To the Graduate Council:

I am submitting herewith a dissertation written by Jong-Yoon Chun entitled "Two Novel Genes of Bradyrhizobium japonicum Involved in Nodule Formation and Function". I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy with a major in Life Science.

Gary Stacey, Major Professor

We have read this Dissertation and recommend its acceptance:

Accepted for the Council

Associate Vice Chancellor and
Dean of the Graduate School

TWO NOVEL GENES OF *BRADYRHIZOBIUM JAPONICUM* INVOLVED IN NODULE FORMATION AND FUNCTION

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Jong-Yoon Chun

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ABSTRACT

Two new symbiotic genes, nfeC and hsfA, essential for nodulation competitiveness and host specific nitrogen fixation, respectively, were identified and sequenced from the soybean root nodule bacterium, Bradyrhizobium japonicum. A Tn5 insertion (NAD14) in nfeC did not affect nitrogen fixation but caused a significant delay in soybean (Glycine max) nodulation. In addition, this mutant exhibited a reduction in its competitive ability to nodulate soybean when co-inoculated with the wild type. DNA sequence analysis of the mutated region revealed that the NAD14 Tn5 insertion mapped within an open reading frame of 825 bp. Primer extension using B. japonicum mRNA from three different growth conditions, aerobic, anaerobic, and bacteroids (i.e. symbiotic form), indicated that the upstream region of the gene contained two promoters, which were differentially regulated in response to the growth conditions. One promoter was active in bacteroids, but not under aerobic or anaerobic free-living conditions. The other promoter was functional only under aerobic conditions. Therefore, the regulation of the nfeC gene appears to be unique. The bacteroid-specific promoter has a 5' consensus sequence suggesting a requirement for RpoN (i.e., σ^{54}). A bacteroid- specific activator of nfeC is postulated based on comparison to other RpoN-dependent promoters.

A mutation in hsfA exhibited a host specific nitrogen

fixation phenotype. This mutant induced nitrogen-fixing nodules on Glycine max, G. soja, and siratro (Macroptilium atropurpureum), but ineffective nodules on cowpea (Vigna unguiculata). Microscopic analysis of a deletion mutant, lacking hsfA, revealed that, while some bacteroids were found in the infected cowpea nodule cell cytoplasm, they appeared to be readily degraded and usually failed to develop into mature bacteroids. On soybean, nodule cells infected by this strain differentiate bacteroids in a manner similar to wild-type infected cells. These results suggest that hsfA may play a role maintaining the integrity of B. japonicum in cowpea but not soybean nodules. DNA sequence analysis of the hsfA region showed an open reading frame of 96 amino acids. Primer extension results indicated that hsfA also has a bacteroid-specific RpoN-dependent promoter.

This work extends the genetic characterization of the nodulation region of B. japonicum by identifying two new genetic loci involved in nodule formation and function.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Introduction

Bacteria of the genera Rhizobium, Bradyrhizobium, and Azorhizobium (collectively referred to here as rhizobia) have the ability to establish a symbiotic relationship with leguminous plants. These bacteria induce the plant to develop a new organ, the nodule (on root or stem), where they fix nitrogen from the atmosphere into ammonia which is then assimilated by the plant; in return, the plant supplies the bacteria with carbon sources for their active metabolism and for supporting the energy required for nitrogen fixation. Symbiotic nitrogen fixation is complex processes, involving interactions between the bacterium and the host plant at a number of defined steps. The association of the rhizobia with the host plant shows a high degree of specificity. For example, a particular rhizobial species can nodulate only certain plant host species.

Rhizobia were initially classified into cross-inoculation groups in terms of the range of plants they nodulate. However, in recent years the use of modern methods such as 16 rRNA sequence similarity, nucleic acid hybridization, and sequencing led to the definition of three

genera, Rhizobium (fast growing), Bradyrhizobium (slow growing), and Azorhizobium (stem-nodulating rhizobia).

The degree of specificity varies greatly among rhizobia (see Young and Johnston, 1989, for review) (Table 1). Some rhizobia have a narrow host range. For example, Rhizobium meliloti strains elicit nitrogen-fixing nodules only on species of the genera Medicago, Melilotus, and Trigonella, and R. trifolii only on species of Trifolium (clover). In contrast, some other rhizobia are able to fix nitrogen with a diverse variety of legume hosts. For example, some tropical Bradyrhizobium strains nodulate legumes in different tribes and subfamilies, and Rhizobium sp. strain NGR234 can nodulate at least 20 different legume genera (Stanley and Cervantes, 1991). The mechanism by which host specificity is achieved has long been an object of study for researchers in this field.

Rhizobia strains have been reported to form effective (i.e., nitrogen fixing) nodules on one plant species (or genus) and ineffective ones on another, showing that specificity is not limited to nodulation. Therefore, the host specificity of rhizobia is manifested not only in the early steps of infection and nodulation (host-specific nodulation) but also in the late stages of nodule development (e.g., the establishment of a nitrogen fixing symbiosis or host-specific nitrogen fixation).

A further examination of such host specificity could

Table 1: Nodulation host range of various Rhizobium, Bradyrhizobium and Azorhizobium species

Rhizobial species	Plant hosts
Rhizobium meliloti	Alfalfa (Medicago)
Rhizobium leguminosarum	
biovar viciae	Pea (Pisum), vetch (Vicia)
biovar trifolii	Clover (Trifolium)
biovar phaseoli	Bean (Phaseolus)
Rhizobium loti	Lotus
Rhizobium fredii	Soybean (Glycine)
Rhizobium sp. NGR234	Broad host range; Tropical legumes, Parasponia (nonlegume)
Bradyrhizobium japonicum	Soybean, Siratro, Mungbean, Pigeon pea, cowpea
Azorhizobium caulinodans	Sesbania (stem-nodulating)

lead to means to improve legume productivity under agricultural conditions. Legume productivity may be improved by inoculation with selected highly effective nitrogen-fixing rhizobial strains. However, inoculation with efficient rhizobial strains often failed to improve yield due to the inability of inoculant strains to compete against indigenous strains in the soil for nodule occupancy (see Dowling and Broughton, 1986; Triplett and Sadowsky, 1992, for review). This problem is termed the rhizobium competition problem. As an attempt to solve this competition problem, the host specificity of rhizobia is studied with the long term goal being to develop rhizobium strains which can nodulate a very strain-specific host-legume.

Molecular genetic studies have led to the identification of a number of bacterial genetic determinants of host specificity and nodulation competitiveness. In this work, we present two additional symbiotic genes involved in nodulation competitiveness and host-specific nitrogen fixation (Figure 1).

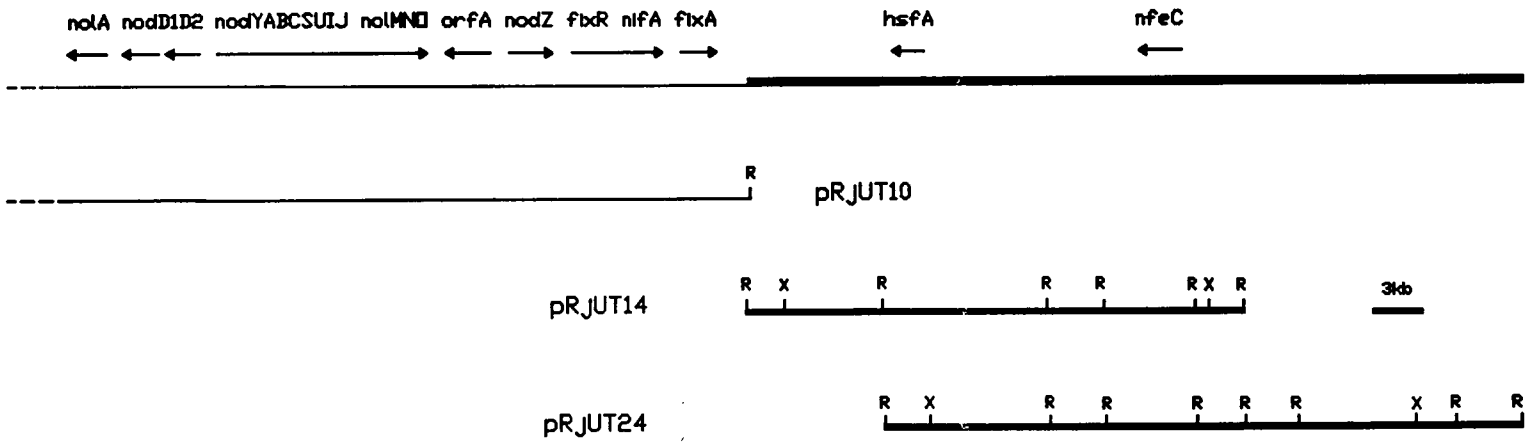
Nodule development process

The development of nitrogen-fixing nodules is a complex process requiring interaction between the bacterium and the plant host. The first step in the establishment of the

Figure 1. Genetic map of the known symbiotic genes of B. japonicum strain USDA110 found in cluster II (see Fig. 2). The two new symbiotic genes identified in the present work are also shown. pRjUT10, pRjUT14 and pRjUT24 are pHC79 cosmid clones isolated from USDA110 genomic library.

Bradyrhizobium japonicum (USDA110)

Cluster II



symbiosis is the recognition and invasion of the appropriate legume by the appropriate rhizobia. Rhizobia respond positively to exudates from plant roots and show chemotaxis toward the root exudates (Caetano-Anolles et al., 1988; Maxwell and Phillips, 1990; Barbour et al., 1991; Kape et al., 1991). The flavonoids in root exudates induce the transcription of bacterial nodulation genes (described below) (Peters et al.,1986; Spaink et al., 1987; Banfalvi et al., 1988; Smit et al., 1992). For the induction of nodulation genes, the bacteria preferentially recognize particular flavonoids and this recognition is an important determinant of rhizobial-host specificity (Horvath et al., 1987; Kondorosi et al., 1989; Spaink et al., 1989).

After chemotaxis, rhizobia attach to root hairs all over the root of their host, but the root hairs that are the most responsive are those that have recently emerged (Bhuvaneswari et al., 1980). An early hypothesis to explain rhizobial attachment to roots was that host plant lectins mediated the specific recognition and binding of the bacteria to the root surface (see Halvorson and Stacey, 1986, for review). However, there are several reports challenging this hypothesis. For example, several rhizobial species bind to lectins isolated from plants which they do not infect or nodulate (Law and Strijdom, 1977; Wong, 1980; Seegers and LaRue, 1984). Another hypothesis was also proposed that the binding of rhizobia to root hairs

may be mediated by a protein on the bacterial surface known as rhicadesin (Dazzo et al., 1984; Smit et al., 1987; 1989). Rhicadesin is a calcium-binding protein that appears to be common among Rhizobiaceae. Subsequent to rhicadesin-mediated attachment, tighter adherence occurs by means of cellulose fibrils (Smit et al., 1987). Fimbriae have also been suggested to mediate rhizobial attachment to root surfaces (Vesper and Bauer, 1986).

