bacteria to meet th energy demands required by nitrogen fixation.

Some bradyrhizobial strains can be made to fix nitrogen in free-living culture under specialized conditions (Keister, 1975; et al., 1975; Kurz and Larue, 1975; McComb et al., 1975; Tjepkem Evans, 1975). These conditions include requirements for both a of fixed nitrogen (preferably glutamate) and the presence of oxy an electron acceptor for oxidative phosphorylation. The require oxygen in these systems presents a difficulty since the nitroge enzyme is inactivated by oxygen. Oxygen partial pressures must maintained at a level which is high enough to allow the oxidati phosphorylation levels necessary to provide the 12-15 ATPs requ N_2 reduced (Ljones and Burris, 1972), and low enough to prevent irreversible inactivation of nitrogenase (Mortenson and Thornle

Nitrogen metabolism during asymbiotic nitrogen fixation by bradyrhizobia is similar to that observed in bacteroids since m the fixed nitrogen is not used to support growth but is instead into the medium (O'Gara and Shanmugan, 1976; Bergerson and Turn Ludwig, 1980). For at least some bradyrhizobial strains this f assimilate the fixed nitrogen is partly due to a decrease in gl synthetase activity (Ludwig, 1980; Bergerson and Turner, 1978).

regulation of glutamine synthetase activity in rhizobia and bradyrhizobia is complicated by the fact that these bacteria contain two unique forms of the enzyme, GSI and GSII (Darrow and Knotts, 1977). These two distinct nitrogen assimilatory enzymes are encoded by separate genes that are regulated by different mechanisms. Transcription of the gene encoding GSI (glnA) is relatively constant under all conditions (Carlson et al., 1985). This enzyme is regulated post-translationally via adenylylation such that activity is decreased during growth in nitrogen rich media (Bishop et al., 1976; Ludwig, 1978). No post-translational modification of GSII has been described. Transcription of the gene encoding GSII (glnII) is controlled by a mechanism that resembles the Ntr system (Carlson, 1986) as described for enteric bacteria (Magasanik, 1982). Under nitrogen limiting growth conditions transcription of the glnII gene is increased presumably to increase the nitrogen assimilatory capacity of the cell.

We are interested in understanding the mechanisms through which gln and nif gene expression are controlled by Bradyrhizobium japonicum, the soybean symbiont, during symbiotic nitrogen fixation as well as in free-living cultures. We have begun to examine this regulation by quantitatively determining the relative steady-state mRNA levels for the nifH, nifDK, glnA, and glnII transcription units in bradyrhizobial cells grown under a variety of conditions. For the nifH, nifDK, and glnII transcription units, expression is coordinately induced in response to both symbiotic development and microaerobic growth. This response is not mediated through fixed nitrogen supply but instead is primarily controlled either by oxygen availability or by developmentally-specific signals. The physiological mechanisms by which these signals are

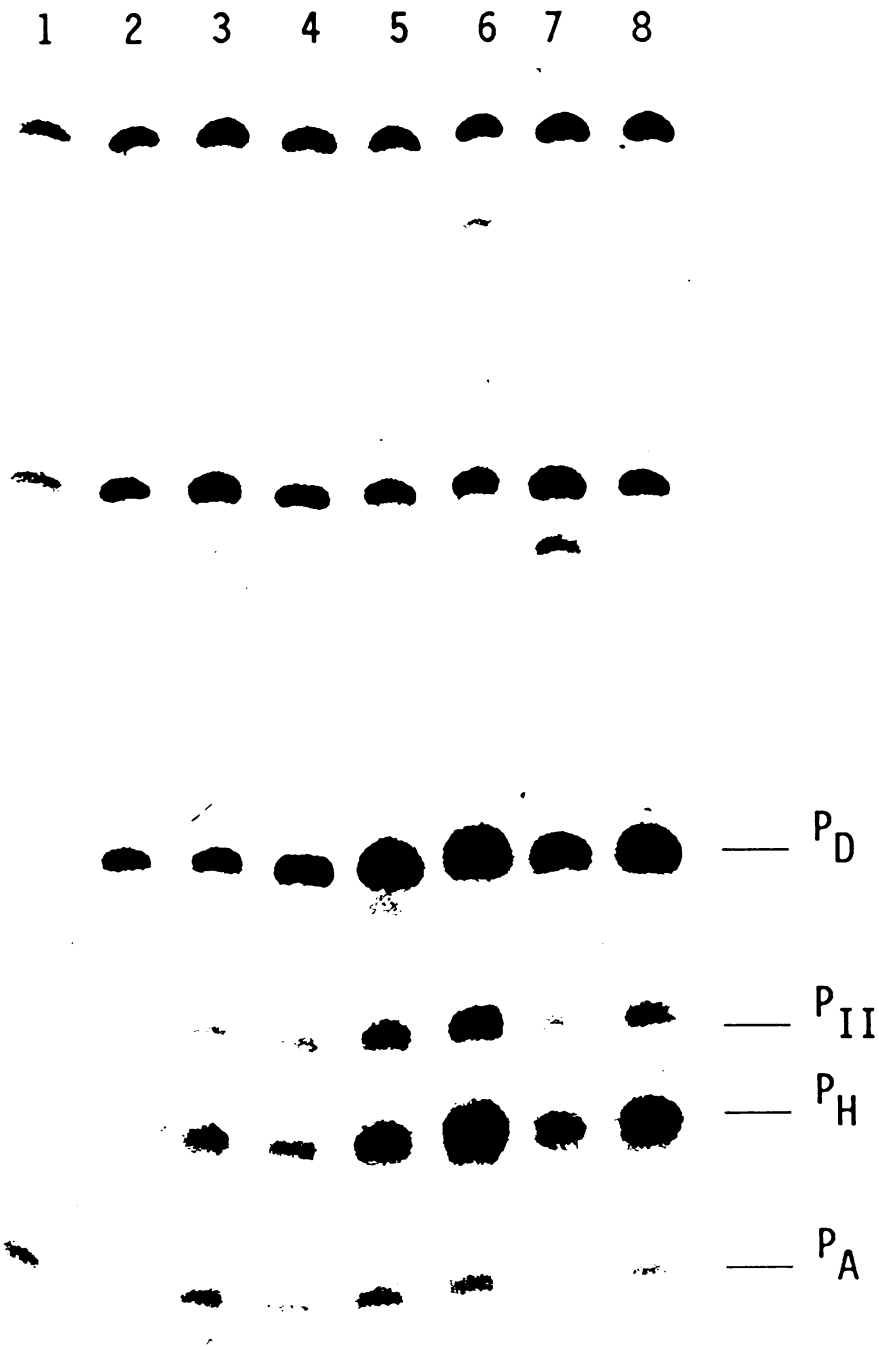
transduced to change gene expression will be discussed.

RESULTS

Effects of O_2 concentration on B. japonicum gene expression. To study the role of oxygen in regulating nif and gln gene expression for B. japonicum, we have examined total cellular RNA isolated from B. japonicum cultures grown in rich media under a variety of oxygen concentrations. Results for one such set of experiments are shown in Figure 1. The activities of the nifH, nifDK, glnII, and glnA genes were monitored using a quantitative S1 protection assay system. Equal quantities of four single-stranded, 5' end-labelled DNA probes specific to the nifH, nifDK, glnII, and glnA promoters were mixed and hybridized to total cellular RNA under conditions where the probes were in excess to the respective RNAs (Experimental Procedures). After hybridization, the mixtures were treated with S1 nuclease and the protected fragments separated by gel electrophoresis. Hybridization probes were designed such that the protected fragments would be well resolved on the gel. This allows the simultaneous measurement of transcript abundance for all four genes using a single RNA sample. The radioactive content for each band on the gel was determined directly (Experimental Procedures).

When B. japonicum is grown in a standard rich medium (YEMN) which is being continuously sparged with air, no transcript for the nifH, nifDK, or glnII genes is observed (Fig. 20, Lane 1; Tab. 3). The glnA gene, in contrast, is transcriptionally active. As reported previously (Carlson et al., 1985), we have observed only small variations between

Figure 20. Abundance of nifH, nifDK, glnII, and glnA transcripts in B. japonicum grown under a variety of atmospheric oxygen concentrations (with a gas balance of N_2). Quantitative analyses were by the S1 protection method described in the Experimental Procedures. The migration positions of the four protected fragments are indicated by P_D, P_{II}, P_H, and P_A. The other major radioactive products observed represent undigested probe DNA. The total cellular RNAs (2 ug RNA in each experiment) used in the hybridization reactions were isolate from B. japonicum cultured under the following conditions: 20% O_2 in YEMN (lane 1); 0.2% O_2 in YEMNA (lane 2); 5% O_2 in YEMN (lane 3); 2% O_2 in YEMN (lane 4); 1% O_2 in YEMN (lane 5); 0.4% O_2 in YEMN (lane 6); 0.2% O_2 in YEMN (lane7); and 0.1% O_2 in YEMN (lane 8). All media and growth conditions are described in the Experimental Procedures.


1 2 3 4 5 6 7 8
P_D
P_{II}
P_H
P_A

any two cultures in glnA transcript levels. This property of glnA makes it a good control for the integrity of an RNA preparation. If B. japonicum is cultured in this same medium but continuously sparged with a gas mixture of nitrogen and oxygen such that oxygen availability limits growth (5% O_2 or less in these experiments), then transcription of all four gene units is observed. (Fig. 20, Table 3). Similar cultures have been grown either with (YEMN) or without (YEM) added KNO_3. Under microaerobic conditions, KNO_3 helps to stimulate growth by serving as an alternative electron acceptor to oxygen (Daniel et al, 1982; M. L. Guerinot and B.K. Chelm, unpublished). Very little difference in the relative levels of transcript for any of the genes monitored was observed under any of the conditions tested (Table 3). However, a 3- to 4-fold decrease in mRNA abundance for nifH, nifDK, and glnII was observed as O_2 levels were increased above 1% in YEMN medium or above 0.1% in YEM medium. Despite the high levels of the two nif mRNAs observed in these cultures, no nitrogen fixation activity, as measured by the acetylene reduction assay (Hardy et al., 1968), was detectable.

Effect of ammonia on microaerobic growth and gene expression.

Under aerobic growth conditions, glnII transcription is regulated by a mechanism that senses the relative availabilities of carbon and nitrogen (Carlson, 1986). When growth is limited by nitrogen supply, glnII transcription is activated. Presumably this activation is an attempt by the cells to maximize their capacity to assimilate nitrogen. Conversely, carbon limited growth leads to a repression of glnII transcription since increased nitrogen assimilation would not increase growth. This control is similar to the Ntr control system which

Table 3. Effects of Varying O_2 Concentrations on Transcription of *nif* and *gln* Genes.

		oxygen concentration							
Medium[a]	Probe	0.1	0.2	0.4	1.0	2.0	5.0	10.0	20.0
YEM	*nifD*	380[b]	56	36	78	-	-	-	-
	nifH	295	22	20	23	-	-	-	-
	glnII	101	27	9	45	-	-	-	-
	glnA	44	12	11	18	-	-	-	-
YEMN	*nifD*	161	175	231	211	110	59	0[c]	0
	nifH	133	72	157	151	59	75	0	0
	glnII	51	43	72	63	24	25	0	0
	glnA	34	23	34	37	29	35	31	18
YEMNA	*nifD*	-	62	-	-	-	-	-	-
	nifH	-	42	-	-	-	-	-	-
	glnII	-	11	-	-	-	-	-	-
	glnA	-	21	-	-	-	-	-	-

[a] media and growth conditions were as described in Experimental Procedures with carbon and nitrogen sources abbreviated as follows: YE, yeast extract; M, mannitol; N, nitrate; A, Ammonia.

[b] cpm of hybridization probe protected from S1 nuclease digestion per ug of input total cellular RNA. This indicates the relative steady state mRNA level for each specific gene. Data represent a mean value determined from two separate experiments.