The first visible plant response to rhizobia is a curling and deformation of root hairs after the attachment of rhizobia. The rhizobia become entrapped in the curl of the root hair and invade the plant by a newly formed tubular structure, the infection thread. These infection threads start in the curl of the root hair. Concomitant with the infection process, cells in the root cortex start to divide and form the developing nodule primordium. Growing infection threads enter individual primordium cells and are then released from the infection thread(s) into the cytoplasm by an endocytotic mechanism (Dart, 1977; Goodchild, 1978). The intracellular bacteria are enclosed within a membrane of plant-derived origin termed the peribacteroid or symbiosome membrane (Robertson et al., 1978; Roth et al., 1988; Bassarab et al., 1989). The membrane-enclosed bacteria are termed bacteroids. When the bacteria undergo conversion to bacteroids, massive changes in the cellular protein composition take place (Werner,

1992). The structure encompassing the membrane-enclosed bacteroids, which include the bacteroid, the symbiosome space, and the peribacteroid (symbiosome) membrane, has been termed a symbiosome and represents the 'organelle' (functional unit) of nitrogen fixation (Roth et al., 1988).

Nitrogen fixation

The culmination of this complex developmental process of nodule formation is nitrogen fixation.. The biological reduction of dinitrogen (N_2) to ammonia is catalyzed by nitrogenase. This process is energy-demanding because the nitrogenase enzyme has an absolute requirement for ATP and the reduction of 1 mole of N_2 to ammonia utilizes 16 moles of ATP. Nitrogenase is a complex metalloenzyme composed of two separate components. Component I (also designated MoFe protein) is a tetramer made of two pairs of nonidentical subunits (α and β). It contains two types of metal centers that are involved in the redox reaction of the N_2 reduction process: P centers organized as four [4Fe:4S] clusters and two identical FeMo cofactors (FeMoco), which are known to be the sites for N_2 binding and reduction. Component II (also called Fe protein), which serves as an electron donor to the component I, is a dimeric protein complex with one [4Fe:4S] cluster. During catalysis, the Fe protein binds two MgATP molecules, is reduced, associates with the MoFe protein, and

donates a single electron to the MoFe protein in a reduction coupled to ATP hydrolysis and component protein dissociation. Nitrogenase is also able to reduce substrates other than N_2 and H^+. Most notable among these is C_2H_2, which can be reduced by two electrons to yield C_2H_4 (Dilworth et al., 1966; Schollhorn and Burris, 1967). Acetylene reduction to ethylene is frequently used as an assay for nitrogenase activity because C_2H_2 and C_2H_4 are easily separated and quantitated by gas chromatography (Dilworth et al., 1966; Schollhorn and Burris, 1967).

With the given complexity of the nitrogenase system it is not surprising that rhizobia and other diazotrophic bacteria possess a large number of genes involved in the N_2 fixation process. Genes important to nitrogen fixation are generally identified by the fact that mutations that disrupt their function result in an inability to fix nitrogen (i.e., Fix^-) (Beringer, 1980). However, in the case of rhizobia, a Fix^- phenotype may also indicate an inability of the bacteria to develop into bacteroids since nitrogen fixation naturally only occurs *in planta* (the exception to this is Azorhizobia, de Bruijn et al., 1988). Genes for nitrogen fixation in rhizobia (i.e., with no other apparent metabolic effect) are generally designated by either of two names; that is, those that are homologous to genes identified previously in free-living nitrogen fixing bacteria, such as *Klebsiella*, (referred to as *nif* genes), and those that are

required for symbiotic nitrogen fixation, but whose function does not appear to be analogous to the genes characterized in free-living bacteria, (referred to as *fix* genes) (see Merrick, 1992, for review). Mutants defective in *nif* and *fix* gene functions are able to induce nodule development, but the nodules do not fix nitrogen (Nod^+ Fix^-).

Organization and function of nitrogen fixation genes

The location and organization of *nif* and *fix* genes vary among rhizobia (Sanjuan et al., 1993). The structural *nif* genes of *R. meliloti*, and other *nif* and *fix* genes, are located in several clusters dispersed on the symbiotic megaplasmid (i.e., pSym). The *R. leguminosarum* biovars appear to have more tightly linked plasmid-encoded *nif* and *fix* genes. In *B. japonicum*, the *nif* and *fix* genes as well as other symbiotic genes are largely located in two separate chromosomal gene clusters.

The *nif* genes have been identified through either hybridization with the identified *nif* genes of *K. pneumoniae* and *A. vinelandii* or sequence analysis of DNA regions believed to carry *nif* genes because of linkage to known *nif* genes (see Gussin et al., 1986; Dean and Jacobson, 1992, for review). The *nif* genes include the nitrogenase structural genes, *nifHDK* (e.g., Corbin et al., 1983; Fischer and Hennecke, 1985; Fuhrmann et al., 1985; Quinto et al., 1985) and a regulatory gene, *nifA* (e.g., Szeto et al., 1984). The

nifA gene product is required for transcription of other nif operons (see Merrick, 1992, for review). Other genes found in R. meliloti, R. leguminosarum, and B. japonicum are nifN, nifE, nifB, which are involved in the biosynthesis or processing of the FeMo cofactor required for activity of the nitrogenase component I (Fuhrmann et al., 1985; Norel et al., 1985; Shah et al., 1986; Buikema et al., 1987; Ebeling et al., 1988). In addition, the nifS gene has been identified in B. japonicum (Ebeling et al., 1987). NifS may be required for nitrogenase maturation.

The fix genes have been identified in several ways such as, through random mutagenesis and sequencing, through mutagenesis and sequencing of DNA adjacent to identified nif genes, or by mapping DNA regions identified by hybridization to bacteroid RNA (examples below). The biochemical functions of most fix gene products are not known. The fixABC genes have been found in Rhizobium and Bradyrhizobium (Fuhrmann et al., 1985; Earl et al., 1987; Gronger et al., 1987) and are proposed to function in electron transport to nitrogenase. The fixLJ genes were first reported in R. meliloti (David et al., 1988) and were shown to be transcriptional regulators of nifA and fixK expression. The fixR gene has only been identified in B. japonicum and is found within the same operon as nifA (Thöny et al., 1987). Its function is not known, but the predicted FixR protein sequence shows similarity to a NAD-dependent dehydrogenase

(Baker, 1989). Therefore, it was suggested that FixR may be involved in an oxidation/reduction process. In addition to these *fix* genes, several additional *fix* loci have been identified by mutagenesis but remain to be fully characterized (e.g., Regensburger et al., 1986; Ward et al., 1989).

Regulation

Regulation of nitrogen fixation can be achieved by a number of mechanisms. In all organisms studied so far, control is exerted at the level of transcription of *nif* and *fix* genes. In free-living organisms, the bacterial *nif* genes are controlled by various environmental parameters, including the availability of combined nitrogen in the medium (see Merrick, 1992, for review). For example, in *K. pneumoniae* the global nitrogen circuit acts through *ntrC* to control the transcription of *nifA*, which regulates the expression of all other *nif* operons (Wong et al., 1987; Minchin et al., 1988). NtrC is an activator protein for *nifA* gene expression and is regulated by the N-status of the cells (Keener and Kustu, 1988; Stock et al., 1989). When cells are N-limited, NtrC is phosphorylated by NtrB. The phosphorylation of NtrC stimulates its DNA-binding properties and renders it able to function as a transcriptional activator. Under conditions of N-excess, NtrB no longer has its kinase activity and promotes the

dephosphorylation of NtrC. As a result, the activator and DNA-binding properties of NtrC are diminished and expression from NtrC-dependent promoters is switched off. The ntrB and ntrC genes are present in many other bacteria, including rhizobia (see Merrick, 1992, for review). Their products are members of a large family of bacterial two component regulatory proteins, all of which probably function in a similar manner (Stock et al., 1989). In most cases a pair of proteins acts in concert to coordinate the regulation of a group of genes in response to an environmental stimulus. One of the pair is a histidine protein kinase that acts as "a sensor protein", and the partner protein or "response regulator" is usually (but not always) a transcriptional activator.

In Rhizobium, the NtrC-mediated control pathway does not exert major control over nitrogen fixation in bacteroids (see Merrick, 1992, for review; e.g., Szeto et al., 1987). Rather, nif and fix gene expression is controlled by oxygen concentration in the nodule environment (see Merrick, 1992, for review; e.g., Anthematten and Hennecke, 1991; Kahn and Ditta, 1991; Agron et al., 1992; Weinstein et al., 1992). For example, in R. meliloti, low oxygen triggers nif/fix gene expression through a regulatory cascade involving FixL and FixJ, a two component regulatory couple (David et al., 1988; Monson et al., 1993). Under low O_2 concentrations, FixLJ induces nifA expression. In contrast, FixLJ are also

responsible for nifA repression in the presence of high O_2 (David et al., 1988). In B. japonicum, oxygen also controls the level of the nifA expression (Thöny et al., 1987; 1989). However, unlike R. meliloti, the fixLJ genes are not involved in the regulation of the B. japonicum nifA gene (Anthamatten and Hennecke, 1991). In B. japonicum, nifA is expressed under aerobic conditions, but the NifA protein that is produced under these conditions is inactive (Thöny et al., 1989). Under microaerobic conditions and in planta, nifA expression is enhanced by the presence of active NifA. Therefore, the expression of the B. japonicum nifA is dually controlled by NifA-independent activation and by NifA-dependent autoregulation in response to cellular O_2 status (Thony et al., 1989). Active NifA is required for nif and fix gene expression in B. japonicum. Activation of nif and fix genes by NifA in B. japonicum is dependent on the product of another gene, rpoN (also called ntrA or glnF), which encodes an alternative sigma factor, σ^{54} (Thöny et al., 1987). All RpoN-dependent promoters have the -24/-12 conserved sequence (TGG-N_8-TTGCA), which is the site recognized by σ^{54} (see Thony and Hennecke, 1989, for review). Recently, in B. japonicum, two separate, strongly conserved rpoN genes were identified (Fischer et al., 1990). Mutagenesis of either rpoN gene gave a Nod^+ Fix^+ phenotype, whereas a rpoN1-rpoN2 double mutant had a Nod^+Fix^- phenotype. These results suggest that both rpoN genes are

active and can functionally complement one another. In addition, many (but not all) RpoN-dependent genes possess an upstream activator sequence (UAS) which is the target site for the binding of an activating protein that is required for maximal expression. For example, some nif and fix promoters in B. japonicum are preceded by one or more copies of an UAS (TGT-N_{10}-ACA), which is the NifA binding site (e.g., Alvarez-Moales et al., 1986; Buck et al., 1986; Gubler, 1989). This sequence is not essential for expression of these genes but is responsible for strong enhancement of their transcription (e.g., Buck et al., 1987; Buck et al., 1989).

Different RpoN-dependent operons are controlled by different environmental conditions (e.g., oxygen or nutritional limitation). Therefore, they need to have adequate sensory systems which transduce the appropriate signals to the corresponding transcriptional regulators. This means that the activating proteins themselves are subject to different controls at the level of transcription. The most well studied transcriptional regulatory proteins are NtrC and NifA. In addition to these two, the DctD, XylR, and HydG proteins have been characterized as being able to positively control expression from a RpoN promoter.

Other genes related to symbiotic nitrogen fixation in rhizobia.

In addition to *nif* and *fix* genes, other genetic determinants involved in symbiotic nitrogen fixation have been identified from *Rhizobium* and *Bradyrhizobium* species. Examples are genes for the synthesis of cell surface polysaccharides of rhizobia (e.g., Chua et al., 1985; Finan et al., 1985; Leigh et al., 1985; Vandenbosch et al., 1985; Dylan et al., 1986; Noel et al., 1986), genes for bacterial respiration (e.g., Thöny-Meyer et al., 1989), and genes for the transport of C4 dicarboxylic acids (*dct*) (e.g., Ronson et al., 1981; Finan et al., 1983). A range of Fix$^-$ mutants has been isolated. Some of these mutants form empty nodules that have either no infection threads or contain aborted infection threads, demonstrating that the process of nodulation can be uncoupled from bacterial invasion of the plant host. These Fix$^-$ mutants have been shown to have alterations in the composition of extracellular exopolysaccharides (Finan et al., 1985; Leigh et al., 1985), lipopolysaccharides (Noel et al., 1986) or in β-glucan synthesis (Dylan et al., 1986).

The respiratory demands imposed by nitrogen fixation in a microaerobic environment appear to be met by the induction of an alternative, nodule-specific bacterial respiratory chain with a high-affinity terminal oxidase that functions at nanomolar concentrations of free oxygen (Thöny-Meyer et al., 1989; Bergersen and Turner, 1990). Accordingly, mutations in respiratory genes can result in a Fix$^-$

phenotype (e.g., Thöny-Meyer et al., 1989). Dicarboxylic acids are the most likely carbon sources for respiration and N_2 fixation by bacteroids (see Stowers, 1985; McDermott et al., 1989, for review; e.g., Gardiol et al., 1982; Waters et al., 1985). Mutations in the dct genes required for the transport of C4 dicarboxylic acids result in a Fix^- phenotype (e.g., Ronson et al., 1981; Finan et al., 1983).

Host specificity in nitrogen fixation

Bacterial host specificity in symbiotic nitrogen fixation was reported by the observation that some wild-type rhizobial strains form effective (Nod^+ Fix^+) nodules on a subset of the legumes but they form ineffective (Nod^+ Fix^-) nodules on other hosts (e.g., Pankhurst et al., 1979; Trinick, 1980; Cregan and Keyser, 1986; Bromfield and Barran, 1990). For example, the broad host range Rhizobium strain NGR234 forms an effective symbiosis with a wide variety of legume hosts but induces ineffective nodules on other legumes (Trinick, 1980). B. japonicum strain USDA110 forms ineffective nodules with some commercial varieties of soybean (Cregan and Keyser, 1986). Rhizobium loti strain NZP2037 forms nitrogen fixing nodules on both Lotus tenuis and Lotus pedunculatus, but strain NZP2213 forms effective nodules on L. tenuis but ineffective tumor-like structures on L. pedunculatus (Pankhurst et al., 1979). Recently, Bromfield and Barran (1990) showed that thirty-three

isolates of indigenous Rhizobium meliloti induced nodules which were symbiotically ineffective on Trigonella foenum-graecum (100% of plants nodulated) and Phaseolus vulgaris (40 to 100% of plants nodulated). However, all but two isolates were symbiotically effective with Medicago sativa.

Additionally, nitrogen fixation mutants have been isolated which have lost the ability to fix nitrogen with some, but not all of the normal legume hosts of the wild-type strain (Stacey et al., 1982; Chen et al., 1985; Wilson et al., 1987; Ward et al., 1989; Hotter and Scott, 1991). In a broad host range Rhizobium strain NGR234, Tn5-induced mutants with altered exopolysaccharide production exhibited host-specificity in nitrogen fixation; i.e., they were Fix^+ on some hosts but Fix^- on others (Chen et al., 1985). A transposon (Tn5) mutant of wide host range Bradyrhizobium sp. (Arachis) strain NC92 was isolated which formed an ineffective symbiosis with pigeonpea, but effective symbioses with groundnut and siratro (Wilson et al., 1987). Ward et al. (1989) also reported that Tn5-induced mutants of Rhizobium loti strain NZP2037 defective in release from the infection threads or in normal bacteroid development were Fix^- on L. pedunculatus but Fix^+ on L. corniculatus.

In all cases of host-specific fixation the inability of the rhizobia to form an effective symbiosis with a particular host must reside in a step subsequent to nodule induction. Moreover, since some hosts are Fix^+, the trait

involved must not affect the intrinsic ability of the rhizobia to fix nitrogen. Whether a defect in rhizobia-legume interaction leading to the Fix$^-$ phenotype involves loss of specific signalling or is due to a simple physiological incompatibility is unclear.

Although several host-specific nitrogen fixation mutants were isolated from Rhizobium and Bradyrhizobium, little has been reported concerning the gene affected or the possible functions of the mutated regions. A host-specific nitrogen fixation (hsf) gene, hsfA, of B. japonicum strain USDA110 has been identified from our laboratory and is presented in this work. Mutations in this gene result in a Fix$^-$ phenotype on cowpea, but Fix$^+$ on G. max, G. soja, and siratro.

Nodulation

Nodule development consists of several stages determined by genes both in the rhizobia and the respective host plant. The bacterial genes important for nodulation are the nodulation (nod) genes. Due to the fairly high number of nodulation genes, the terminology has used up all letters of alphabet. Therefore, the recently identified nod genes are designated as nol genes. Numerous nod/nol genes have been identified and sequenced from the three rhizobial genera, leading to the description of over 44 distinct

nodulation genes.

Organization and function of nodulation genes

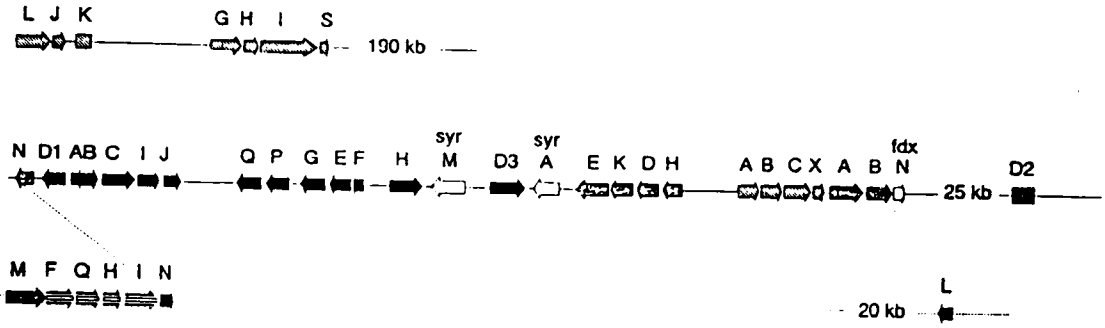
In the genus Rhizobium, the nod genes are localized on large indigenous plasmids (pSym) in the vicinity of the nitrogen fixation genes. In Bradyrhizobium and Azorhizobium the nod/nol genes are located on the chromosome. In all cases the nod/nol genes are found in one or in a few gene clusters (Figure 2). The nodulation genes are classified into two general categories, the common and the specific, according to their ability to complement mutations of similar genes in other rhizobial species.

i) Common nodulation genes

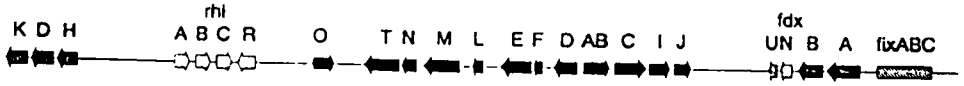
The common nodABC genes have been found in all Rhizobium, Bradyrhizobium, and Azorhizobium species (Goethals et al., 1989; Stacey, 1990). These genes are functionally interchangeable among Rhizobium and Bradyrhizobium species (see Barbour et al., 1992; Long, 1992, for review). Mutations in these genes totally block root hair deformation and curling and, consequently, nodulation (Kondorosi et al., 1984; Rossen et al., 1984; Djordevic et al., 1985; Russell et al., 1985; Bender et al., 1987; Dudley et al., 1987). Two additional genes, nodIJ, seem to be conserved among all rhizobia as part of the nodABC operon (Evans and Downie, 1986; Nieuwkoop et al.,

Figure 2. Major symbiotic clusters from R. meliloti strain AK631, R. leguminosarum bv. viciae strain 248, R. leguminosarum bv. trifolii strain ANU843 and B. japonicum strain USDA110. Taken from Sanjuan et al. (1993).

Rhizobium meliloti RCR2011 plasmid pSym [strain AK631]

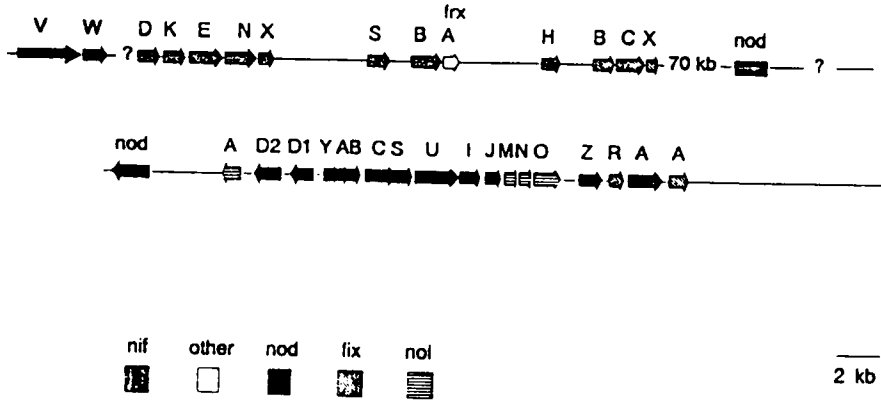


R. leguminosarum biovar *viciae* 248 plasmid pRL1JI



R. leguminosarum biovar *trifolii* ANU843 plasmid pRt843a

Bradyrhizobium japonicum USDA110



1987; Spaink et al., 1987; Göttfert et al., 1990). Mutations in nodIJ result in a delayed nodulation phenotype on vetch with R. leguminosarum bv. viciae (Canter Cremers et al., 1990), but have no detectable effect with B. japonicum (Göttfert et al., 1990).