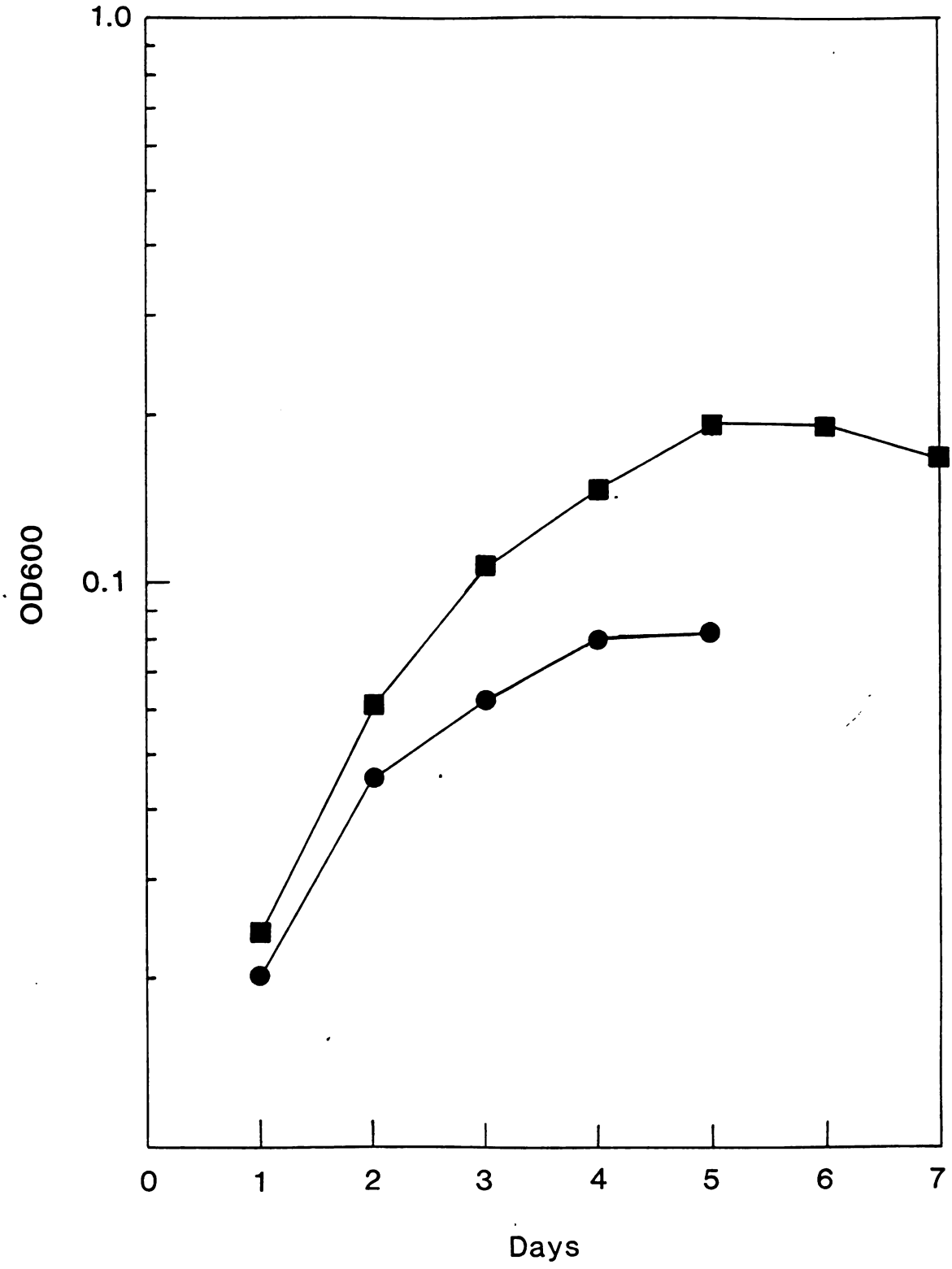
[c] 0 implies that no transcript was detected

operates in the enteric bacteria (Magasanik, 1982). For enteric diazotrophs, Ntr regulation is also involved in nif gene induction (Dixon, 1984). Some Ntr-like control of nitrogenase expression has also been described for bradyrhizobia (Ludwig, 1980; Bergerson and Turner, 1978).

For B. japonicum, ammonia is an especially good nitrogen source, being capable of supporting the highest rates of growth. Under most aerobic culture conditions, addition of ammonia to the medium leads to a repression of glnII transcription (Carlson, 1986). To determine whether the low oxygen induction of glnII and nif gene transcription is under Ntr-like control, B. japonicum was grown under microaerobic conditions in YEMN medium with 10 mM NH_4Cl. Comparison of growth rate and total growth for this culture with a second culture grown under precisely the same conditions except without added ammonia indicates that in this microaerobic culture medium, growth can be stimulated by addition of nitrogen in the form of ammonia (Fig. 21). This demonstrates that the microaerobic cultures are nitrogen limited. Microaerobic induction of glnII as well as nifH and nifDK could therefore be due to an Ntr-like response similar to aerobic glnII induction. When total cellular RNA was isolated from these cultures and assayed for glnII, nifH, nifDK and glnA expression, transcript levels for all but glnA were significantly reduced in cultures with ammonia (Table 3) supporting the conclusion that microaerobic induction of these genes may be under some level of Ntr control.

Growth phase dependence of microaerobic nif and gln transcription. Previous studies on the role of microaerobiosis in nonsymbiotic bradyrhizobial nif gene induction have relied upon the expression of

Figure 21. Growth properties of B. japonicum cultured under 0.2% oxygen (balance N_2) with and without ammonia. The data represent optical density measured at 600 nm.


1.0
0.1
OD600
0
1
2
3
4
5
6
7
Days

nitrogenase enzyme activity. These studies indicate that the presence of nitrogenase activity in free-living cultures of bradyrhizobia usually requires that the cells have stopped growing (Ludwig, 1984). For this reason, the cultures used for the experiments described above were grown for two days into stationary phase prior to harvesting (with the notable exceptions of the 1, 2, and 5% O_2 cultures which were harvested earlier; see Experimental Procedures). To determine whether the microaerobic transcriptional induction of glnII and nif genes has a growth phase requirement like that for nitrogenase activity, cells were harvested at different times during microaerobic growth and total cellular RNA prepared. As shown in Table 4, nifH, nifDK, and glnII transcripts are all observed within 24 h of inoculation. There is no discernible pattern to the variation in transcription levels observed throughout the growth curve for any of the genes monitored.

Transcription of nif and gln genes during symbiotic development. In order to evaluate the role of bacteroid development in nif and gln transcription, RNA was isolated from the bacteria within B. japonicum induced soybean root nodules. Two weeks after sowing and inoculation, transcript levels for both nif operons are about 2 to 4-fold higher in the nodule bacteria population than under any of the microaerobic growth conditions tested (Table 5). Transcript abundances for the nifH and nifDK genes are approximately equal under these conditions. In contrast, bacterial RNA from four week old soybean root nodules has an increased ratio of nifH to nifDK message with nifH transcript about twice as plentiful as nifDK. This is accomplished mainly through an increase in the steady-state mRNA levels for nifH. Whether these

Table 4. *nif* and *gln* Gene Transcription During Growth at 0.2% Oxygen.

	hours after culture inoculation[a]				
Probe	24	48	72	96	120
nifD	93[b]	48	80	83	63
nifH	101	43	118	78	36
glnII	42	23	39	35	27
glnA	67	12	23	14	14

[a] see figure 2 for growth curve.

[b] cpm of hybridization probe protected from S1 nuclease digestion per ug of input total cellular RNA. Data represent mean values obtained in duplicate experiments.

Table 5. mRNA Abundance of *nif* and *gln* Genes During Symbiotic Development.

RNA Source	mRNA Abundance[a]			
	nifH	*nifDK*	*glnII*	*glnA*
2 week old nodule bacteria	498	243	63	29
4 week old nodule bacteria	636	205	66	15
"Nodule Bacteria"[b]	579	181	34	23
"Transforming Bacteria"[b]	767	166	34	25
"Bacteroids"[b]	442	171	19	31

[a] cpm of hybridization probe protected from S1 nuclease digestion per ug of input total cellular RNA. Data represent mean values determined from two identical experiments.

[b] "developmental" cell types isolated from the total population of bacterial cells within a soybean root nodule as described in Experimental Procedures.

differences represent changes in transcription rate or changes in mRNA stability has not yet been determined. glnII transcript levels in these same nodule bacterial cell populations are approximately equal to those seen in microaerobic culture.

A method for fractionating the mixed populations of bacteria isolated from within soybean root nodules has been described (Ching et al., 1977). This technique relies on the change in buoyant density which occurs for bacteroids due to the accumulation of polyhydroxybutyrate granuoles. The expression of certain other "bacteroid-specific" properties (including nitrogenase enzyme activity) parallel this PHB accumulation (Ching et al., 1977). RNA was isolated from the three bacterial populations distinguished by this method (Experimental procedures). No significant differences in nifH, nifDK or glnII gene expression patterns were observed (Table 5).

DISCUSSION

Under specialized environmental conditions, several bradyrhizobial strains, including Bradyrhizobium japonicum USDA 110, are able to reduce dinitrogen to ammonia in free-living culture (Keister, 1975; Pagan et al.., 1975; Kurz and Larue, 1975; McComb et al., 1975; Tjepkema and Evans, 1975). In this paper we have demonstrated that the requirements for transcriptional induction of the genes encoding the nitrogenase structural polypeptides are not as stringent as for achieving enzyme activity. Optimal conditions for asymbiotic nitrogen fixation include requirements for organic acids such as succinate or malate as carbon and

energy sources; the presence of a growth promoting nitrogen source; and most importantly, extreme microaerobiosis. Microaerobic conditions are required for nitrogen fixation because the nitrogenase enzyme is inactivated by intracellular oxygen. Some oxygen is required for growth however, since rhizobia are obligate aerobes. The highest free-living nitrogen fixation rates by bradyrhizobia are achieved at dissolved oxygen concentrations of about 1 uM or less (Ludwig, 1984). Little or no nitrogenase activity is observed above 4 uM oxygen (Rao et al., 1978). For B. japonicum, transcription of the nifH and nifDK genes is observed in cultures which are being continuously sparged with gas mixtures containing as high as 5% oxygen (2 mM, balance N_2) at a rate of 30 liters/hr. The transcript levels for nifH and nifDK were only slightly different in cultures grown under these intermediate O_2 levels than in similar cultures grown with a 0.1% O_2/99.9% N_2 atmosphere. No expression was observed when cultures were sparged with air at the same rate. Nitrogen fixation activity was never observed with any of the media or aeration conditions used.

Induction of nif gene transcription takes place less than 24 hours after inoculation of the microaerobic culture medium described. This contrasts with a four day microaerobic incubation requirement for expression of translational fusions between the nifH or nifD genes from B. japonicum and the E. coli lacZ gene (Alvarez-Morales et al., 1986). Some differences exist between the cultural conditions described by Alvarez-Morales et al. (1986) and those described here. This might account for the large time lag necessary for expression of the translational fusions described. Alternatively, the difference in the times required for transcriptional or translational activity of nifH and

nifDK might result from a translational control mechanism for nitrogenase expression in B. japonicum. No translational control of nitrogen fixation processes has ever been reported, however, post-translational regulation of nitrogenase activity has been described in the Rhodospirillaceae (Ludden and Burris, 1979). A related regulatory mechanism in B. japonicum would not resolve the conflict between our results and those of Alvarez-Morales et al. (1986).

In addition to inducing nif gene transcription, the low O_2 conditions described here result in the accumulation of high levels of glnII messenger RNA, the gene encoding the nitrogen assimilatory enzyme glutamine synthetase II. As with the nif genes, glnII was not transcribed in the same medium under fully aerobic conditions. Thus expression of glnII appears to be activated coordinately with nifH and nifDK in response to low oxygen. Previous studies by Rao et al. (1978) indicate that glutamine synthetase II activity is decreased during microaerobiosis for some bradyrhizobia. This inhibition of glutamine synthetase II activity occurs at the same oxygen tension that nitrogenase activity first becomes apparent (about 0.4 % O_2 in the experiments described; Rao et al., 1978). Since no nitrogenase activity was observed in the experiments we describe, it is possible that at lower oxygen concentrations glnII transcription would have been repressed. However, we observed similar levels of glnII transcript in total cellular RNA populations isolated from the bacteria in soybean root nodules and bacteria grown in microaerobic non-nitrogen fixing culture. We therefore have no explanation for the discrepancy between our results and those of Rao et al. (1978).

The observation that transcription of the gene encoding the nitrogen assimilatory enzyme glutamine synthetase II is activated coordinately with nif gene expression both microaerobically and in bacteroids is surprising. Other workers have proposed that bradyrhizobial nitrogen-fixation is uncoupled from nitrogen assimilation (O'Gara and Shanmugan, 1976; Bergerson and Turner, 1967). The large majority of the ammonia produced by bradyrhizobial nitrogenase is exported (Bergerson and Turner, 1967; O'Gara and Shanmugan, 1976; Bergerson and Turner, 1978; Ludwig, 1980). Why then is glnII transcribed at high levels in cultures which express nif? Ludwig (1984) proposed that asymbiotic nitrogen-fixing cultures of Bradyrhizobium sp. 32HI contain mixed populations of cell types that grow cooperatively. One population terminally differentiates into nonviable nitrogen fixing cells while the other grows utilizing the ammonia produced from nitrogen fixation by the first population. It is possible that the glnII transcription observed here occurs in a different cell subpopulation than does nif expression. If this were the case, one might expect some change in relative transcript levels for glnII, nifH and nifDK during the growth curve since the population of nonviable cells increases as the culture enters stationary phase (Ludwig, 1984). No such change was observed (Tab. 4). In addition, glnII transcript levels were the same in all three of the "developmental" cell types isolated from nodules leaving us to conclude that either the export of ammonia does not require complete GS repression or that GSII is repressed by some as yet unknown post-transcriptional mechanism.

It has been noted previously that the promoter for the glnII gene shares sequence homology in the -10 to -25 region with several nif

promoters in B. japonicum . This region is also related to promoters from other bacteria that are controlled by nitrogen availability (Ntr control; Magasanik, 1982). In aerobic culture, transcriptional activity of glnII is mediated by an Ntr-like system (Carlson, 1986; Adams et al., In Press). When B. japonicum is grown aerobically under nitrogen limiting conditions, transcription of the glnII gene is induced and when nitrogen nutrition does not limit growth, glnII is not transcribed (Carlson, 1986). The relationship between oxygen availability and nitrogen limitation in bradyrhizobia is complex. Addition of ammonia to microaerobic cultures of B. japonicum in YEMN media increases both growth rate and final cell density. Similar changes in growth properties can be achieved by increasing atmospheric oxygen levels indicating that the amino acids present in yeast extract do not limit aerobic growth. These data imply that growth limitation during microaerobiosis results from nitrogen limitation brought about through changes in the cells ability to utilize amino acids as a source of nitrogen. Oxygen limitation has been shown to induce an ammonia export system in Bradyrhizobium sp. 32HI(Ludwig, 1980; Gober and Kashkett, 1983). If such a system were induced here, nitrogen limitation could occur even if amino acids were catabolized normally since active ammonia excretion would prevent its incorporation. Addition of ammonia to these cultures might then partially relieve nitrogen limitation and stimulate growth by shifting the intracellular/extracellular ammonia equilibrium concentrations. We find that transcript levels for nif genes and for glnII are significantly reduced by addition of ammonia, indicating that nitrogen limitation might be at least partially responsible for causing an Ntr-like induction of glnII and/or nif genes. Similar results have

been observed by others in studying ammonia effects on nitrogenase activity (Ludwig, 1980; Bergerson and Turner, 1978).

Transcript levels for the nifH and nifDK genes differ quantitatively between microaerobically grown cells and bacteria isolated from soybean root nodules. Steady-state mRNA levels for the nifDK operon are approximately 2 to 3-fold higher during symbiotic nitrogen fixation than in microaerobic culture. For nifH this increase in message level is closer to 5-fold. The differences in transcript levels observed might result from either changes in transcription rates or varied rates of mRNA stability under the different conditions. Recently we described a B. japonicum gene (odcA) that is required for transcription of nifH, nifDK and glnII both microaerobically and during symbiosis (Chapter 6). Mutations in this gene have severe effects on symbiotic development for both the plant and the bacterium even at very early times after nodule initiation indicating that the gene is required long before the onset of nitrogen fixation. Since nodules induced by the odcA mutant strain fail to develop normally, it is impossible to tell whether this gene is responsible for nif transcription late in nodule development. It is possible that a second regulatory gene is required to give the increased transcript levels observed during symbiotic nitrogen fixation.

EXPERIMENTAL PROCEDURES

Bacterial strains, media, and growth conditions. All of the experiments in this paper used a small-colony derivative of B. japonicum

3I1b110 isolated described (Kuykendall and Elkan, 1976; Meyer and Pueppke, 1980). Bacteria were grown in YEM (0.04% yeast extract, 1% mannitol, 3 mM K_2HPO_4, 0.8 mM $MgSO_4$, 1.1 mM NaCl), YEMN (YEM with 10mM KNO_3), or YEMNA (YEMN with 10 mM NH_4Cl) media. 10 liter cultures of B. japonicum were grown using Microferm Fermenters (New Brunswick Scientific, Edison, New Jersey) agitated at a rate of 200 rpm and sparged with gas mixtures of oxygen and nitrogen at rates of 500 ml/minute. Gas flow rates were controlled using thermal mass flowmeters (Brooks Instruments, Hatfield Pennsylvania, Model 5850 C) to give the atmospheric oxygen concentrations reported.

Isolation of bacteria from soybean root nodules. Total bacterial populations from frozen soybean nodules were prepared as described previously (Chapter 2, Adams and Chelm, 1984). Separation of these bacteria into the three developmental fractions discussed was by centrifugation through a discontinuous sucrose gradient using a zonal ultracentrifuge rotor (Beckman 14 Ti, Beckman Instruments, Fullerton, California) as described (Carlson et al., 1985).

Nucleic acid Techniques. RNA isolations and S1 nuclease protection experiments were as described previously (Chapters 2 and 6; Adams and Chelm, 1984). Probes for the quantitative S1 protection experiments described were synthesized by primer extension (Chapter 6; Carlson, 1986). The partially protected fragments from S1 nuclease protection experiments with these probes are 120 bp (glnA), 150 bp (nifH), 170 bp (glnII), and 200 bp (nifDK).

CHAPTER 6

Characterization of a Bacterial Gene Required for the Normal Differentiation of the Bradyrhizobium japonicum / Soybean Symbiotic Interaction

INTRODUCTION

Rhizobia and bradyrhizobia can associate with leguminous plants to establish a symbiotic relationship in which photosynthetic products made by the plant provide the energy required for bacterial nitrogen fixation and, in exchange, the bacteria provide ammonia to the plant. The successful outcome of this interaction requires a series of developmental steps for both the bacterium and the host plant, and results in the development of root nodules, specialized organs in which both plant and bacterial cells are highly differentiated. This coordinate differentiation requires extensive regulation of gene expression, the best characterized examples of which are the induction of bacterial genes involved in nodule initiation (nod) and nitrogen fixation (nif) (Mulligan and Long, 1985; Corbin et al., 1982; Paau and Brill, 1983) and the activation of plant genes involved in respiratory control (e.g. leghemoglobin) and nitrogen assimilation (e.g. glutamine

synthetase) (Verma and Bal, 1976; Lara et al., 1983). The symbiotic mechanisms controlling this differentiation are not understood, although some bacterial mutants which are affected in this complex process have been identified (Vincent, 1978; Noel et al., 1982; Chua et al., 1985; Stanley et al., 1986; Regensburger et al., 1986). One factor that has been experimentally implicated in the control of differentiation is oxygen limitation. Low oxygen levels presumably exist during the early stages of nodule development due to multiple diffusion barriers and the active respiration by both the plant and the bacteria. Oxygen limitation has been shown to cause a small but detectable induction of uricase, an enzyme which is normally nodule-specific, in non-nodule soybean tissue (Larsen and Jochinsen, 1986). In addition, nif gene expression can be induced in a number of bradyrhizobial strains by growth under oxygen limiting conditions (Keister, 1975; Scott et al., 1979). The importance of this oxygen-mediated gene regulation to symbiotic differentiation has not, however, been directly tested.

Efforts to understand the symbiotic coordination of gene expression have initially focused on the analysis of bradyrhizobial genes whose expression is induced during nodule development. The transcriptional initiation sites for several nif operons from Bradyrhizobium japonicum, the soybean symbiont, have been determined (Adams and Chelm, 1984; Fuhrmann et al., 1985), and these sequences closely resemble those identified for nif operons in other diazotrophs including Klebsiella pneumoniae and Rhizobium meliloti. In these other organisms a positive regulatory gene, nifA, has been shown to be necessary for the transcriptional activation of several nif operons (Dixon et al., 1980; Szeto et al., 1984; Ausubel, 1984). For K. pneumoniae, where the

regulation is best characterized, the expression of nifA is dependent upon the more general nitrogen regulation system (Ntr) through the action of the ntrC gene product (for review see Magasanik, 1982; Dixon, 1984). nifA and ntrC have many structural and functional similarities (Ow and Ausubel, 1983; Buikema et al., 1985; Drummond et al., 1986). In addition, the promoters for genes normally controlled by either nifA or ntrC share a common consensus sequence (Ow et al., 1983; Beynon et al., 1983) and under certain conditions some of these promoters can be activated by the product of either regulatory gene (Ow and Ausubel, 1983; Merrick, 1983; Buck et al., 1985). Physiologically, however, these genes can be distinguished by their role in either the global control of genes involved in nitrogen assimilation (ntrC) or the specific control of genes encoding the nitrogen fixation process (nifA).

The K. pneumoniae nifA gene product is able to activate transcription from the B. japonicum nifH and nifDK promoters when overexpressed in the heterologous E. coli system (Alvarez-Morales and Hennecke, 1985; Alvarez-Morales et al., 1986), supporting the idea that B. japonicum nif gene expression may be modulated via a similar regulatory gene product. I previously reported the identification of two separate regions in the B. japonicum genome which hybridize to the K. pneumoniae nifA gene and suggested that these regions might encode proteins having either ntrC- or nifA-like properties (Adams et al., 1984). Further characterization of these regions has demonstrated that the first of these homologous regions (designated hnal for homology to nifA) was incorrectly identified due to the unexpected presence of nifB sequences in the hybridization probe utilized (Fuhrmann et al.,1985; my unpublished results). Sequence analysis of the second region (hna2)

has confirmed that this region does indeed contain an open reading frame having extensive nifA homology (Chapter 4). Here I report on the identification of additional nifA-homologous regions in B. japonicum DNA and present a detailed phenotypic analysis of one of these. This nifA-like gene has a pleiotropic effect on the symbiotic differentiation of both the plant and bacterium. The defective interaction becomes apparent after the bacteria are released into the host plant cells of the nodule cortex. Further analysis showed that this gene is necessary for the regulation of expression of both the B. japonicum nif genes and the gene encoding glutamine synthetase II, glnII (Carlson and Chelm, 1986). This effect was seen both in association with the plant host and in response to oxygen-limited growth ex planta, supporting the proposed importance of oxygen limitation in the B. japonicum / soybean symbiosis. The wild type allele of this locus limits the final cell density achieved in cultures grown under controlled oxygen limitation, indicating that growth control might also play an important role in the plant-bacterial interaction process. I propose to term this gene odcA to indicate its pleiotropic regulatory role in oxygen and developmental control.

RESULTS

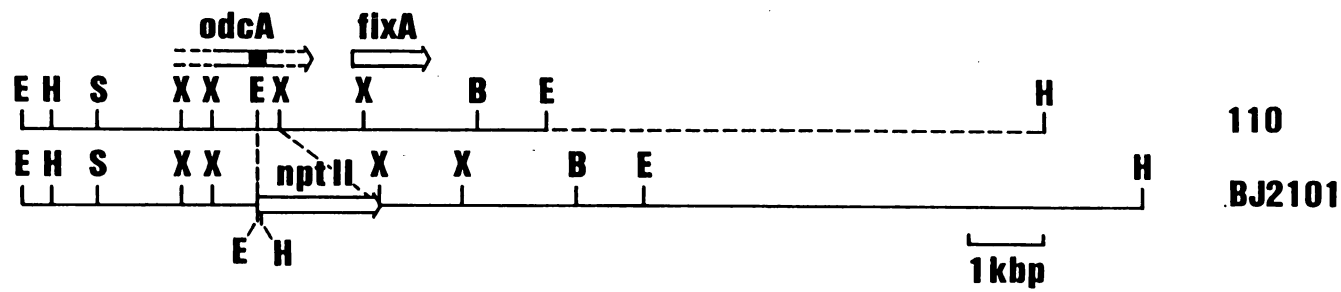
Identification and Mutagenesis of the odcA Gene. B. japonicum contains several different regions with sequence homology to the nifA gene of K. pneumoniae. A 950 bp PstI-EcoRV fragment which is entirely internal to the K. pneumoniae nifA gene (Buikema et al., 1985) was

radioactively labelled and hybridized to Southern transfers of EcoRI digested B. japonicum genomic DNA (see Fig. 14, Chapter 5). Several restriction fragments in addition to the one which was previously identified and tentatively labelled hna2 (11 kbp; Adams et al., 1984) hybridize to the probe. The region which was previously identified as hnal (Adams et al., 1984) does not hybridize in these experiments (Chapter 5, Fig.14, lane 9) confirming that the isolation of hnal was actually due to contamination of the probe used in those experiments with nifB sequences (Fuhrmann et al., 1985) and that the probe utilized here is free of nifB contamination. In order to isolate the additional nifA-homologous regions described here, I screened a cosmid library (Adams et al., 1984) with the probe described above. Several recombinant cosmids which contain nifA-homologous sequences were identified and further characterized into distinct classes based on restriction site patterns. A cosmid from one such class, pRJcos7-36, contains two EcoRI restriction fragments of 3.2 and 3.8 kbp with sequences homologous to nifA (Chapter 5, Fig. 14, lane 8).. A restriction endonuclease map of the region containing these two fragments is illustrated in Figure 22a. The nifA-like gene localized in this region has been termed odcA for the reasons elaborated below.