ii) Host specific nodulation genes

In contrast to the common nod genes, the other group of nodulation genes are not functionally or structurally conserved among rhizobia since mutations in these genes are not fully complemented by DNA from other rhizobial species or biovars (e.g., Kondorosi et al., 1984; Djordjevic et al., 1985; Debellé et al., 1986; Horvath et al., 1986). These genes are found only in certain strains or genera and help to determine the host range of the bacteria. Therefore, these genes have been termed host-specific nodulation genes, and include nodFEGPQ, nodH, nodL, and nodM in R. meliloti (e.g., Debellé et al., 1986; Fisher et al., 1987; Faucher et al., 1989; Baev and Kondorosi, 1992), and nodFEL, nodM, and nodO from R. leguminosarum bv. viciae (Shearman et al., 1986; Canter et al., 1989; de Maagd et al., 1989), and nodZ and nodVW from B. japonicum (Göttfert et al., 1990; Stacey et al., 1994). While mutations in the common nodABC genes totally block nodulation, mutations in these host specific nod genes cause a delay in nodulation or a decrease in the number of nodules formed. For example in R. leguminosarum

and R. meliloti, individual mutations in the host-range nodF, E, L, M and N genes do not block nodulation. However, these genes are collectively essential because the deletion of all of the host-specific genes results in a completely Nod$^-$ phenotype on vetch (Downie and Surin, 1990; Downie et al., 1991). Mutations in some host specific nod genes narrow or may even extend host range. For example, in B. japonicum, the nodZ and nodVW genes appear to be involved in host range determination (Göttfert et al., 1990; Stacey et al., 1994). Mutations in these genes result in a narrowing of host range. Mutants in nodZ or nodVW lose the ability to nodulate siratro but not soybean. Rhizobium sp. NGR234 nodSU mutants are Nod$^-$ on Leucaena leucocephala but Nod$^+$ on siratro (Lewin et al., 1990). In R. trifolii, in contrast to the wild-type strains, nodFE$^-$ mutants nodulate white and red clovers poorly but have acquired the ability to infect and nodulate peas (Djordjevic et al., 1985). In R. meliloti, nodH mutants are unable to nodulate the homologous host alfalfa but acquire the ability to infect and nodulate heterologous hosts such as vetch (Faucher et al., 1989). In contrast, nodQ mutants can infect both alfalfa and vetch (Faucher et al., 1989)

Regulation

Nodulation genes are typically not transcribed in free-living rhizobia with the exception of the nodD gene, which

is constitutively expressed. The nod genes are induced by host-secreted flavonoids (Spaink et al., 1987; Zaat et al., 1987; Banfalvi et al., 1988; Smit et al., 1992). In addition to these flavonoids, nod gene expression also requires a functional nodD gene product (Rossen et al., 1985; Spaink et al., 1987; Banfalvi et al., 1988). The NodD protein is a transcriptional activator that binds to the nod box, a conserved cis-acting promoter element (Kondorosi, 1992). Binding of NodD to the nod box does not require the presence of the flavonoid inducers (Hong et al., 1987; Fisher et al., 1988). It seems that upon addition of the appropriate inducer, the NodD bound to the nod box with its DNA-binding domain undergoes a conformational change, resulting in a form which activates transcription of genes lying downstream.

The nod gene expression is negatively regulated in some R. meliloti strains (Kondorosi et al., 1989). A repressor was identified that bound to the overlapping nodD1 and nodA promoters and to the nodD2 promoter (Kondorosi et al., 1989). Recently, combined nitrogen (such as ammonia) has been shown to repress the expression of inducible nod genes; that is, nodD3 in R. meliloti (Dusha et al., 1989) and nodYABC in B. japonicum (Wang and Stacey, 1989).

Host-specific nodulation

Host-specific nodulation is determined by at least two steps of signal exchange between host and symbiont. In the first step, flavonoids excreted by the host plant induce the transcription of the bacterial nodulation genes. As described above, host specificity of this process involves the bacterial NodD protein, a transcriptional regulatory protein that presumably interacts directly with the flavonoids (Horvath et al., 1987; Spaink et al., 1987). In the second step, the bacterium, by means of the nod genes, produces lipooligosaccharide Nod factors that induce morphological changes in the plant root (described below). These compounds possess a β-1,4-linked N-acetylglucosamine backbone, with modifications that vary according to the species and host range of the bacterium (see Dénarie and Cullimore, 1993; Spaink, 1992, for review).

Both regulatory nodD proteins and Nod factors produced by nod genes are major determinants of nodulation host range.

Regulatory nod genes

The regulatory nodD genes are found in all of the strains of Rhizobium, Bradyrhizobium, and Azorhizobium (see Long, 1989; Kondorosi, 1992, for review). Some species like R. leguminosarum biovars viciae and trifolii have only one nodD gene (Kondorosi, 1992), while R. meliloti (Göttfert et al., 1986; Honma et al., 1987), R. phaseoli

(Davis and Johnston, 1990), R. fredii (Appelbaum et al., 1988), and B. japonicum (Göttfert et al., 1989) carry two or three copies of nodD. Different NodD proteins have different inducer specificities. For example, the R. meliloti nodD1 gene does not restore the ability to nodulate siratro in a nodD1 mutant of the broad host range strain Rhizobium sp. NGR234 (Horvath et al., 1987). Similarly, the R. meliloti nodD1 gene does not restore the ability to nodulate red clover in a nodD mutant of R. trifolii (Spaink et al., 1987). This lack of complementation results from the inability of R. meliloti nodD1 to respond to the compounds exuded by siratro and red clover. Phillips et al. (1993) reported that compounds released by alfalfa induce R. meliloti nod genes by interacting with different NodD proteins. Surprisingly, two of the compounds are betaines (i.e., trigonelline and stachydrine), which are structurally distinct from flavonoids. Since previous work on isolating natural nod gene-inducing compounds has been clearly biased toward flavonoids, the structural flexibility in the type of compounds that are active suggests that there may be additional chemical compounds that act as nod gene-inducers.

In addition, mutations in various combinations of nodD alleles in R. meliloti alter the range of inducers (Györgypal et al., 1988; Honma et al., 1990) and also affect host range (Györgypal et al., 1988). Furthermore, R. meliloti or R. trifolii strains carrying the nodD1 gene of

Rhizobium sp. NGR234 exhibit an extended host range including siratro (Horvath et al., 1987). Therefore, all these data clearly suggest that individual inducers activate different NodD proteins for the induction of common and host specific nod genes; therefore, the inducer-NodD interactions appear to be a first critical level of host-symbiont specificity.

In addition to the nodD genes, other nod regulatory genes have been suggested as determinants of host specificity in B. japonicum. B. japonicum nodV and nodW genes have been identified (Göttfert et al., 1990). Several lines of evidence indicate that NodV and NodW are regulatory proteins. For example, sequence comparisons revealed that the NodV and NodW proteins are members of the two component family of transcriptional regulators; NodV protein has similarity to membrane-bound sensors, and NodW has similarity to DNA-binding regulators (Göttfert et al., 1990). In B. japonicum, a double mutant of nodD1 and nodD2 is still Nod^+ (Göttfert et al., 1989), suggesting that there may be other regulators besides nodD, presumably nodVW. Mutants defective in nodVW exhibit delayed nodulation on soybean but have lost the ability to nodulate cowpea, mungbean, and siratro (Göttfert et al., 1990). Therefore, these two genes appear to be determinants of host specificity. Recently, the nodW gene product was found to be required for the induction of the nodYABC and nodD1

operons in B. japoncium (Sanjuan et al., 1994, in press).

The interaction between nod regulatory proteins and plant signals contributes to the specificity of the symbiotic relationship. However, the transfer of the appropriate nodD gene is not generally sufficient to transfer the host range from one Rhizobium species to another (Horvath et al., 1987; Spaink et al., 1987). The transfer of host-specific structural nod genes is usually required, showing that these genes play a major role in the control of host specificity.

Nod factors

One of the first indications that rhizobia produce chemical signals was the observation that the sterile supernatants of rhizobial cultures contained molecules that caused changes in root or root hair morphology (Yao and Vincent, 1969). The use of various plant bioassays has revealed that the nod gene products synthesize nodulation signals (so-called Nod factor) (see Denarie et al., 1992, for review). Using a root hair deformation bioassay, an extracellular nodulation signal molecule was first purified from induced cultures of R. meliloti strain 2011, genetically engineered to overexpress the nod genes. The signals purified were lipooligosaccharides consisting of an oligomer of chitin (a β-1,4-linked polymer of N-acetyl glucosamine), N-acylated and O-acetylated on the nonreducing

end and sulfated at the reducing end (Lerouge et al., 1990; Roche et al., 1991). Nodulation factors have subsequently been characterized from several other rhizobia (Spaink et al., 1991; 1992; Sanjuan et al., 1992; Schultze et al., 1992; Mergaet et al., 1993). All of these compounds are a N-acetylglucosamine backbone with modification that vary according to the species and host range of the bacterium. For example, the R. meliloti factors carry a C16:2 N-acyl modifications on the nonreducing end and a 6-O-sulfate on the reducing end residue (Lerouge et al., 1990; Schultze et al., 1992). R. leguminosarum bv. viciae molecules carry a C18:4 acyl group and no sulfate (Spaink et al., 1992). Broad host range Rhizobium strain NGR234 factors are variously decorated with carbamoyl, fucosyl, acetyl, and sulfate moieties. B. japonicum factors are modified with a 2-O-methyl fucose at the reducing end and a C18:1 N-acyl group attached the terminal, non-reducing residue (Sanjuan et al., 1992).

Structural elucidation of Nod factors has allowed greater insight as to the possible functions of common and host-specific nod gene products. Since all rhizobia possess the common nodABC genes, they are expected to play a role in the synthesis of the common N-acetylglucosamine backbone. A R. leguminosarum strain containing only the common nodABC genes excretes mono-acylated chitin oligomers. This suggests that the common nodABC genes are sufficient for the

production of the basic lipooligosaccharide (Spaink et al., 1991). The sequence of the NodC protein shows strong similarity to chitin synthases suggesting that it functions as an N-acetylglucosaminyl transferase that synthesizes the oligosaccharide backbone of the lipooligosaccharides (Bulawa, 1992). Recently, John et al. (1993) reported that NodB from R. meliloti is involved in the removal of the acetate moiety from the C-2 residue of the nonreducing sugar, an activity that may be a necessary prerequisite for the N-acyl transfer (John et al., 1993). Recent evidence strongly suggests that NodA mediates this N-acylation (Long, personal communication)

The particular decorations present on the chitosan backbone may determine the host specificity of the compound. By comparing the factors produced by rhizobia strains mutated in host-specific nod genes with those produced by the corresponding wild-type strains, it has been possible to make a direct link between the genetic determinants of host specificity and the structure of the lipooligosaccharides. In R. meliloti, the nodH and nodPQ genes are the major host range determinants. Mutants in nodH are able to nodulate the normal host alfalfa but gain the ability to nodulate vetch (Debellé et al., 1986; Horvath et al., 1986). The sterile culture filtrate of nodH mutants is active on vetch but not on alfalfa, implying that the nodH gene is modifying the specificity of the Nod factor (Faucher et al., 1988).

Analysis of the factors produced by these mutants has shown that they lack the sulfate moiety when compared to the wild type (Roche et al., 1991). Mutants in nodPQ produce both sulfated and non-sulfated factors (Roche et al., 1991) and show an extension of host range to include both vetch and alfalfa (Schwedock and Long, 1990). nodP and nodQ, homologues of E. coli cysD, cysN and cysC, encode ATP sulphurylase and APS kinase (Fisher and Long, 1992). The NodH protein sequence shows significant similarity to sulfotransferases (Roche et al., 1991). Therefore, these gene products are probably directly involved in catalyzing the sulfation of R. meliloti Nod factor. For R. leguminosarum bv. viciae it is the structure of the acyl moiety that seems to be most important for nodulating its hosts, pea and vetch (Spaink et al., 1991). The nodE gene is a major host-range determinant in this species (Spaink et al., 1989). NodF has a 4'-phosphopantetheine prosthetic group (Geiger et al., 1991), analogous to that of acyl carrier proteins involved in fatty acid biosynthesis. NodE shares homology with a group of β-ketoacylsynthases such as the E. coli condensing enzyme of fatty acid biosynthesis, FabB (Bibb et al., 1989). Therefore, it seems likely that the nodFE genes encode enzymes directly involved in the synthesis of the fatty acid. A nodE mutant of R. leguminosarum bv. viciae secretes Nod factors that are N-acylated by vaccenic acid (C18:1), whereas the wild-type

strain also secretes Nod factor with a highly unsaturated C18:4 acyl chain (Spaink et al., 1991). Therefore, the nodE gene likely specifies the synthesis of the highly unsaturated acyl chain moiety of the Nod factors. Other host-specific genes whose products are known to play a role in Nod factor biosynthesis include the nodL and nodM genes from R. leguminosarum bv. viciae. NodL appears to be involved in the transfer of an O-acetyl group to C-6 of the nonreducing sugar (Spaink et al., 1991). NodM has homology to D-glucosamine synthase and can complement a glmS mutant of E. coli (Marie et al., 1992).

In summary, the nodABC genes are required for the synthesis of the nodulation factors and are similar with regard to sequence and function in all rhizobia (e.g., Spaink et al., 1991; Fisher and Long, 1992; John et al., 1993). The host-range nod genes mediate the decoration of the nodulation factors with various substituents (N-acyl chain, sulfate, acetate and carbamoyl groups, sugars) to make them plant host-specific (e.g., Roche et al., 1991; Spaink et al., 1991; Sanjuan et al., 1992). Thus, the wide variety of host ranges found among rhizobia could be determined by a variety of diverse substituents on the common lipooligosaccharide nodulation signal.

Nodulation competition

The ability of certain strains of Rhizobium and Bradyrhizobium to dominate nodulation in a multistrain environment has been termed competitiveness (see Dowling and Broughton, 1986; Triplett and Sadowsky, 1992, for review). Despite the importance of interstrain competition for the successful use of inoculants, the molecular mechanism of competition is poorly understood. Previous studies have shown that many factors can influence competitiveness. Among these are biological factors such as cell surface components, motility, and saprophytic competence (see Dowling and Broughton, 1986; Triplett and Sadowsky, 1992, for review; e.g., Parker and Grove, 1970; Ames and Bergman, 1981; Bhagwat et al., 1991). Environmental conditions such as temperature, soil pH, soil type, soil moisture, and salinity can also influence competitive ability (e.g., Bowen and Rovira, 1976; Bromfield and Jones, 1980; Boonkerd and Weaver, 1982). The multiplicity of factors influencing competition makes it difficult to design a genetic approach to address the problem.

Two approaches are being used by geneticists to examine interstrain competition among rhizobial strains (Triplett, 1990). One approach is to construct inoculum strains with an altered host range and/or to use legume genotypes with restricted host range. The strategy is to manipulate the interaction such that a particular strain-host interaction is favored; thus one strain gains a competitive advantage.

Another approach is to construct strains with an increased ability to compete for nodule occupancy. However, in order for this second approach to be successful, bacterial genetic determinants involved in nodulation competitiveness must be identified and characterized. In this section, these two approaches will be reviewed.

One approach to overcome interstrain competition is to take advantage of the naturally occurring host restriction of nodulation in certain plant genotypes. For example, strains of B. japonicum serogroup USDA123 dominate nodule occupancy even if they are not present in higher concentrations in soil than other competing strains (Moawad et al., 1984; Schmidt et al., 1986). Soybean lines that restrict nodulation by this B. japonicum serogroup (Cregan et al., 1986; Sadowsky et al., 1987) have been identified. Therefore, these genotypes can be used to overcome the competition from serogroup USDA123 strains because they may permit nodulation by the inoculant while preventing nodulation by the usually dominate 123 strains. This approach can be used in other hosts such as alfalfa and clover because dominant serogroups have also been identified in R. meliloti and R. leguminosarum bv. trifolii (Demeza and Bottomley, 1984; Jenkins and Bottomley, 1985; Shishido and Pepper, 1990). In most cases, a single dominant plant genetic locus has been identified as determining the restriction phenotype (e.g., Phillips and Teuner, 1992). In

some groups of bacterial strains (e.g., serogroup USDA123 of B. japonicum) that are restricted for nodulation of certain soybean genotypes, the absence of a single bacterial gene, nolA, has been demonstrated to cause restricted nodulation (Sadowsky et al., 1991). Other such genotype specific nodulation genes have been reported. The first report of such a gene was in R. leguminosarum bv. viciae strain TOM, where a single gene, nodX, was identified that allowed this strain to nodulate Afghanistan pea (Davis et al., 1988). In R. fredii strain USDA257, a single gene, nolC, controls nodulation of a commercial soybean cultivar McCall (Heron et al., 1989). This strain nodulates soybean cultivar Peking normally but cannot nodulate cultivar McCall. Mutations in nolC allow R. fredii to nodulate cultivar McCall.

Another approach towards the elucidation of the molecular mechanism of nodulation competitiveness has focused on the search for specific genetic determinants in various rhizobial species. A number of gene have been identified by the effect of mutations that decreased competitive ability when compared to the wild-type strain (Triplett and Barta, 1987; Triplett, 1988; Triplett, 1990; Bhagwat et al., 1991; Sanjuan and Olivares, 1991). Common usage in the field suggests that in order to be termed a gene involved in nodulation competition, the gene must be generally dispensable for the establishment of nitrogen-fixing symbiosis. However, the gene must somehow affect the

nodulation ability of the strain when tested in a multistrain environment.

Although there are many reports regarding genes for nodulation competition, relatively little has been done to characterize these genes at the molecular level. Some strains of rhizobia produce bacteriocins that have been implicated in interstrain competition for nodulation (e.g., Schwinghamer and Belkengren, 1968; Joseph et al., 1983; Hodgson et al, 1985). The most well characterized antirhizobial bacteriocin is trifolitoxin, a small peptide produced by R. leguminosarum bv. trifolii strain T24 (Triplett, 1987; Triplett et al., 1988). T24 was found to be highly competitive for nodulation when coinoculated with a bacteriocin-sensitive strain of R. leguminosarum bv. trifolii (Schwinghamer and Belkengren, 1968). Mutants of T24 defective in trifolitoxin production lost their competitive advantage for nodulation when coinoculated with a trifolitoxin-sensitive strain (Triplett and Barta, 1987). Trifolitoxin inhibits strains of R. leguminosarum bvs. phaseoli, trifolii, and viciae, as well as R. fredii (Triplett and Barta, 1987).

The integrity of the cell-surface composition also appears to play a role in nodulation competitiveness. For example, Tn5 mutants of R. leguminosarum bv. phaseoli and B. japonicum deficient in exopolysaccharide (EPS) production are less able to compete for nodule occupancy than the

corresponding wild-type strain (Araujo and Handelsman, 1990; Bhagwat et al., 1991). In other strains, such mutations have a more drastic effect. For example, EPS$^-$ mutants in R. meliloti do not infect alfalfa, the normal host plant (e.g., Finan et al., 1985; Leigh et al., 1985; Doherty et al., 1988).

Several reports suggest that motility provides a competitive advantage in multistrain nodulation tests (Ames and Bergman, 1981; Mellor et al., 1987; Caetano-Anollés et al., 1988; Liu et al., 1989). Ames and Bergman (1981) first reported that motility-deficient mutants of R. meliloti were less competitive than the wild-type strain for nodulation of alfalfa roots. Such mutants were identical to the wild-type strain in growth rate, timing, and number of nodules formed. Similar nonmotile mutants from B. japonicum, R. leguminosarum bv. trifolii, and R. meliloti were isolated by transposon mutagenesis or spontaneous mutation (Mellor et al., 1987; Caetano-Anollés et al., 1988; Liu et al., 1989). All these mutants were less competitive for nodulation when compared to their corresponding wild-type stains.

Recently, in R. meliloti strain GR4, a DNA region involved in nodulation efficiency and competition on alfalfa roots was identified (Toro and Oliveres, 1986; Sanjuan and Olivares, 1989). The nucleotide sequence of this region revealed the presence of four major open reading frames (ORF) (Soto et al., 1993). Two were termed nfe1 (nodule

formation efficiency) and nfe2 because mutations in these ORFs caused a delay in nodule formation as well as a reduction in nodulation competitive ability in comparison to the wild type. In addition, the expression of the nfe genes was dependent on the NifA-RpoN regulatory system, although nfe1 could also be transcribed in a NifA-independent manner. The putative Nfe1 protein had a region of sequence similarity with the nifH gene product, with strong similarity between the first 30 amino acid residues of Nfe1 and the amino terminus of NifH. However, the rest of the Nfe1 protein diverged completely from the nifH-encoded ferroprotein. The other two ORFs, ORFA and ORFB, were transcribed in the opposite orientation to the nfe genes. The putative ORFB protein showed sequence similarity with the tnpA gene product of some bacterial class II transposable elements. However, the phenotype of this ORF was not studied. Comparison of the ORFA or nfe2-encoded proteins with sequences in the data bases showed no significant similarities.

Bhagwat et al. (1991) recently identified a DNA region of B. japonicum strain USDA110 that, when mutated by Tn5, resulted in a reduction in nodulation efficiency and competition on soybean plants. However, the sequence and possible function of this region has not been reported. Another nfe-like gene, nfeC, of B. japonicum strain USDA110 involved in nodulation competitiveness on soybean plants has

been identified from our laboratory and is presented in this work.