To verify that the two EcoRI fragments in pRJcos7-36 that have homology to nifA exist as a contiguous stretch of DNA in the bacterial genome, these fragments were individually recloned into plasmid vectors, radioactively labelled and used to probe Southern transfers of various restriction digests of B. japonicum genomic DNA (data not shown). All of the hybridization data is in complete agreement with the restriction map presented in Figure 22a. The approximate position of the region

Figure 22. A) Restriction map of the B. japonicum strain BJ110 odcA genomic region. Also shown is the genomic map for the odcA deletion strain BJ2101 in which a region internal to odcA has been deleted and replaced with the nptII gene. Restriction sites indicated are E, EcoRI; H, HindIII; X, XhoI; S, SalI; B, BamHI. (B) Comparison of the nucleotide and inferred amino acid sequence of regions of the K. pneumoniae nifA gene (Buikema et al., 1985) and the B. japonicum odcA gene. The nifA sequence starts at nucleotide 802 and the odcA sequence originates from the location indicated by the blackened box in (A). Identical amino acids are indicated by the colons between the sequences. (C) Restriction map of the nifDK region of the B. japonicum strain BJ110 genome (Adams et al., 1984). Also shown is the genomic map of the nifDK deletion strain BJ702 in which part of the nifD gene and all of the nifK gene have been deleted and replaced with the nptII gene.

A



B

```
K.p. 802   AACTGCGCGGCGCTGCCGGACAACCTGCTGGAGAGCGAGCTGTTTGGTCATGAGAAAGGCGCGTTTACCGGCGCGGTGCGCCAGCGGAAA
           N  C  A  A  L  P  D  N  L  L  E  S  E  L  F  G  H  E  K  G  A  F  T  G  A  V  R  Q  R  K
           :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :
           N  C  A  A  L  P  E  T  V  L  E  S  E  L  F  G  H  E  K  G  A  F  T  G  A  V  R  R  P  Q
B.j.       AATTGCGCGGCGCTCCCCGAGACGGTCCTGGAATCGGAATTGTTTGGCCATGAGAAAGGAGCCTTTACCGGTGCTGTCAGGCGCCCGCAA

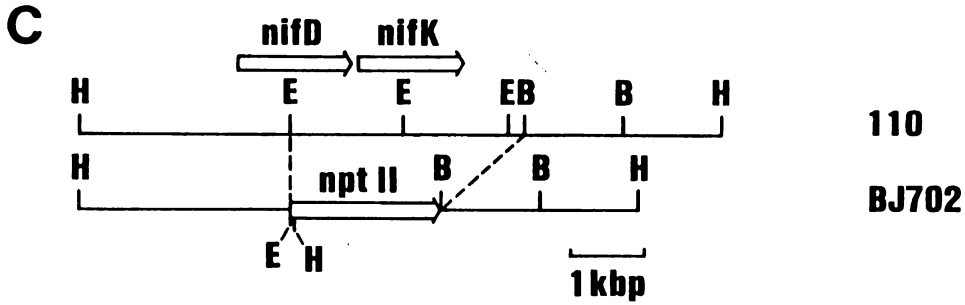
K.p. 892   GGCCGCTTTGAGCTGGCGGACGGCGGCACCTTATTCCTCGATGAGATCGGCGAAAGCAGCGCCTCGTTTCAGGCTAAGCTACTGCGTATT
           G  R  F  E  L  A  D  G  G  T  L  F  L  D  E  I  G  K  S  S  A  S  F  Q  A  K  L  L  R  I
           :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :
           G  R  F  E  L  A  D  K  G  T  L  F  L  D  E  I  G  K  I  S  P  P  F  Q  A  K  L  L  R  V
B.j.       GGGCGCTTCGAGCTTGCTGACAAAGGGACGCTATTTCTTGACGAGATCGGAGAGATCTCACCTCCGTTCCAGGCGAAGTTGCTGCGAGTT

K.p. 982   CTGCAAGAGGGGGAGATGGAGCGCGTCGGCGGCGACGAAACCCTGCGGGTCAACGTGCGCATTATCGCGGCGACCAACCGCCATCTGGAA
           L  Q  E  G  E  M  E  R  V  G  G  D  E  T  L  R  V  N  V  R  I  I  A  A  T  N  R  H  L  E
           :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :
           L  Q  E  Q  E  F  E  R  V  G  S  N  H  T  I  K  V  D  V  R  V  I  A  A  T  N  R  M  L  E
B.j.       CTGCAAGAGCAGGAGTTCGAGCGCGTCGGCAGCAATCACACGATCAAAGTCGATGTTCGGGTGATAGCTGCGACCAACAGGAACCTTGAA

K.p.1072   GAGGAGGTGCGGCTGGGTCATTTCCGCGAGGATCTATACTACCGCCTGAACGTAATGCCTATCGCGCTGCCGCCGCTGCGCGAGCGCCAG
           E  E  V  R  L  G  H  F  H  K  D  L  Y  Y  R  L  N  V  M  P  I  A  L  P  P  L  H  E  R  Q
           :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :
           E  A  V  A  H  S  K  F  H  A  D  L  Y  Y  H  I  S  V  V  P  L  L  L  P  P  L  H  K  W  H
B.j.       GAGGCTGTGGCAAGGAGCGAATTCCGCGCGGACCTCTACTATCGTATTAGCGTAGTTCCCTTGTTGTTGCCGCCGCTTCGCGAAAGACGC

K.p.1162   GAGGATATCGCCGAGCTGGCG   CACTTTCTG
           E  D  I  A  E  L  A  -  H  F  L
           :  :  :  :  :  :  :     :  :
           S  D  I  P  L  L  A  H  E  F  L
B.j.       AGTGATATTCCGCTGCTCGCAAGAGAGTTCCTC
```

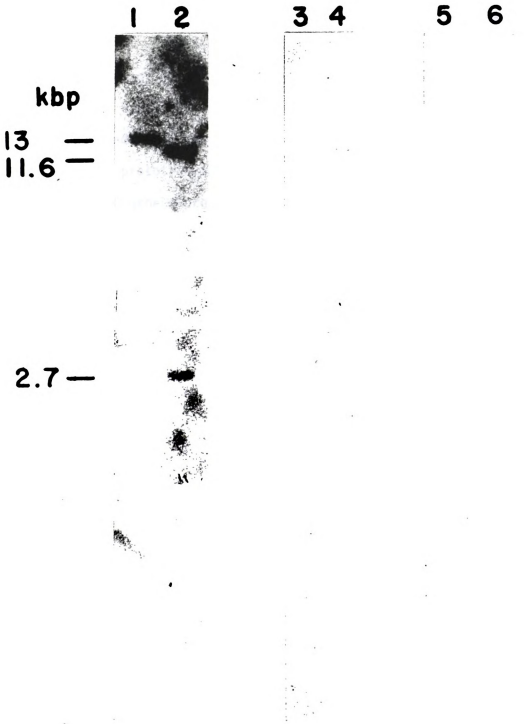
C



homologous to nifA was determined by a combination of Southern hybridization and DNA sequence analyses. The partial DNA sequence and the inferred protein sequence (Fig. 22b) indicate that the observed hybridization is due to an open reading frame containing substantial sequence homology to the K. pneumoniae nifA gene. Examination of the restriction map of the 3.8 kbp EcoRI fragment containing the 3' end of the nifA-homologous region suggested that it may be the same as the region identified by Fuhrmann et al. (1985) as containing a gene termed fixA. We confirmed this localization of fixA by sequence analysis (data not shown) and the approximate location of the fixA gene next to odcA is included on the map we present.

In order to analyze the role of odcA in symbiotic nitrogen fixation a mutant strain was constructed using the techniques described by Guerinot and Chelm (1986). The pBR322 derivative pBJ210, which contains a large deletion in the nifA-homologous open reading frame of the odcA gene (Fig. 22a), was conjugally transferred into wildtype B. japonicum strain BJ110. For purposes of manipulation, the deleted region was replaced by a fragment containing the nptII gene, which confers kanamycin resistance to B. japonicum. B. japonicum strains in which this deletion replaced the wildtype copy of the odcA gene were isolated as described (Guerinot and Chelm, 1986). In order to verify that the correct gene replacement had occurred, total genomic DNA was extracted from several putative recombinants, and Southern transfers of restriction endonuclease digests were hybridized to various DNA probes (Fig. 23). As expected, the 13 kbp wild type HindIII fragment is absent in the mutant strain, and is replaced with the 2.7 and 11.6 kbp fragments predicted for the gene replacement (Fig. 23, lanes 1 and 2).

Figure 23. Genomic hybridization analyses of the B. japonicum strains BJ110 (lanes 1, 3 and 5) and BJ2101 (lanes 2, 4 and 6). All lanes contain HindIII digestions of total cellular DNA. Hybridization probes were the BJ110 region illustrated in Figure 2A (lanes 1 and 2), the nptII-containing plasmid pKC7 (lanes 3 and 4), and pBR322 (lanes 5 and 6).


1 2
3 4
5 6
kbp
13
11.6
2.7

This same 11.6 kbp HindIII fragment hybridizes to the nptII region (Fig. 23, lane 4) but not to pBR322 (Fig. 23, lane 6). This odcA deletion derivative of B. japonicum BJ110 has been labelled strain BJ2101.

odcA Control of Bacterial Growth and Gene Expression. The odcA deletion strain BJ2101 grows as well as the parental strain BJ110 on minimal media under both aerobic and microaerobic conditions, indicating that it remains prototrophic. However, a number of differences between BJ2101 and BJ110 gene expression were observed under the various growth conditions examined. In some cases, BJ2101 gene expression patterns were also compared to a BJ110 Nif^- derivative, BJ702, which contains a deletion spanning the 3' end of the nifD gene and most of the nifK gene as shown in Figure 22c. This deletion leaves the 5' end of the nifDK operon intact, allowing the regulation of its expression to still be monitored. The construction of BJ702 is described in the Experimental Procedures. The results of quantitative analyses of the levels of expression of the nifH, nifDK, and glnII transcription units in cells grown under a variety of conditions are illustrated in Figure 24 and summarized in Table 6. These measurements of mRNA levels were made using S1 protection assays. Equal quantities of three single-stranded, 5' end-labelled probes were mixed and hybridized to total cellular RNA under conditions where the probes were in excess to the respective mRNAs (see Experimental Procedures). Following hybridization, the mixtures were treated with S1 nuclease and the protected fragments were separated by gel electrophoresis. The hybridization probes were designed such that the three protected fragments were well resolved, allowing for the simultaneous measurement of the three transcripts in a single RNA

Figure 24. Abundance of nifH, nifDK and glnII transcripts in B. japonicum from a variety of growth conditions. Quantitative analyses were by an S1 protection method described in the Experimental Procedures. The migration positions of the three probes (lane 1) are indicated as glnII, nifDK and nifH. Ten times as much probe was used in the experimental lanes (lanes 2-19) than in the marker lane (lane 1). The migration positions of the three protected fragments are indicated by P_D (lane 3), P_{II} (lane 2) and P_H (lane 4). The total cellular RNAs utilized in the hybridization reactions were isolated from the following strains and growth conditions: BJ110 in aerobic XG (1 ug, lane 5); BJ110 in aerobic XGN (1 ug, lane 6); BJ2101 in aerobic XG (1 ug, lane 7); BJ2101 in aerobic XGN (1 ug, lane 8); BJ110-induced nodules (0.2 ug, lane 9; 0.4 ug, lane 10); BJ702-induced nodules (0.2 ug, lane 11; 0.4 ug, lane 12); BJ2101-induced nodules (2 ug, lane 13); BJ110 in microaerobic YEMN (1 ug, lane 14; 2 ug, lane 15); BJ702 in microaerobic YEMN (1 ug, lane 16; 2 ug, lane 17); BJ2101 in microaerobic YEMN (4 ug, lane 18); BJ110 in aerobic YEMN (4ug, lane 19). Media are as described in Table 6.