Rationale for present work

This work was initially started with two previously constructed mutants, NAD14 and NAD163, of B. japonicum strain USDA110 that were linked to the known nodulation gene cluster (i.e., nodYABC; Deshmane, 1988). Both mutants exhibited delayed nodulation phenotypes on soybean. Since many known host-specific nodulation genes show similar delayed nodulation phenotypes, these two mutants were further characterized to identify new symbiotic genes that are essential for an effective nitrogen fixing symbiosis on soybean. A molecular and phenotypic characterization of the mutated regions allowed the identification of new symbiotic genes involved in nodulation competitiveness and host specific nitrogen fixation. The regulation of these genes was determined by primer extension and RNA hybridization using B. japonicum mRNA from three different growth conditions, aerobic, anaerobic, and bacteroids (i.e., the symbiotic form).

CHAPTER II

MATERIALS AND METHODS

Bacterial strains, plasmids and phage

Bacterial strains, plasmids and phage used in this work are listed in Table 2. Details of newly constructed strains and plasmids are given in the text.

Bacterial culture media and growth conditions.

B. japonicum strains were cultured at 30^{0}C in minimal medium containing HM salts plus arabinose and gluconate (Cole and Elkan, 1973) or RDY medium (Nieuwkoop et al., 1987). E. coli strains were grown in LB medium (Miller, 1972) at 37^0 C. For single-stranded template DNA preparation, XL1-Blue cells harboring pBluescript II (+/-) phagemid derivatives were grown in Superbroth as described by the manufacturer (Stratagene, La Jolla, CA, 1990). When appropriate, antibiotics were added to the medium at the following final concentrations (micrograms per milliliter): B. japonicum, kanamycin (Km), streptomycin (Sm), spectinomycin (Sp), or tetracycline (Tc) (150 each); E. coli, ampicillin (Ap)(50), kanamycin (50), tetracycline (20), streptomycin (30), spectinomycin (30), and

Table 2: Bacterial strains, plasmids, and phage

Strains	Relevant characteristics	Source/reference
Bacteria strains		
B.japonicum		
USDA110	Wild type; colony type I110	Kuykendall and Elkan 1976
NAD163	USDA 110 (Δ pRjUT14, Km^r)	Deshmane 1988
NAD164	USDA 110 (Δ pRjUT24, Km^r)	Deshmane 1988
NAD14	USDA 110 (pND63::Tn5, Km^r, Sm^r)	Deshmane 1988
ZB940	USDA 110 (pZB886::Tn5::lacZ, Km^r)	This laboratory
ZB941	USDA 110 (pZB891::Tn5::lacZ, Km^r)	This laboratory
AN218	USDA 110 (pAN34::Tn5, Km^r, Sm^r)	This laboratory
JC143	USDA 110 (pND143::Tn5, Km^r, Sm^r)	This study
JC890	USDA 110 (pZB890::Tn5::lacZ, Km^r)	This study
BjjC208	USDA 110 (Δ pJY208, Sm^r, Spc^r)	This study
BjjC211 -1,-2,-3	USDA 110 (pJY211::Ω, Sm^r, Spc^r)	This study
Bj2101	USDA 110 (fixRnifA, Km^r)	Chelm and Adams
E.coli		
S17-1	hsdR thi pro recA; RP4-2 kan::Tn7 tet::Mu integrated in the chromosome	Simon et al. 1983
XL1-blue	recA1 lac endA1 gyrA96 thi hsdR17 supE44 relA1 (F' proAB $lacI^q$ lacZΔM15 Tn10)	Stratagene
Plasmids		
pBluescript II SK + and -	Ap^r, Sequencing vector	Stratagene

Table 2 (continued)

Strains	Relevant characteristics	Source/reference
pUC18	Ap^r, cloning vector	Vieira and Messing 1982
pSUP202	Ap^r Cm^r Tc^r, Mob^+	Simon et al. 1983
pUC4-KISS	Km^r cassette	Pharmacia
pUC4-KIXX	Km^r cassette	Pharmacia
pHP45Ω	Sm^r, Spc^r cassette	Fellay et al. 1987
pRjUT14	pHC79 clone of B.japonicum	Russell et al. 1985
pRjUT24	pHC79 clone of B.japonicum	Russell et al. 1985
pRK2013	Km^r, Helper plasmid	Figurski and Helinski 1979
pZB890, pZB886 pZB891	Tn5::lacZ insertion in 7.0kb EcoRI fragment of pRjUT14	This laboratory
pND63, pND143, pAN34	Tn5 insertion in 7.0kb EcoRI fragment of pRjUT14	This laboratory
pRi676	amp^r, nifDK	Fisher and Hennecke 1984
pJY400	1.4kb EcoRI fragment containing parts of nifD and nifK from pRj676 subcloned in pBluescriptII SK+	This study
pJY14-7	7.0kb EcoRI fragment from pRjUT14 subcloned in pSUP202	This study
pJY100	3.8kb ClaI-SacI fragment from pJY14-7 subcloned in pBluescriptII SK+	This study
pJY101	2.0kb NruI-SacI fragment from pJY100 subcloned in pBluescriptII SK+	This study

Table 2 (continued)

Strains	Relevant characteristics	Source/reference
pJY102	2.0kb HindIII-SacI fragment from pJY101 subcloned in pUC18	This study
pJY103	2.0kb HindIII-EcoRI fragment from pJY102 subcloned in pBluescriptII SK-	This study
pJY104	1.4kb NruI fragment from pJY100 subcloned in pBluescriptII SK-	This study
pJY105	2.6kb SfuI-SacI fragment from pJY100 subcloned in pBluescriptII SK+	This study
pJY107	0.6kb SalI fragment from JY101 subcloned in pBluescriptII SK-	This study
pJY108	0.4kb NruI-SalI fragment from pJY101 subcloned in pBluescriptII SK-	This study
pJY200	5.8kb XhoI fragment from pRjUT14 subcloned in pBluescript II SK+	This study
pJY201	1.6kb EcoRI-BamHI fragment within 5.8kb of pJY200 subcloned in pBluescript II SK+	This study
pJY202	1.6kb EcoRI-BamHI fragment within 5.8kb of pJY200 subcloned in pBluescript II SK-	This study
pJY2021	0.3kb SacI-EcoRI fragment from pJY202 subcloned in pBluescript II SK-	This study
pJY2022	1.3kb BamHI-SacI fragment from pJY202 subcloned in pBluescript II SK+	This study
pBluescript II SK+ BR$^-$	BamHI-EcoRI fragment in polylinker site of pBluescript II SK+ deleted	This study
pBluescript II SK- BR$^-$	BamHI-EcoRI fragment in polylinker site of pBluescript II SK- deleted	This study

Table 2 (continued)

Strains	Relevant characteristics	Source/reference
pJY204	5.8kb XhoI fragment from pJY200 subcloned in pBluescript II SK- BR	This study
pJY205	5.8kb XhoI fragment from pJY200 subcloned in pBluescript II SK+ BR	This study
pJY206	1.6kb BamHI-EcoRI fragment within 5.8kb XhoI of pJY205 replaced with 45Ω cassette	This study
pJY207	6.2kb XhoI fragment from pJY206 subcloned in XhoI site of pUC4-KIXX	This study
pJY208	6.2kb EcoRI fragment from pJY207 subcloned in pSUP202	This study
pJY209	5.8kb XhoI fragment from pJY200 subcloned in SalI site of pSUP202	This study
pJY210	45Ω cassette subcloned in SmaI site of pUC4-KISS	This study
pJY211	3.3kb SacI fragment containing 45Ω cassette from pJY210 inserted into SacI site within 5.8kb of pJY209	This study
Phage		
L408	Helper phage for single-stranded DNA isolation	Stratagene

chloramphenicol (Cm)(30). Anaerobic growth of B. japonicum was achieved in KNO_3 medium containing 10 mM KNO_3 (Stacey et al., 1994).

Bacterial genetic techniques

Bacterial transformation

Transformations of plasmids into E. coli were performed as follows. Appropriate E. coli strains were grown overnight in 5 ml of LB medium (with or without appropriate antibiotics) at 37^0C. The culture was diluted 100-fold in 100 ml of LB medium (with or without antibiotics) and grown until an OD_{600} of 0.3 was reached. Cells were pelleted at 8000rpm for 10 min. at 4^0C and resuspended in 1/10th volume of 0.1M $MgCl_2$. The cells were again pelleted at 3500rpm for 10 min. at 4^0C and then resuspended in 1/5th volume of 0.1M $CaCl_2$. The cell suspension was then placed on ice for 30 min. prior to addition of plasmid DNA. DNA was then added to 150 μl aliquots of cells and the cells were placed on ice for 30 min. The cells were then heat shocked at 37^0C for 5 min. One ml of LB medium was then added. The cells were incubated at 37^0C for 60 min. and then plated to an appropriate selective medium.

Bacterial conjugation

Bacterial conjugations were done by the triparental

mating system as described by Ditta et al. (1980). B. japonicum strains, as recipient, were grown in HM medium at 30°C to early stationary phase. E. coli helper strain (pRK2013) and donor strains were grown overnight at 37°C in 5 ml of LB broth containing the appropriate antibiotics. One ml of each culture was pelleted at 4000 rpm for 5 min. The cells were resuspended in 1 ml of HM medium and re-centrifuged. Cells were resuspended in 100 µl of HM medium, mixed, and spotted onto a HM agar plate. The plate was incubated at 30°C for 48 hours. Bacteria were washed from the surface using an HM salt solution (HM medium minus carbon source) and plated to appropriate selective media. Donors, helpers, and recipients were plated individually to selective media as controls.

DNA manipulation

DNA isolation

Plasmid DNA was isolated on a small scale by using the alkaline lysis method as described by Sambrook et al. (1989) or by using the Magic™ Minipreps DNA Purification System (Promega Corporation, Madison, WI). For large scale isolation of plasmid DNA, the Triton-lysozyme lysis method described in Sambrook et al.(1989) or the Magic™ Maxipreps DNA Purification System (Promega Corporation, Madison, WI) was used.

Chromosomal DNA from B. japonicum was isolated on a small scale as follows. Cultures were grown in RDY medium until late log phase was reached. Three ml of the culture was pelleted and washed with 1.5 ml of TNE buffer (Sambrook et al. 1989) containing 0.5% sarcosyl. The pellet was resuspended in 0.45 ml of TE buffer. 0.15 ml of a 5 % SDS solution and 0.15 ml of a 2.5 mg/ml solution of Proteinase K were then added to the resuspended cells, mixed, and incubated at 37^{0}C for one hour. The solution was then extracted with phenol/chloroform 3 - 5 times and DNA was precipitated by adding two volumes of ethanol.

DNA fragment extraction

DNA restriction endonuclease fragments used in subcloning were isolated by electrophoresis in agarose gels and extraction by the Geneclean II kit (Bio 101 Inc., La Jolla, CA).