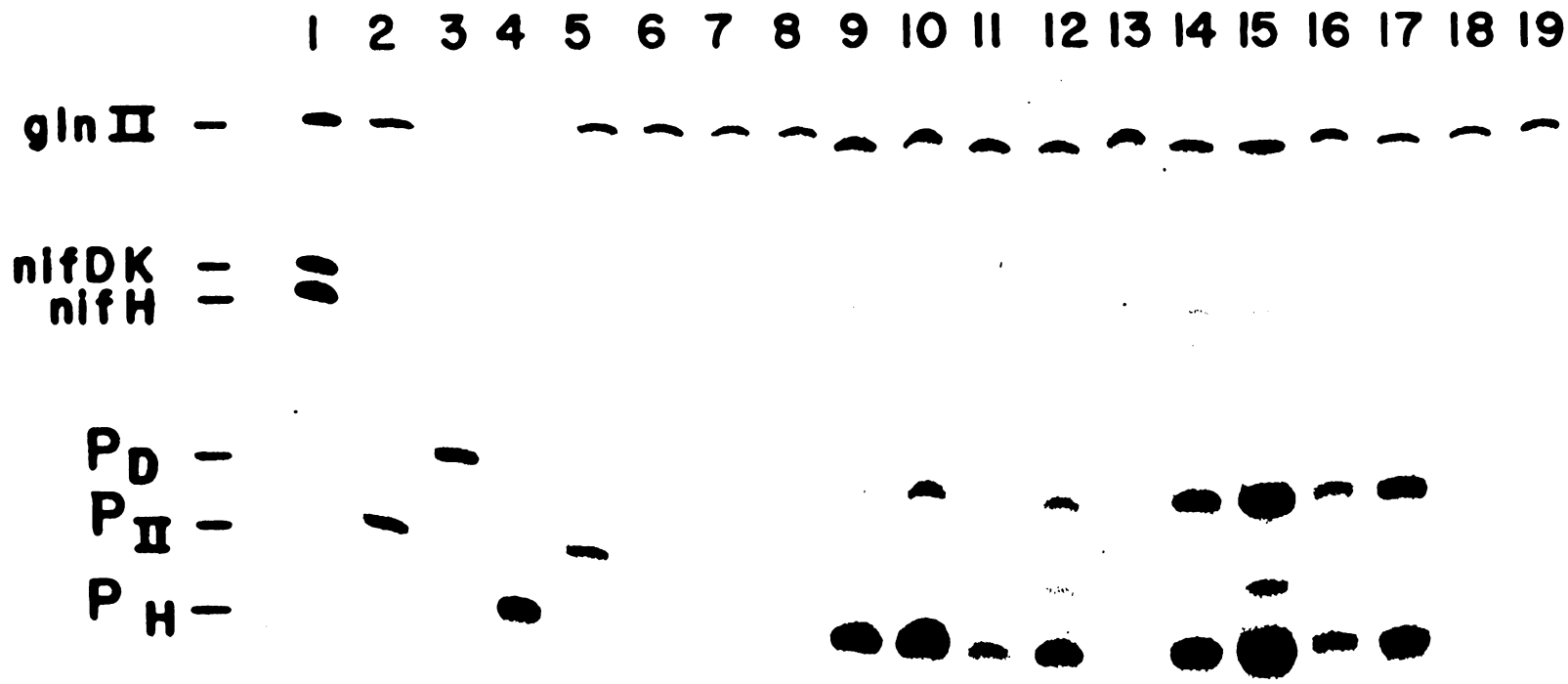

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
glnII
nifDK
nifH
PD
PII
PH

Table 6. Abundance of nifH, nifDK and glnII Transcripts in B. japonicum Grown Under Various Conditions

Strain	Medium[b]	Aeration	mRNA Abundance[a] nifH	nifDK	glnII
BJ110	XG	Air	0[c]	0	100
	XGA	Air	0	0	0
	YEMN	Air	0	0	0
	YEMN	0.1% O_2	300	154	39
BJ2101	XG	Air	0	0	50
	XGA	Air	0	0	0
	YEMN	0.1% O_2	0	0	0
BJ702	YEMN	0.1% O_2	135	92	19

a cpm of hybridization probe protected from S1 nuclease digestion per ug of input total cellular RNA

b media and growth conditions are as described in Experimental Procedures with carbon and nitrogen sources as follows: X, xylose; G, glutamate; A, ammonium; N, nitrate; YE, yeast extract; M, mannitol

c 0 implies that no transcript was detectable

sample. The radioactive content of each protected fragment band reflects the steady state mRNA level for a given gene in the total cellular RNA sample.

When B. japonicum is grown under aerobic conditions, no nitrogen fixation activity is detected and, as expected, no transcription of the two nif operons is observed (Fig. 24 and Table 6). Under these aerobic growth conditions, the gene encoding glutamine synthetase II, glnII, is controlled in response to the quality of the available nitrogen source, being expressed with glutamate, a poorer nitrogen source, but not with ammonia or the mixture of amino acids found in yeast extract, better nitrogen sources. This control is similar to the Ntr control system found in the enterics (Magasanik, 1982). This aerobic Ntr control in B. japonicum is not dependent on the functional odcA gene as indicated by the normal pattern of aerobic glnII expression seen in BJ2101. Similar experiments were performed using a hybridization probe specific for the B. japonicum glnA gene (Carlson et al., 1985), which was reported to be transcribed at a relatively constant level under the conditions used in this study (Carlson et al., 1985). The level of expression of glnA was also constant in the BJ2101 mutant strain (data not shown).

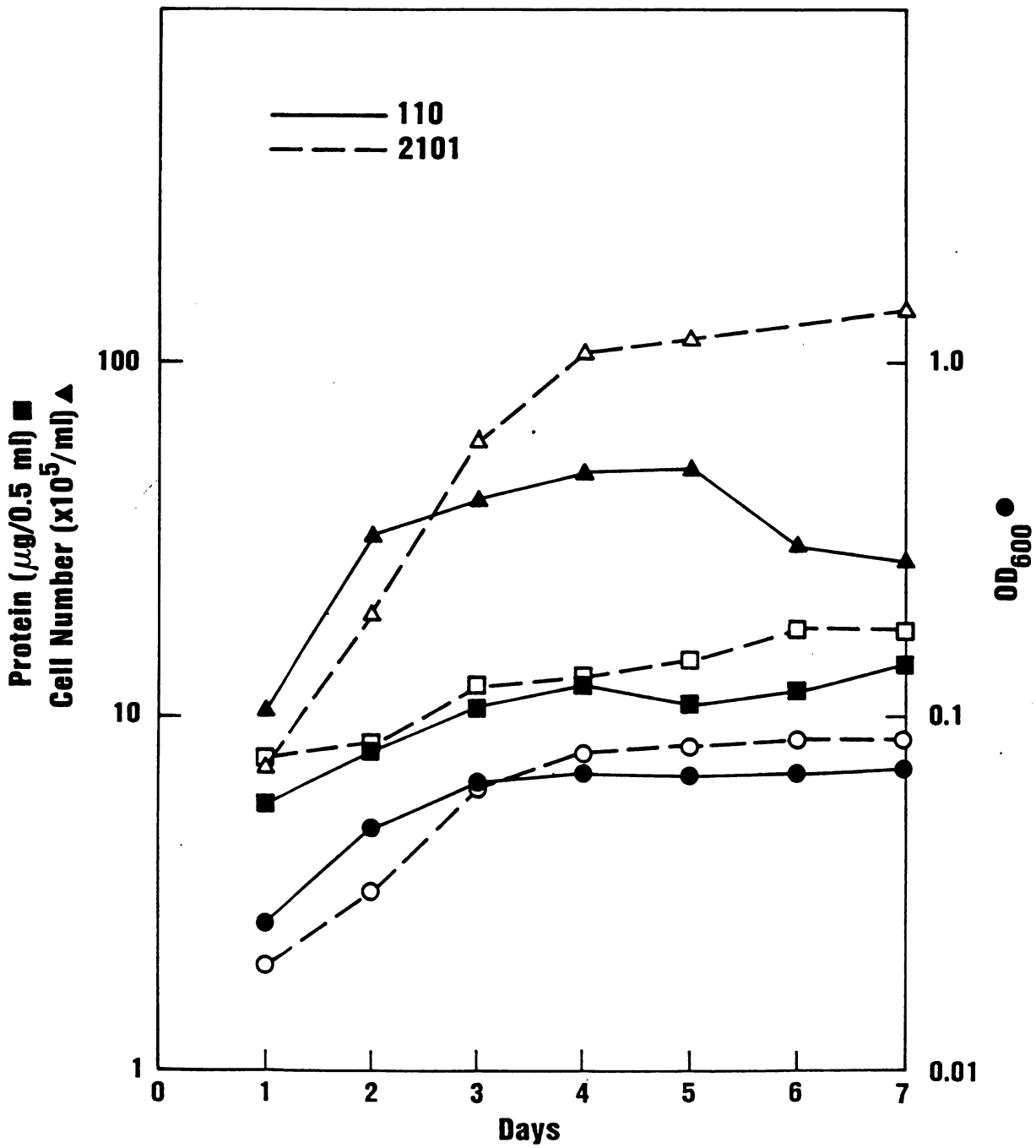
When B. japonicum BJ110 is grown in a standard rich medium with limiting oxygen (0.1 % oxygen in these experiments), transcription of the nifH and nifDK operons is induced (Fig. 24 and Table 6), and this induction is enhanced when 10 mM KNO_3 is included in the media. Nitrate also helps to stimulate growth under these conditions by serving as a terminal electron acceptor for respiratory metabolism (Daniel et al., 1982; M.L. Guerinot and B.K. Chelm, unpublished). Despite the high levels of nifH and nifDK mRNA seen in these cultures, no nitrogen

fixation activity, as measured by the acetylene reduction assay (Hardy et al., 1968), is detectable. In addition to nif gene expression, the glnII gene is transcribed under these conditions. No expression is observed for any of these genes when B. japonicum is grown under well aerated conditions in this same medium, indicating that the expression of these genes requires the microaerobic condition. When the odcA mutant strain BJ2101 was grown under limiting oxygen in this medium, nifH, nifDK or glnII transcripts were not detectable (Fig. 24 and Table 6). These defects are not observed for the nifDK deletion strain BJ702, indicating that these results are not due to a secondary effect of the lack of nitrogenase expression.

The odcA mutant BJ2101 also has modified growth properties under oxygen limiting culture conditions. Data for cultures of the wild type BJ110 and the odcA deletion BJ2101 grown at 0.1% oxygen are illustrated in Figure 25. At low oxygen the odcA mutant grows to similar turbidity and protein concentrations as the wildtype strain. However a large difference is found for the viable cell density, with BJ2101 reaching about a three-fold higher viable cell density than BJ110 under these conditions. These differences indicate that the microaerobically grown BJ2101 cells have a modified control of cell division or viability when compared to wildtype cells grown under oxygen limited conditions. No differences have been detected between BJ2101 and BJ110 in normal aerobic cultures or between BJ702 and BJ110 in either environment.

It is unlikely that the observed regulatory phenotype of the odcA deletion strain BJ2101 is due to polar effects of the nptII insertion used in its construction since the fixA gene and a fixA promoter have been mapped just past odcA (Fig. 22a; Fuhrmann et al., 1985) indicating

Figure 25. Growth properties of B. japonicum strains BJ110 (solid figures) and BJ2101 (open figures) under limiting oxygen conditions. The data represents the optical density at 600 nm (circles), protein concentration (squares), and viable cell density (triangles) determined by measurement of colony forming units.


110
2101
Protein (μg/0.5 ml) ■
Cell Number (x10⁵/ml) ▲
OD600 ●
100
10
1
1.0
0.1
0.01
0
1
2
3
4
5
6
7
Days

that odcA is at the 3' end of its transcription unit. In addition, fixA mutant strains have been reported to have a normal developmental phenotype (Fischer et al., 1986). Therefore, these experiments indicate that odcA encodes a product that functions directly in a positive control system for nif and glnII, and in some manner on low oxygen growth control.

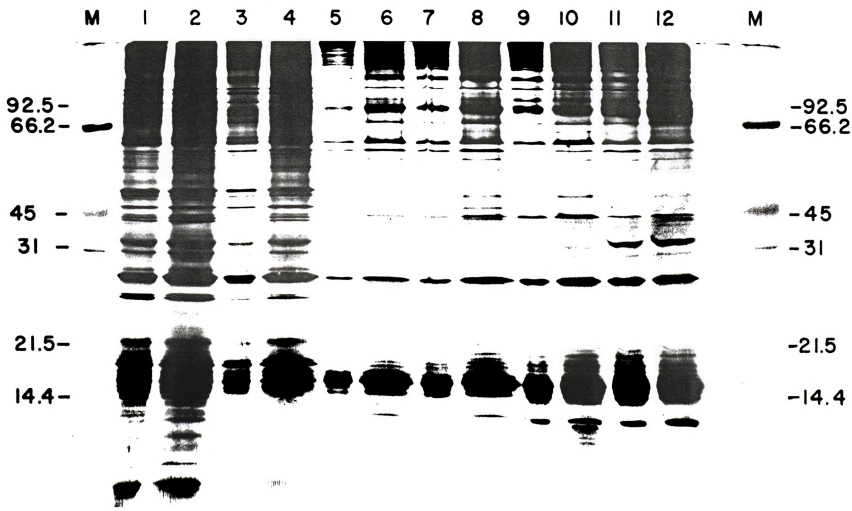
odcA Control of the Plant-Bacterial Interaction. The odcA mutation has a dramatic effect on the interaction between B. japonicum and its normal symbiotic host, the soybean plant. Surface sterilized soybean seeds were inoculated with wildtype BJ110, the odcA mutant BJ2101 or the nifDK mutant BJ702 and grown in modified Leonard jars (Vincent, 1970) with a nitrogen-free nutrient solution. After four weeks of growth the plants inoculated with either of the two mutant strains are small and chlorotic when compared to those inoculated with wildtype B. japonicum, and no nitrogen fixation activity can be detected by the acetylene reduction assay (Hardy et al., 1968) for plants inoculated with either of the mutant bacterial strains. Examination of the nodules formed by infection with BJ2101, BJ702, or the parental strain BJ110 suggests that the effects produced by the deletion of odcA are more pleiotropic than one would expect if this gene simply regulated bacterial nif or gln gene expression. The number of nodules induced by BJ2101 is always at least twice as high as with the wildtype BJ110 or the nifDK$^-$ mutant BJ702. In addition, plants inoculated with a mixture of BJ110 and BJ2101 form many more nodules than plants inoculated with the wildtype strain alone, indicating that the odcA mutant may override the plant's internal regulation of effective nodulation events (Pierce and Bauer, 1983). The

nodules formed following infection with BJ2101 are also visually different than those induced by either wildtype or nifDK$^-$ bacteria, being smaller in size and lacking the normal pink color of the wildtype and nifDK$^-$ induced nodules.