Southern blotting and hybridization

Transfer of electrophoresed DNA to Nylon filters (Amersham Corporation, Arlington Heights,IL) was done as described by Southern (1975). Hybridizations were carried out at 65^{0}C as recommended by the Amersham protocol. For high stringency washes, the filters were washed twice for 15 min at 65^{0}C with 2X SSC, once for 30 min at 65^{0}C with 2X SSC, 0.1% SDS, and finally for 10 min at 65^{0}C with 0.1X

SSC. Low stringency washes were done as above but at 45^{0}C. The filters were air-dried and autoradiographed with Kodak X-OMAT AR film at -80^{0}C. ^{32}P-labelled radioactive probes were prepared by nick translation using α ^{32}P-dATP (ICN Biochemical, Irvine, CA) as recommended by the manufacturer.

All B. japonicum mutants constructed in this study were confirmed by Southern hybridization.

Colony hybridization

Colony hybridization of B. japonicum was performed as recommended by the Amersham protocol with minor modification. The colonies were transferred to Nylon filters (Amersham Corporation, Arlington Heights, IL) by laying the filters on the plates at 30^{0}C for 3 - 5 hours. The filters with adhering colonies were placed on Whatman paper saturated with denaturation solution (0.5 N NaOH) for 7 min, and then transferred to Whatman paper saturated with neutralization solution (1M Tris pH 7.5) for 1 minute. This final step was repeated twice. The blots were air-ydried and fixed under UV light for 3 min. Hybridizations were carried out at 65^{0}C as described previously for Southern hybridization.

RNA isolation and dot blot hybridization

RNA isolation

B. japonicum strains were grown with shaking at 200 rpm in 500 ml flasks containing 100 ml of RDY medium with appropriate selection. Anaerobic growth in the presence of 10mM KNO_3 was performed in 15 ml Falcon tubes that were filled to the top with nitrate medium inoculated with approximately 10^7 cells. When the cultures were in the late log phase, cells were harvested and frozen under liquid nitrogen, then stored at -70^{0}C for later use. B. japonicum USDA110 bacteroids were kindly provided by Dave Weaver (University of Tennessee). Bacteroids were isolated from soybean nodules 21 days after plant inoculation. Nodules (approximately 1 g) were macerated with a homogenizer in 0.5 ml of bacteroid extraction buffer (i.e., 0.5M mannitol, 20mM sodium succinate, 5mM sodium dithionite, 25mM Tris-HCl [pH 7.5]). Plant debris was collected by centrifugation at 1,500 rpm for 5 min.(Beckman SS-34 rotor). The supernatant was then transferred to a clean tube and centrifuged at 14,000 rpm for 10 min. to collect the bacteroids. Total RNA from B. japonicum free-living culture cells (aerobic or anaerobic cultures) and the isolated bacteroid cells were isolated using the hot phenol extraction method as previously described (Wang et al., 1991). Any contaminating DNA in the RNA samples was removed by digestion with RNase-free DNase I (Promega Corp., Madison, WI).

RNA dot blot hybridization

RNA (0.5 - 10 μg) was blotted onto nitrocellulose or Nylon filters (Amersham Co., Arlington Heights, IL) as described by Sambrook et al. (1989). Hybridization was done at 42^{0}C in a solution of 10% dextran sulfate, 50% formamide, 5X SSPE, and 5X Denhart's reagent as recommended by the Amersham protocol. After hybridization for 48 hours, RNA filters were washed for 20 min at room temperature in 1X SSC, 0.1% SDS, followed by three washes of 20 min each at 68^{0}C in 0.2X SSC, 0.1% SDS. Autoradiography was done with Kodak X-OMAT AR film at -80^{0}C. The blots were subsequently stripped by washing in 5 mM Tris-HCl, pH8.0, 2 mM Na_2EDTA, 0.1 X Denhardt's solution for 2 hours at 65^{0}C. These blots were then rehybridized with a DNA fragment coding for the 23S ribosomal RNA from Pseudomonas aeruginosa (Festl et al., 1986).

Enzymes and isotopes

DNA restriction endonucleases, DNA polymerases, and DNA ligase were obtained from Promega Corp. (Madison, WI), U.S.Biochemical (Cleveland, OH), Bethesda Research Laboratories (Gaithersburg, MD), or New England BioLabs (Beverly, MA). Reaction conditions for these enzymes were used as recommended by the manufacturer. Radioisotopes were obtained from either ICN Biomedical (Irvine, CA) or New

England Nuclear (Wilmington, DE).

DNA sequencing

DNA sequences were determined by the dideoxy chain termination method (Sanger et al., 1977) using ^{35}S-ATP. A 2269 base pair (bp) DNA fragment encompassing the nfeC gene region and a 1584 bp DNA fragment containing the hsfA gene region were sequenced in both orientations using Sequenase™ or Taquence™ polymerases (United States Biochemical, Cleveland, OH) according to the manufacturer's protocol. The sequences of transposon insertion positions were determined by sequencing the Tn5-flanking regions of B. japonicum mutants using a Tn5-specific oligonucleotide, 5' CAGGACGCTACTTGT 3', as primer.

Cloning procedure

To sequence the region important for interstrain competition for nodule formation (i.e., nfeC), a 7 kb EcoRI fragment from pRjUT14 was cloned into the EcoRI site of the vector pSUP202 to produce plasmid pJY14-7. This 7 kb fragment included the insertion site of the NAD14 mutation. A 3.8 kb ClaI/SacI fragment from the 7.0 kb insert of pJY14-7 was subcloned into the ClaI/SacI sites of pBluescript II SK+, resulting in plasmid pJY100. Restriction mapping of the 3.8 kb insert of pJY100 indicated

that an internal 2.0 kb NruI/SacI fragment contained the locations of the various Tn5 insertions found in strains NAD14, ZB940, ZB941, and JC143. Therefore, this fragment was cloned into the EcoRV/SacI sites of pBluescript II SK+. This gave rise to plasmid pJY101. To sequence the opposite strand of pJY101, the plasmid pJY101 was digested with SacI and HindIII (from the polylinker site of vector pBluescript I SK+). The 2.0 kb SacI/HindIII fragment from pJY101 was cloned into the SstI/HindIII sites of pUC18 (resulting in plasmid pJY102) and subsequently digested with EcoRI and HindIII, which cut within the polylinker region, releasing a 2 kb fragment. This fragment was cloned into the EcoRI-HindIII sites of pBluescript II SK- to produce plasmid pJY103. Further sequencing of the region required the subcloning of a 1.4 kb NruI fragment and a 2.6 kb SfuI/SacI fragment from the 3.8 kb insert of pJY100 into the EcoRV site and the SacI/ClaI sites of pBluescript II SK- (yielding plasmid pJY104) and SK+ (plasmid pJY105), respectively.

To sequence the region responsible for host specific nitrogen fixation on cowpea, a 5.7 kb XhoI fragment from pRjUT14 was cloned into the XhoI site of pBluescript II SK+ resulting in plasmid pJY200. Restriction mapping of the 5.7 kb insert of pJY200 indicated that an internal 1.6 kb EcoRI/BamHI fragment encompassed the locations of the various Tn5 insertions found in strains AN218, NAD122, NAD124, and NAD150. Therefore, this fragment was cloned

into the EcoRI/BamHI sites of pBluescript II SK+ (yielding plasmid pJY201) and SK- (plasmid pJY202). The plasmid pJY202 has two SacI sites; one in the polylinker region of the vector and the other within the 1.6 kb insert DNA. A 1.3 kb SacI fragment in pJY202 was removed and the plasmid religated. This resulting clone contains only a 0.3 kb SacI/EcoRI insert and is called plasmid pJY2021. Plasmid pJY202 was digested with BamHI and SacI releasing a 1.3 kb fragment which was subcloned using the same enzymes into pBluescript II SK+ resulting in plasmid pJY2022.

Construction of a nested set of deletions

Plasmids pJY101 and pJY103 encompassing the nfeC region and plasmids pJY201 and pJY2022 including the hsfA region were used for the construction of a nested set of deletions to aid in sequencing. Deletions were generated by the exonuclease III digestion procedure of Henikoff (1984) with minor modifications. Each clone was first digested with a suitable enzyme to generate a unique 3' overhang and subsequently with another enzyme to produce a unique 5' or blunt end that must lie between the insert DNA and the 3' site chosen. The DNA was then treated with Exonuclease III (15 unit / 1 μg DNA) (USB, Cleveland, OH) at 37^{0}C. After adding ExoIII to the reaction, 2 μg DNA (20 μl) was removed at each of the time intervals (30 - 60 sec.) and transferred directly to a tube containing diluted S1 nuclease buffer

(175 μl) (Sambrook et al., 1989). The tube was immediately heated at 68°C for 15 min and then placed on ice. Each tube was then amended with 40 units (5 μl) of S1 nuclease and incubated for 30 min at 37°C. The reaction (200 μl) was stopped by addition of 1/10th volume (22 μl) of S1 Stop buffer (Sambrook et al., 1989) followed by heating at 68°C for 15 min. The DNA was phenol:chloroform extracted, ethanol precipitated, and resuspended in 10 μl of TE buffer. Two μl of the resuspended DNA was used for gel electrophoresis. 0.5 μg of the remaining DNA was directly used in the ligation reaction. After incubation at 4°C overnight, the ligation mixture was added to competent E. coli (XL1-Blue) cells prepared as previously described and plated on LB plates containing antibiotics (Ap/Tc).

Single-stranded or double-stranded template DNA isolation

Single-stranded template DNA was isolated as follows. A single colony from a Tc/Ap plate was grown overnight in 3 ml of LB medium with tetracycline and ampicillin. The culture was diluted 100-fold in 10 ml of Superbroth (Per liter: Bacto-tryptone 10g, Bacto-yeast extract 20g, NaCl 5g, adjusted to pH 7.5 with NaOH) (without antibiotics) and grown with vigorously shaking (225 rpm) until an OD_{600} of 0.3 was reached. Helper phage R408 was added to the culture at a multiplicity of infection of 20:1 (phage : cells). The infected culture was then incubated with shaking for 8 hours

at 225 rpm. Cells were pelleted at 8000rpm with a JA14 rotor for 15 min and the supernatant was tranferred into a 30 ml OakRidge tube. The bacteriophage particles were precipitated by adding 0.25 vol. of phage precipitating buffer (20% polyethylene glycol (PEG8000) in 3.75 M ammonium acetate, pH7.5), incubating at room temperature for 15 min, and centrifuging for 15 min at 10,000 rpm. The pellet was resuspended in 700 μl of TE buffer (pH8.0). The resuspended solution was extracted with phenol/chloroform 3-5 times followed with chloroform 2 times. The DNA was precipitated by adding an equal volume of 7.5 M ammonium acetate,pH7.5 and 2 volumes of ethanol.