To investigate whether plant gene expression is different in BJ2101-induced nodules than in those induced by BJ110 or BJ702, the plant nodule proteins were examined for each nodule type. The plant protein profiles in nodules induced by BJ110, BJ2101 or BJ702 are similar but not identical when examined by one dimensional denaturing polyacrylaminde gel electrophoresis. The most notable difference observed in the plant proteins from BJ2101-induced nodules is the complete absence of the leghemoglobin protein. This is evident in coomassie brilliant blue stained gels (data not shown) but since this protein does not stain effectively with silver, it is not apparent in Figure 26. Leghemoglobin is the major protein in the BJ702- and BJ110-induced nodules, in agreement with the pink color of these nodules. There are several protein bands which are present in BJ2101 but absent in both the BJ110- and BJ702-incited nodules (Fig. 26). An additional difference between these tissues is that the BJ2101-induced nodule extracts contain less than 10% of the glutamine synthetase activity found in the wildtype BJ110-induced nodule extracts; however since the nifDK$^-$ BJ702-induced nodule extracts contain only 25% of the glutamine synthetase activity found in wildtype nodules, this decrease might not be odcA specific.

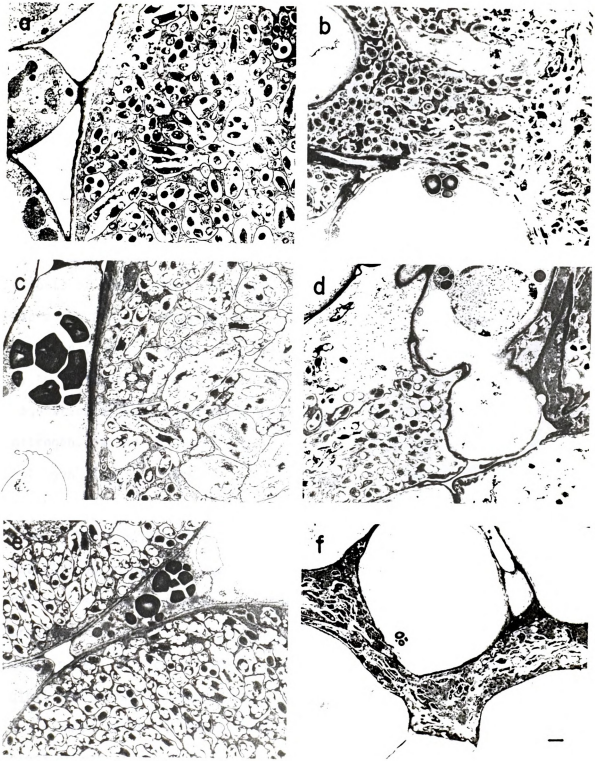
The nodules induced by BJ2101 are also abnormal at the ultrastructural level, as illustrated in representative electron micrographs shown in Figure 27. Two weeks after sowing and inoculation,

Figure 26. SDS-PAGE of plant protein isolated from root nodules incited by B. japonicum strains BJ2101 (lanes 1-4), BJ702 (lanes 5-8), and BJ110 (lanes 9-12). 35 ug (odd numbered lanes) or 70 ug (even numbered lanes) of protein were loaded. Nodules were harvested at 19 days (lanes 1,2,5,6,9,10) or 29 days (lanes 3,4,7,8,11,12) after inoculation and sowing of seeds. Molecular weight markers are shown in lanes M. The heavier staining of the protein from BJ2101-induced nodules is due to the loading of equal total protein and the absence of a major protein, leghemoglobin, in those lanes (not shown).


M
1
2
3
4
5
6
7
8
9
10
11
12
M
92.5-
66.2-
45 -
31 -
21.5-
14.4-
-92.5
-66.2
-45
-31
-21.5
-14.4

the nodules induced by the wildtype BJ110 contain the normal nodule cortex in which a high percentage of the plant cells have been infected and are densely packed with bacteroids contained within peribacteroidal envelopes (Fig. 27a). In contrast, the cortical regions of odcA mutant BJ2101-induced nodules contain few infected plant cells, and the densely packed bacteria found in these infected cells are not surrounded by peribacteroidal envelopes (Fig. 27b). These BJ2101-infected plant cells appear to be undergoing a degradative response, having lost the integrity of their cell walls and organelles and their distinct shape. Four weeks after sowing and inoculation the nodules induced by BJ110 have enlarged, and their ultrastucture continues to appear normal; the only difference from the younger tissue is the increased accumulation of polyhydroxybutyrate granules in the bacteroids (Fig. 27c). After four weeks the BJ2101-induced nodules have not enlarged further, and the infected cells continue to degenerate (Fig. 27d). In addition, the four week old BJ2101-induced nodules contain a large number of bacteria which are not within plant cells but are instead embedded within a darkly staining matrix in intercellular spaces (Fig. 27f). Bacteria in the process of cell division can easily be found within these spaces. The nifDK deletion strain BJ702 serves as a control in these experiments, confirming that the effects of the odcA mutation are not due to the lack of expression of nitrogen fixation. No significant developmental differences are found between nodules induced by wildtype BJ110 or nifDK$^-$ BJ702 (Fig. 27e). The only structural difference detected is the increased accumulation of glycogen granules in the BJ702-induced nodules. This is likely to be a result of the energy excess condition

Figure 27. Typical electron micrographs of nodule tissue incited by B. japonicum strain BJ110 (a and c), BJ2101 (b, d and f) or BJ702 (e). The tissue was harvested either 2 weeks (a, b and e) or 4 weeks (c, d and f) after seed inoculation and sowing. All micrographs were taken at the same magnification with the bar in panel f representing 1 um.


a
b
c
d
e
f

resulting from the lack of energy utilization by the nitrogen fixation process.

Bacterial gene expression is also abnormal in nodules incited by the odcA mutant BJ2101 when compared to the wildtype BJ110 and the nifDK mutant BJ702 (Fig. 24 and Table 7). Bacteria isolated from nodules incited by BJ702 contain the nifH, nifDK and glnII transcripts, although their relative abundances are slightly modified from those seen in nodules incited by BJ110. In contrast, no transcripts from either nifH, nifDK or glnII can be detected in bacteria isolated from nodules incited by the odcA mutant BJ2101. It is interesting to note the quantitative effects of these mutations on the expression of the glnII gene, which is expressed at approximately three-fold higher levels in nodules incited by the nitrogen fixation deficient nifDK mutant than in nodules incited by the wildtype bacteria. This effect may be analogous to the Ntr regulation system observed for B. japonicum ex planta and may indicate a nitrogen starved condition within the nodule resulting from the lack of nitrogen fixation. These results agree with measurements of glutamine synthetase activity in other Nif$^-$ rhizobial strains (Werner et al., 1980). The complete absence of glnII transcription in the BJ2101-induced nodules suggests two alternative explanations. Under oxygen limiting conditions, the Odc system, acting through the odcA product, rather than the Ntr system, controls the expression of the glnII gene. This could also be the case in nodules. Alternatively, glnII expression in nodules may require some aspect of the proper nodule environment which the BJ2101-induced nodules lack due to the earlier block in nodule development.

Table 7. Abundance of *nifH*, *nifDK* and *glnII* Transcripts in *B. japonicum* Isolated From Soybean Nodules

Bacterial Strain	mRNA Abundance[a]		
	nifH	nifDK	glnII
BJ110	1450	276	49
BJ702	468	169	146
BJ2101	0[b]	0	0

[a] as in Table 6

[b] 0 implies that no transcript was detected

DISCUSSION

In this paper we report the isolation, physical description and phenotypic characterization of a regulatory gene from the bacterium Bradyrhizobium japonicum. This gene and the protein it encodes have sequence homology to both the nifA and ntrC genes of Klebsiella pneumoniae, although functionally it is not strictly analogous to either of these genes. The Klebsiella nifA gene product functions only as a positive transcriptional regulator of other nif genes, while the ntrC-encoded protein has a more general role in controlling the expression of gene systems involved in nitrogen assimilation (for review see Magasanik, 1982; Dixon, 1984). Phenotypically, K. pneumoniae mutants in either gene are Fix^-, but only ntrC mutations have additional phenotypes, being unable to utilize a number of nitrogen sources. The B. japonicum gene described in this report is required for the transcription of nitrogen fixation genes, at least one gene necessary for ammonia assimilation, and for some aspect of the control of cell division when cultures are grown under oxygen limiting conditions. This gene is also essential for proper cellular differentiation during the symbiotic association of B. japonicum with its soybean host. Plants which have been inoculated with a B. japonicum strain defective in this gene initiate root nodule development normally, but the interaction becomes abnormal upon release of the bacteria into the host cortical cells. These abnormal nodules lack some nodule-specific plant proteins, such as leghemoglobin, and contain some additional proteins not normally found in nodule tissue.

Recently, mutations in this gene were described by Fischer et al.

(1986), who have labelled it nifA due to its homology with the Klebsiella gene as well as its requirement for nif gene expression. These criteria can also be met by the Klebsiella ntrC gene which has a more general function than nifA. Since our results demonstrate that the B. japonicum gene is also of more general function than K. pneumoniae nifA, we propose to call this new gene odcA in order to indicate its general regulatory role under oxygen limiting conditions and during symbiotic development.

Bacterial mutants have previously been used to study the rhizobial and bradyrhizobial interactions with their host plants. One class of mutants that have been extensively characterized are completely defective in nodulation at the earliest stages in the interaction with the host plant (Kondorosi et al., 1984; Downie et al., 1985; Jacobs et al., 1985). The genes which have been identified in those studies are termed nod genes, and their mechanism of action is currently under investigation (Mulligan and Long, 1985). Other studies, notably those of Noel et al. (1982), Chua et al. (1985) and Regensburger et al. (1986), have identified genetic loci by mutagenesis which are necessary for the proper progession of the later stages of nodule differentiation. This report, however, is the first to correlate a defect in nodule differentiation with a bacterial deficiency in a specific physiological regulatory system.

It was not completely unexpected that the regulation of the bacterial response to oxygen limited growth conditions would be important for nodule function, since the induction of nitrogen fixation had previously been described during oxygen limited growth in some bradyrhizobia (Keister, 1975; Scott et al., 1979). Oxygen limitation

has also been shown to cause a small but detectable induction of uricase, a nodule-specific enzyme, in soybean tissue (Larsen and Jochinsen, 1986). It is obvious that the proper control of oxygen availability is essential to nodule function since molecular oxygen irreversibly inhibits the nitrogenase reaction and yet is essential for the continued oxidative respiration of both the plant and bacteria. This is accepted as the reason for the accumulation of the oxygen binding protein leghemoglobin, which presumably supplies a high flux of molecular oxygen at low partial pressure (for review see Appleby, 1983). However, it was not expected that the elimination of a bacterial low oxygen response system would have the dramatic effect on nodule differentiation described in this report. The results presented here imply a crucial role for the response to a lowered oxygen availability at a stage well before the induction of leghemoglobin accumulation, possibly even before the release of bacteria from the infection thread into the host cells. It is likely that the bacteria are oxygen limited at this stage in the developmental process since they are actively respiring while embedded in the mucilaginous matrix of the infection thread. Ludwig (1984) has presented evidence that, upon a shift to low oxygen conditions, Bradyrhizobium sp. RC3200 can induce the ability to fix nitrogen and coordinately differentiates into nongrowing cells which lose their colony-forming capacity. The limited oxygen growth control phenotype described here for BJ2101 when compared to BJ110 is reminiscent of that reported by Ludwig (1984). This might indicate that the Odc regulatory system we describe is responsible for the differentiation to non-viability described by Ludwig (1984) and thereby is crucial for proper nodule development.

The results presented here give rise to several further questions. What is the mechanism by which odcA controls the expression of B. japonicum genes in response to oxygen limitation? The odcA gene product could act directly as a positive regulator of genes such as nif, glnII, and genes involved in the growth control and developmental phenotypes. Alternatively, the odcA product could act indirectly through the induction of a regulatory cascade involving at least one secondary regulator. The major precedent for a cascade system are the Nif and Ntr systems of the enterics (for review see Magasanik, 1982; Dixon, 1984). In that case the nifA gene product acts directly on the promoters of the genes it activates, and the ntrC product acts indirectly by inducing the expression of the secondary regulator nifA. Since the ntrC, nifA and odcA genes and products share sequence homology (Buikema et al., 1985; Figure 22) both the direct activation and cascade activation mechanisms remain plausible possibilities. Perhaps knowledge of the regulation of the expression of odcA itself will shed light on these alternatives. It has been shown that the B. japonicum nif promoters can be activated by the K. pneumoniae nifA gene product but not by the K. pneumoniae ntrC protein in E. coli (Alvarez-Morales and Hennecke, 1985). However these heterologous system experiments are known to be capable of yielding specificities which do not directly represent those that occur in the homologous system (e.g. Sundaresan et al., 1983; Better et al., 1985). A direct genetic analysis of the possible odcA product / nif or glnII promoter interaction is certainly warranted.

Several aspects of the plant-bacterial symbiotic interaction are perturbed in the odcA mutant strain. In a wildtype interaction, the plant controls the number of effective nodulation events through a

negative feedback response termed autoregulation (Pierce and Bauer, 1983) which apparently occurs after the infection of root hairs at the stage of nodule emergence (Calvert et al., 1984). Both the wildtype strain (BJ110) and a Nif$^-$ derivative (BJ702) seem to elicit such a response and control the number of nodules formed, but the odcA mutant strain (BJ2101) does not. This ability to bypass the internal nodulation control mechanisms even during a mixed infection with BJ110 and BJ2101 indicates that the odcA product is required for the expression of some aspect of recognition which is necessary for this autoregulation. The ultrastructural analyses indicate that during the later stages of infection, BJ2101 is not recognized as a symbiont, but instead appears to be treated more like a pathogen. The infected plant cells exhibit a degradative response which is in some ways reminiscent of the hypersensitive reponses known for many plants during invasion by pathogenic bacteria (for review see Keen and Holliday, 1982). The inability to be recognized as a symbiont could be due either to a lack of expression of some factor(s) or the lack of repression of some free living function(s) which the plant identifies as a characteristic of pathogens. The abnormal oxygen-limited growth control exhibited by the odcA mutant might relate to the latter of these possibilities, with the mutant apparently unable to repress cell division and become non-viable. Along this line, it is interesting to note the identification of a gene which can cause the inhibition of exopolysaccharide production under certain circumstances and which is required for effective nodulation in Rhizobium phaseoli (Borthakur et al., 1985). The odcA gene might be involved in regulating the expression of similar functions during nodulation by B. japonicum. An understanding of the mechanisms through

which odcA mediates plant-bacterial recognition will certainly shed light on the control of this process.

EXPERIMENTAL PROCEDURES

Bacterial Strains. The wildtype B. japonicum strain used in all experiments is a small-colony derivative of B. japonicum USDA 3I1b110 isolated as described (Kuykendall and Elkan, 1976; Meyer and Pueppke, 1980), and is designated here as BJ110. B. japonicum strains BJ2101 and BJ702 are odcA and nifDK deletion derivatives of BJ110 respectively. The Escherichia coli strains ED8654 (galK, galT, trpR, metB, hsdR, supE, supF, lacY) and HB101 (Boyer and Roulland-Dussoix, 1969) were used for routine plasmid construction and maintainance. The B. japonicum genomic DNA cosmid library described previously (Adams et al., 1984) was maintained in the E. coli glutamine auxotroph ET8051 (Δ(rha-glnA), $hutC_k$, rbs, nal_r) which contains a deletion of the entire glnAntrBC operon (Fisher et al., 1981). E. coli JM103 (Messing et al., 1981) was used for manipulations of recombinant M13 phage.

Recombinant Plasmids, Cosmids, and Phage. pRJcos7-36, pRJcos3-44, pRJcos14-28 and pRJcos2-43 are cosmids which were identified in the library described above on the basis of their hybridization to the K. pneumoniae nifA probe. pRJcos1-62 contains the nifH and nifB region of the BJ110 genome (Adams et al., 1984). pBJ150 and pBJ152 are derivatives of pBR328 carrying either the 3.8 or 3.2 kbp nifA-homologous EcoRI fragment from pRJcos7-36 respectively. These plasmids were used

in making the pBR322 derivative pBJ210, which was used for construction of the odcA deletion strain BJ2101 (Fig. 22). The 300 bp EcoRI-XhoI fragment from pBJ150 was deleted and replaced with the 1.6 kbp nptII containing EcoRI-XhoI fragment from pKC7 (Rao and Rogers, 1979). pBJ210 was then constructed by combining the EcoRI inserts from this pBJ150 derivative and from pBJ152 in pBR322. pBJ70 is a pBR322 derivative which was constructed by deleting 3.6 kbp of B. japonicum DNA between the EcoRI and BamHI restriction sites of pRJ676 (Hennecke, 1981) and replacing this with the 2 kbp nptII containing EcoRI-BamHI fragment from pKC7 as shown in figure 22c. The K. pneumoniae nifA internal hybridization probe used in these experiments was isolated from pBJ240 which carries a 947 bp PstI-EcoRV fragment from the K. pneumoniae nifLA plasmid pGR397 (Reidel et al., 1983) in pIC-19R (Marsh et al., 1984).

The following recombinant M13 phage were constructed for use in synthesizing single-stranded DNA hybridization probes for S_1 protection analyses. M13DK contains a 417 bp PstI-SphI restriction fragment from the promoter region of the nifDK operon (Adams and Chelm, 1984) cloned into the polylinker of M13mp19 (Yanisch-Perron et al., 1985). M13H has a 704 bp BamHI-HindIII fragment from nifH and its upstream regions (Adams and Chelm, 1984) cloned into m13mp19. M13II has a 2.1 kbp SalI fragment from the B. japonicum glnII gene (Carlson and Chelm, 1986) carried in M13mp18.

Bacterial Media, Growth Conditions and Strain Construction. B. japonicum was routinely cultured on broth and agar plates in YEX medium (Adams et al., 1984). Derivatives of wildtype strain BJ110 with chromosomal deletions and insertions were constructed by the method

described (Guerinot and Chelm, 1986). Gene-directed mutagenesis was carried out by triparental matings with BJ110, HB101/pRK2013, and either HB101/pBJ70/pDS4101 (nifDK) or HB101/pBJ210/pDS4101 (odcA) by the methods of Guerinot and Chelm (1986) with the one exception that chloramphenicol (25 ug/ml) was used in place of rifampicin for the counterselection against E. coli. Cultures for aerobic gene expression in B. japonicum strains were grown either in formate medium (Manian and O'Gara, 1982) with 0.2% xylose and 10 mM of the designated nitrogen source or on YEMN (0.04% yeast extract, 1% mannitol, 3 mM K_2HPO_4, 0.8 mM $MgSO_4$, 1.1 mM NaCl, 10 mM KNO_3). Oxygen limited cultures of B. japonicum were grown in YEMN medium continuously sparged with 0.1 % O_2 in N_2 at a rate of 500 ml/minute. Gas flow rates were controlled using thermal mass flowmeters (Brooks Instruments, Hatfield Pennsylvania, Model 5850C). E. coli strains were routinely grown on LB medium or M9 minimal salts medium with glucose and thiamine (Miller, 1972).

Plant Tests.

Soybean [Glycine max (L.) Merr. cv. Amsoy 71] seed sterilization, inoculation, and plant growth were all done as previously described (Guerinot and Chelm, 1986) except that the daily light period was 16 hr. Unless otherwise designated, nodules were harvested 28 days after planting. Nitrogen fixation rates were estimated using the acetylene reduction assay (Hardy et al., 1968). No acetylene was reduced to ethylene during a three hour period for strains labelled Fix^-. Following the assay, nodules were immediately frozen in liquid nitrogen and stored at -70°C for later use. Prior to freezing, random nodules

were chosen, surface sterilized (Guerinot and Chelm, 1986) and the bacteria isolated for strain verification.

Nucleic Acids Techniques. Methods for Southern hybridizations, colony hybridization, plasmid and total genomic DNA isolations, and RNA isolations have all been described (Adams and Chelm, 1984; Adams et al., 1984). Single-stranded 5' end-labelled probes for S_1 protection analyses were synthesized using the primer extension method as follows. 80 ng of a gene-specific oligonucleotide was labelled by reaction with T4-polynucleotide kinase at 37°C for 1 hr 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/mmole). In experiments with mixed probes, the specific activity of each 5' end-labelled oligomer was adjusted to approximately 250 cpm/pg with unlabelled oligonucleotide prior to the elongation reaction. The labelled primers were combined with 20 ug of single-stranded M13 recombinant phage, ethanol precipitated, suspended in 50 ul of 10 mM Tris-HCl, pH 8.5 and 10 mM $MgCl_2$, heated for 5 minutes at 90°C, and allowed to hybridize for 1 hr at 37°C. The hybridized oligonucleotide primer was extended using 3 units (Bethesda Research Laboratory) of the large fragment of E. coli DNA polymerase (Klenow fragment) for 1 hr at 37°C in 100 ul of the same buffer with 0.6 mM each of ATP, CTP, GTP, and TTP. The reaction was stopped by heating at 65°C for 5 minutes and the double stranded DNA was ethanol precipitated and resuspended in the appropriate restriction endonuclease buffer. The probes specific for the nifH and nifDK promoters were prepared by digesting the product of the extension reaction with BstNI for 3 hr at 58°C, ethanol

precipitated, and suspended in 200 ul of 80 % formamide with tracking dyes. The glnII probe was prepared by digestion with SalI for 3 hr at 37°C. Extended primers were denatured by incubation at 100°C and purified by electrophoresis on 8.4 M Urea, 4 % polyacrylamide gels. The labelled probe was detected by autoradiography and eluted by the crush and soak method (Maxam and Gilbert, 1980).

Oligonucleotides for primer extension were synthesized and purified as described previously (Carlson and Chelm, 1986). The sequences of each primer were as follows: 5'-TGCTCTCCATCAACCGA (nifH); 5'-CTTCACCCCGCAGTCCG (nifD); and 5'-CGACGCGAATTCCTTGA (glnII). The partially protected fragments from S_1 protection experiments with each of the probes synthesized with these primers are 150 bp, 200 bp, and 170 bp respectively. For mixed probe experiments, approximately 6 fmoles of each 5' end-labelled single-stranded fragment was included in the hybridization. For the driver RNA quantities used in these experiments, this is an excess of probe since increasing RNA concentrations results in proportional increases in the amount of hybrid protection (e.g. Fig. 24). Hybridization, S_1 digestion, and identification of partially protected products were as described (Adams ad Chelm, 1984).

DNA sequence analysis of the odcA gene region utilized the methods of Maxam and Gilbert (1980). The sequence of the region presented was determined from analysis of both DNA strands.

Protein Analysis. Soluble cytoplasmic proteins from root nodules were prepared as described by Legocki and Verma (1980). Approximately 0.2 g of nodule tissue was homogenized in 500 ul of buffer containing 50 mM Tris-HCl, pH 6.8 and 5 % (v/v) B-mercaptoethanol. Bacteria and plant

cell debris were removed by centrifugation at 20,000 x g. Protein concentration was determined by a modification of the Lowry procedure (Markwell et al., 1978). Proteins were separated by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) on 10 to 15 % polyacrylamide gradient gels and were visualized by silver staining (BioRad).

Microscopy.

Nodules were harvested from roots, rinsed in deionized water, sliced with a razor to pieces of approximately 1 mm in the largest dimension and then immediately fixed in 4 % glutaraldehyde in 0.15 M sodium cacadylate, pH 7.2 for 2 hr under vacuum and then at 4^{o}C overnight. Following fixation, the tissue was rinsed three times over a period of 3 hr at room temperature with Buffer A (Maupin and Pollard, 1983) containing 0.2% (w/v) tannic acid and then for 15 min in Buffer A alone. Postfixation was in 1% (w/v) osmium tetroxide in Buffer A for 1 hr at room temperature. The tissue was then dehydrated through a graded ethanol series, cleared in propylene oxide and embedded in VCD/HSXA ultra low viscosity medium (Ladd Research Industries, Burlington, VT). Thin sections were cut on an LKB Ultrotome III with a Dupont diamond knife. Sections were subsequently stained with uranyl acetate and lead citrate and examined in a Philips EM300 electron microscope. For light microscopy, 2-3 um sections were stained with 0.5% toluidine blue.

CHAPTER 7

SUMMARY AND CONCLUSIONS

For many nitrogen-fixing bacteria, the control of the expression of genes for nitrogen fixation and nitrogen assimilation are linked through overlapping regulatory systems (Magasanik, 1982; Dixon, 1984). In this way, the atmospheric dinitrogen reduced via nitrogenase to ammonia is utilized to support growth. For rhizobia and bradyrhizobia, nitrogen fixation and nitrogen assimilation are apparently not so tightly coupled since most of the ammonia produced by nitrogenase is exported from the bacteria (Bergerson and Turner, 1967; O'Gara and Shanmugan, 1976). For at least some rhizobial strains, this failure to assimilate fixed nitrogen is partly due to a decrease in glutamine synthetase activity (Bergerson and Turner, 1967; O'Gara and Shanmugan, 1976; Ludwig, 1980; Brown and Dilworth, 1975) and to the induction of an ammonia transport system (Gober and Kashkett, 1983). These properties must be related to the roles of Rhizobium and Bradyrhizobium in symbiotic nitrogen fixation. In this dissertation, I have described the isolation of three transcription units (nifH, nifDK, and nifB) that are required for nitrogen fixation by Bradyrhizobium japonicum strain USDA 110 (BJ110). I have examined the physiological and developmental parameters required for transcription of these genes. In addition, I have characterized the transcriptional regulation of B. japonicum glutamine synthetase genes

(glnA and glnII) under conditions that induce nif gene transcription. I found that in contrast to enzyme activity data reported previously, transcription of the gene encoding GSII, glnII, is induced coordinately with nif gene transcription. Finally, I have described four separate B. japonicum genes that have sequence homology to both the nifA and ntrC genes of K. pneumoniae. One of these genes (odcA) is required for both microaerobic and symbiotic induction of B. japonicum nif and glnII gene expression. This gene, however, is not required for the aerobic induction of glnII in response to nitrogen limitation.

Much of what is known about the regulation of nitrogen fixation and nitrogen assimilation genes in B. japonicum comes from comparison to the well characterized Ntr and Nif regulatory systems of K. pneumoniae. The transcription of Klebsella nif genes is controlled by each of these two distinct regulatory systems. This links the expression of nitrogen fixation activity to nitrogen assimilation through the general nitrogen control pathway (Ntr) known for several enteric bacteria (Magasanik, 1982). The addition of a second regulatory mechanism that is specific for nif gene control prevents the expression of the oxygen labile nitrogenase under aerobic conditions. The current model for regulation of K. pneumoniae nif genes involves the concerted action of the ntrA, ntrB, and ntrC gene products (NTRA, NTRB, and NTRC) on the transcription of the nifLA operon (Dixon, 1984). The nifL and nifA products (NIFL and NIFA) in turn control expression from other nif operons. Thus, in nitrogen limited culture, NTRC works in conjunction with NTRA, a putative sigma factor for recognition of Ntr-activated promoters (Hirschman et al., 1985; Hunt and Magasanik, 1985), to induce transcription from the nifLA promoter. Under these conditions, NTRC and

NTRA are also needed to activate the transcription of genes involved in nitrogen assimilation including glnA (glutamine synthetase), put (proline utilization genes), aut (arginine utilization genes), and hut (histidine utilization genes). Under anaerobic, nitrogen-limited conditions, NIFA then serves to activate transcription from other nif operons by a mechanism that also requires NTRA. The function of NIFL is to repress nif transcription when oxygen is present. Finally, NTRB acts to modulate the activity of NTRC in response to the availability of fixed nitrogen.

The promoter sequences for nif genes in B. japonicum share a high degree of nucleotide sequence homology with those identified for the NIFA/NTRA and NTRC/NTRA regulated promoters in K. pneumoniae (Chapters 2 and 3) supporting the hypothesis that B. japonicum nif genes may be controlled by similar regulatory mechanisms. I have described the isolation and characterization of four different B. japonicum genes that have sequence homology to both the K. pneumoniae nifA and ntrC genes. One of these potential regulatory genes has been shown to have an effect on the transcription of nif genes both symbiotically and in microaerobic culture. This gene is also required for the microaerobic and symbiotic expression of the gene encoding glutamine synthetase II (glnII), and for some aspect of controlling cell division or cell viability in oxygen limited cultures. This same gene is also needed for normal development of the symbiotic association between B. japonicum and its soybean host. I suggest that this gene is part of a general regulatory system that is necessary for oxygen availability and developmentally mediated control of gene expression. This regulatory system has been termed Odc and the

nifA-homologous gene required for activity of this system has been called odcA as the first gene identified for this regulatory pathway.

Interpreting the effects of the odcA deletion mutation on microaerobic gene expression is complicated by the complex relationship between oxygen availability and nitrogen limitation in B. japonicum. Growth rate and final cell density of microaerobic cultures in YEMN medium can be stimulated by the addition of ammonia. Similar changes in growth properties can be achieved by increasing oxygen levels indicating that the amino acids present in yeast extract do not limit aerobic growth. This implies that growth limitation during microaerobiosis results, at least in part, from nitrogen limitation brought about through changes in the cells ability to utilize amino acids as a source of nitrogen. Thus, the microaeroabic induction of nif and glnII gene transcription might be due to nitrogen limitation, oxygen limitation, or a combination of the two.

It is possible that the expression of odcA activity is induced in microaerobic culture by another regulatory protein in response to the nitrogen limiting growth conditions observed. This would be analogous to the NTRC mediated activation of the nifLA promoter under nitrogen limited growth conditions in K. pneumoniae. If this occurred in B. japonicum, one might expect that microaerobic glnII transcription, like aerobic glnII transcription, would be independent of the odcA gene. This is not the case, however, clarification of this question awaits the isolation of a B. japonicum ntrC-like mutant strain. Alternatively, ODCA activity might be the cause rather than the effect of microaerobic nitrogen limitation. Low oxygen tensions have been shown to induce ammonia export in some wildtype Bradyrhizobium strains (O'Gara and

Shanmugan, 1976; Ludwig, 1980; Gober and Kashkett, 1983). Active ammonia excretion might result in nitrogen limited growth even if cultures were supplied with a utilizable nitrogen source like amino acids. Addition of ammonia to these cultures might partially relieve nitrogen limitation and stimulate growth by shifting the intracellular/extracellular ammonia equilibrium concentrations. In the extreme case, nitrogen limitation brought about by the export of fixed nitrogen, might lead to a loss of cell viability as the cells deplete their internal nitrogen stores such as protein. Ludwig (1984) observed that cells grown microaerobically with a growth limiting nitrogen supply export ammonia and lose cell viability. This loss of cell viability is most dramatic at oxygen tensions that are too high for nitrogen fixation. From these experiments Ludwig (1984) suggested a model for development of bradyrhizobial nitrogen fixation activity. This model implies that during microaeobiosis, a subpopulation of cells in a culture switch their metabolic state to one in which nitrogen is fixed but growth is repressed. This metabolic change could be accomplished if the induction of nif genes were directly coupled to the induction of ammonia transport genes. I propose that ODCA is responsible for the induction of the ammonia export system as well as the transcription of nif genes. Such a role for ODCA could explain the effect of odcA mutation on cell viability in microaerobic culture. The effects of odcA mutation on ammonia export should be examined.

It is difficult to understand why Bradyrhizobium should have evolved a system for ammonia export that is active even under nitrogen limiting growth conditions. Perhaps this is a colonial survival mechanism in which some bacteria export their nitrogen reserves to

supply other bacteria in the colony with fixed nitrogen thus resulting in syntrophic growth. This behavior becomes most useful when, as described by Ludwig (1984), these non-growing ammonia-exporting bacteria also fix nitrogen.

For the model described above, glnII expression could be explained by either the direct action of ODCA in inducing the glnII promoter, or the indirect effect of ODCA on ammonia limitation which then results in a Ntr-like response. A R. meliloti mutant strain that carries a transposon insertion in the glnII gene is unable to fix nitrogen in root nodules (M. Kahn, personal communication). In contrast, the R. meliloti glnA gene is not required for symbiotic nitrogen fixation (Somerville and Kahn, 1983). The need for glnII during symbiotic development could relate to the observation that the gene is induced during microaerobiosis by a mechanism requiring ODCA. High concentrations of ammonia present in nitrogen fixing root nodules would repress the Ntr-like activation of the glnII promoter and lead to the adenylylation of GSI. By providing a second mechanism for the activation of glnII transcription that does not necessarily require nitrogen limitation, GSII activity could be induced in bacteroids and allow some ammonia assimilation for bacterial growth and differentiation. Alternatively, it has been suggested that the bradyrizobial GSII may have some alternative function beyond its role in glutamine synthesis (Ludwig, 1980b). glnII and glnA mutant strains of B. japonicum will be helpful for understanding the respective roles of GSII and GSI in soybean root nodules.

The function of odcA in control of normal root nodule development is not yet clear. The results presented in Chapter 6 indicate that odcA

is essential even at very early stages in nodule differentiation. Ultrastructural analyses demonstrate that BJ2101 (the odcA deletion strain) is treated more as a pathogen than a symbiont when in association with the plant. Infected plant cells exhibit a degenerative response that is reminiscent of the hypersensitive responses described for several plants during invasion by pathogenic bacteria (Keen and Holliday, 1982). The inability of BJ2101 to be recognized as a symbiont could be due either to a lack of expression of some factor(s) or the lack of repression of some free-living function(s) that the plant identifies as characteristic of pathogens. One possibility is that the ammonia export system described above is required for normal symbiosis. Perhaps Bradyrhizobium evades the hypersensitive response by feeding the plant back some of the nitrogen released from amino acid degradation (Kahn et al., 1985). If odcA is required for induction of the ammonia export system then BJ2101 would be unable to accomplish this evasive mechanism.

ODCA is required by B. japonicum for the expression of several genes during microaerobic growth. The mechanism of ODCA action as a positive regulator of genes such as nif and glnII could be either direct at the promoters for these genes, or indirect through the induction of a regulatory cascade involving at least one secondary regulator. The second possibility would be analogous to the situation in K. pneumoniae where NTRC controls nif gene expression indirectly by activating the transcription of nifA. Some evidence for a direct role of ODCA in activating transcription from B. japonicum nif promoters comes from experiments of Fischer et al. (1986) in which they demonstrated that ODCA can activate the B. japonicum nifD promoter in a heterologous E.

coli expression system that requires the E. coli ntrA gene. However, these heterologous system experiments are known to be capable of yielding specificities that do not directly represent those that occur in the homologous system (Better et al., 1985; Sundaresan et al., 1983). A more direct genetic analysis of the possible ODCA / nif or glnII promoter interaction is needed.

The mechanism by which oxygen limitation is sensed in B. japonicum and how this stress is transmitted to ODCA activity is unknown. In K. pneumoniae the activities of both NTRC and NIFA are controlled through other regulatory components that somehow sense relevant aspects of cellular physiology. Hennecke and co-workers (personal communication) have found that ODCA mediated activation of transcription from the B. japonicum nifD promoter in E. coli is sensitive to oxygen. No such oxygen sensitivity has been observed for the K. pneumoniae NIFA protein. This indicates that the ODCA protein may sense oxygen stress, directly. However, these experiments are complicated by the complex physiological differences between aerobic and microaerobic growth. The development of in vitro systems for ODCA mediated transcriptional control should be useful for elucidating this regulatory pathway.

The functions of other nifA-like gene regions in B. japonicum are unknown. Escherichia coli and Rhizobium meliloti also appear to contain multiple nifA-like genes indicating that this property is likely to be generally found in many bacteria. At present, only one nifA-like gene has been described in E. coli (Buikema et al., 1985) and two in R. meliloti (Buikema et al., 1985; Ausubel et al., 1985). It is possible that these different nifA-homologous regions encode a family of NIFA-like regulatory proteins responsible for controlling different gene

systems. The homologies described here might then represent sequences that are required for interaction with NTRA or NTRA-like proteins.

The four separate B. japonicum mutant strains (each with a deletion in a single hna region) described in Chapter 4 have been examined for alterations in Nif-like or Ntr-like transcriptional control systems. Only the hna4 (odcA) mutant discussed above has an easily discernible regulatory phenotype. Of the other hna mutant strains, only BJ1011, the hna2 deletion strain, has any obvious alterations in growth and symbiotic development. Nitrogen fixation activity in soybean root nodules formed by infection with wildtype B. japonicum (BJ110) usually begins about 12 days after sowing and inoculation. The onset of nitrogen fixation in soybean nodules induced by infection with BJ1011 is delayed at least one week in comparison to wildtype infected nodules. Some nif genes are expressed normally during this lag in the initiation of nitrogen fixation suggesting that a general alteration in nif gene control has not occurred. BJ1011 is also perturbed in its free-living growth characteristics and grows more slowly than BJ110 under all media conditions tested. The slow growth phenotype of BJ1011 might account for the observed lag in development for nodules induced by this strain. Alternatively, hna2 might encode a regulatory product that is required for the early establishment of nitrogenase activity. This function would either not be required in older nodules or some other gene product (perhaps HNA3 or HNA5) might be able to replace HNA2. The construction of B. japonicum strains with deletions in more than one hna gene region will be useful in understanding the role of these genes.

Finally, B. japonicum contains other nifA-homologous regions that I have not described in this dissertation. Two of these genes were

isolated recently (G. Martin, unpublished) in our laboratory. These two B. japonicum regions were identified by their homology to a gene isolated from B. parasponia (Nixon et al., In Press). No mutation in the B. parasponia gene has yet been described but this gene does have a very similar derived amino acid sequence to the K. pneumoniae ntrC gene and is linked to a gene that is homologous to ntrB, another gene in the general nitrogen regulatory pathway (Magasanik, 1982). It will be interesting to see the effects of mutations in these genes and other nifA-like and ntrC-like sequences on bradyrhizobial gene expression. The isolation of a B. japonicum mutant strain that is deficient in Ntr-like control of gene expression will be invaluable in answering many of the questions raised by this dissertation.

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