For the isolation of double-stranded DNA template, the alkali denaturation method for supercoiled plasmid DNA was used as recommended by the Promega Corp. (1988) TaqTrack™ Sequencing Systems Technical Manual.

Preparation of sequencing gels

The protocol for the preparation of 0.2 mm sequencing gels is as follows:

1. Wash large (33.5 X 42 cm) and small (33.5 X 39.5 cm) plates in cold detergent solution and place the clean plates on a level surface. The level surface is very important in this method. Dry the plates by swabbing with paper towel soaked in 95% ethanol. Hydrophobic spots can be removed from the plates with a paper towel soaked in 1 M KOH in

ethanol followed by 3 rinses with water.

2. Attach adhesive tape (Highland™ Brand Masking Tape "2600",) as a spacer on both sides and the middle of the large plate. The middle tape spacer makes the gel even and prevents gel smiling during electrophoresis. This tape sticks stably to the plate even during fixation of the gel or washing of the plate. Therefore, this tape can be used repeatedly as a spacer until the tape is wrinkled or torn. If it is necessary to remove this tape from the plate, the tape can be easily removed under hot tap water (about 60 ^{0}C).

3. Make a dam with the same tape at both sides and the bottom of the large plate in order to prevent overflow of the sequencing gel solution.

4. Siliconize the dried small plate by spraying with PAM (American Home Food Products Inc., NY) and then polishing with a dry Kimwipe. It is important to remove the excess oil from the plate because it produces a smeared background.

5. Filter 80 ml of acrylamide stock solution containing 6% acrylamide, 8 M urea and TBE (89 mM Tris-HCl, 89 mM borate, 2 mM EDTA, pH 8.3), through a nitrocellulose filter (Millipore, 0.45 μM pore size, Millipore Co., Bedford, MA) (Once skilled in this procedure, 70 ml of gel solution is enough for a gel of 33.5 X 39.5 cm). Add 0.9 ml ammonium persulfate (100mg/ml) and 30 μl of TEMED (N,N,N',N'-tetramethylethylenediamine) at room temperature (15-20 ^{0}C).

6. Pour this solution immediately and directly onto the ready large plate along the middle tape beginning at the bottom of the plate. Otherwise, air bubbles may form.

7. Spread the solution by sliding the siliconized-small plate slowly from the bottom onto the large plate. Since the dam on the large plate prevents overflow of the gel solution from the bottom and both sides, sliding the small plate onto the large plate is easy and does not produce air bubbles in the gel.

8. Insert the 0.2 mm shark's tooth combs (IBI, 32 teeth, cut from the original 64-teeth comb) between the two plates and on both sides of the middle tape.

9. Place a weight (600g-1000g) on each corner and over the middle tape.

10. Allow time for the gel to polymerize. Time for polymerization varies according to temperature (e.g., 5 - 15 ^{0}C, 40 min - 1 hour; 15 - 30 ^{0}C, 1-4 hours; below 5 ^{0}C, polymerizes while pouring; over 30 ^{0}C, very delayed).

11. After polymerization, remove the weights and tapes used as a dam, and clean any dried gel material from the outside of the gel plates.

12. The sequencing gel is now ready for use.

Fixation and autoradiography of sequencing gels

The protocol for fixation and autoradiography of sequencing gels is as follows:

1. After electrophoresis, remove the siliconized small plate leaving the gel sticking to the large plate. Bake the large plate in a 80^0C oven for 30 min.

2. After the plate has cooled, place it inside a large tray with the top elevated approximately 15^0 degrees off the bottom.

3. Slowly pour 500 ml of a 5% acetic acid and 5% methanol solution from the top of the gel to remove urea from the gel. Repeat this 4 times. The 5% acetic acid and 5% methanol solution can be collected from the tray and reused until the smell of the acetic acid is very weak (about 10 cycles).

4. Dry the gel in a 80^0C oven for 40 min. The dried gel should feel smooth to the touch but not sticky.

5. Once the plate has cooled, place an X-ray (Kodak X-OMAT AR) film directly onto it. Another clean plate is placed on the top of the X-ray film for weight. The film is exposed for 13 - 24 hours at room temperature and subsequently developed. After autoradiography, to remove the dried gel from the plate, place it into a tray containing cold water for 10 - 30 minutes and remove the gel from the plate by rubbing with a sponge. The use of cold water prevents removal of the adhesive tape spacer.

6. Autoradiographs were read mannually and compressions were resolved by substituting a nucleotide analog for dGTP (dITP or 7-deaza dGTP) or using Taquence™ DNA polymerase

instead of Sequenase.

Sequencing analysis

Computer-assisted sequence analysis was done using the Microgenie program (Beckman) and the programs of the University of Wisconsin (Madison, WI) Genetics Computer Group (GCG).

Primer extension.

The transcriptional start sites of the nfeC and hsfA genes were determined using the primer extension procedure described by Kassavetis et al. (1982). The following oligonucleotide primers were used; for nfeC, 5'-GTCTTCCAGGCATTCTCTTCATA-3' (23-mer) and 5'-AATCCGACCACGCTAAGCCATA-3' (22-mer), which are complementary to bases +25 to +47 and +54 to +75 of the nfeC coding sequence, respectively, and for hsfA, 5'-CCAACAGAAATATGCCAAGCT-3', which is complementary to bases +7 to +27 of the hsfA coding sequence. End labeling of the primers was carried out for 1 hour at 37 ^{0}C in a volume of 25 μl with 0.1 μg of DNA primer, 240 μCi of [γ-^{32}P]ATP, and 4 units of T4 polynucleotide kinase using the buffer suggested by the supplier (USB, Cleveland, Ohio). The kinase reaction was stopped by incubating at 65 ^{0}C for 5 min. The unincorporated [γ-^{32}P]ATP was removed by

precipitating twice by the addition of 25 μl of 4M ammonium acetate and 250 μl of ethanol at -70 ^{0}C for 30 min. The labeled primer resuspended in 100 μl of sterile distilled water was hybridized to 20 μg of RNA by precipitating with 4M ammonium acetate and ethanol, resuspending the pellet in 30 μl of hybridization buffer (20mM Tris pH 8.0, 200mM NaCl, 0.1mM EDTA), denaturing at 100 ^{0}C for 3 min, and then incubating at 63 ^{0}C for 1.5 hours. Size standards were obtained by using the same primer in a dideoxy sequencing reaction with plasmid pJY105 (for nfeC) and plasmid pJY201 (for hsfA).

Construction of B. japonicum mutants

For the construction of mutant BjjC208, a 5.8 kb XhoI fragment of pJY200 was subcloned into the XhoI site of pBluescript II SK+BR$^-$, in which the BamHI/EcoRI fragment in the polylinker site was removed, made blunt with Klenow enzyme, and religated. The resulting plasmid was called pJY205. A 1.6 kb BamHI/EcoRI fragment internal to the 5.8 kb XhoI fragment of pJY205 was removed, made blunt with the Klenow enzyme, and replaced with a 2.0 kb SmaI fragment (encoding streptomycin/spectinomycin resistance) from vector 45Ω yielding plasmid pJY206. The 6.2 kb XhoI fragment from pJY206 was subcloned into the XhoI site of pUC-4-KIXX to produce plasmid pJY207. This construct has only two EcoRI

sites located within the polylinker region derived from vector pUC-4-KIXX. Therefore, digestion with EcoRI releases a fragment with an internal 6.2 kb XhoI fragment which was subcloned into the EcoRI site of the mobilizable vector pSUP202 resulting in plasmid pJY208. In this construction the entire region mutated in strains AN218, NAD124 NAD150, and NAD122 was replaced by the streptomycin/spectinomycin resistance cassette.

For the construction of mutant BjjC211, the 5.8 kb XhoI fragment of pJY200 was subcloned into the SalI site of the vector pSUP202 yielding plasmid pJY209. This plasmid has an unique SacI site in the hsfA coding region. The streptomycin/spectinomycin resistance cassette from vector 45Ω was inserted into this SacI site disrupting the hsfA gene and yielding plasmid pJY211. The cassette having the SacI sites at both ends was prepared by cloning the fragment encoding streptomycin/spectinomycin resistance from vector 45Ω by digestion with SmaI and ligation into the SmaI site of vector pUC-4-KISS resulting in plasmid pJY210. Digestion of pJY210 with SacI releases a 3.3 kb fragment containing the antibiotic resistance genes. This is the fragment used to disrupt the hsfA coding region.

The mutations formed in plasmids pJY208 and pJY211 were transferred to the B. japonicum USDA110 genome by homologous recombination following triparental conjugation (see above).

B. japonicum transconjugants were selected on RDY agar plates plus streptomycin/spectinomycin (Sm/Sp). Chloramphenicol (30 μg/ml) for pJY208 or tetracycline (10 μg/ml) for pJY211 was used to counterselect against the E. coli donor and helper strains. To distinguish double cross-over events (true replacements) from single crossover events (co-integrations), in the case of BjjC208 transconjugants, Sm/Sp-resistant transconjugants were screened on RDY medium containing streptomycin, spectinomycin, and tetracycline. Sensitivity to tetracycline was presumptive evidence for gene replacement. However, since pJY211 does not have the tetracycline selection marker, in the case of BjjC211 transconjugants, colony hybridization was used to screen for the absence of pSUP202 DNA using ^{32}P labelled plasmid DNA as a probe. The correct genomic structures of the marker exchange mutants (resulting from double cross-over events) were confirmed by appropriate Southern blot hybridizations.

Plant infection tests

Plants used in this study were soybean (Glycine max cv. Essex and G. soja PI468397), siratro (Macroptilium atropurpureum), and cowpea (Vigna unguiculata cv. Purple Hull). G. max and cowpea seeds were surface sterilized by soaking in a 20% chlorox solution for 10 min. Seeds were

then rinsed three times with sterile distilled water, placed in 0.01 M HCl for 10 min, and rinsed with sterile water five-times. G. soja and siratro seeds were surface sterilized by soaking in 95% ethanol for 30 sec. Following 3 rinses with sterile water, seeds were further soaked in 0.1% $HgCl_2$ for 5 min, and then rinsed with sterile water five-times. Sterilized seeds were germinated on sterile, moist filter paper for 2-3 days in the dark at room temperature. Seedlings were transferred, depending on the experimental purpose, either to sterile 60 ml serum vials containing vermiculite saturated with plant nutrient solution (Wacek and Brill, 1976), to sterile Leonard jar assemblies containing 50% sand, 50% vermiculite (Vincent, 1970), or to sterile plastic growth pouches (Vaughan's Seed Co., Downers Grove, IL). Plants were inoculated using (1ml/plant) cultures of B. japonicum. Actual cell concentrations of the inoculum were checked by viable plate counting. Plants were watered as necessary with a nitrogen-free nutrient solution (Wacek and Brill, 1976). Upon completion of the assays, bacteria were isolated from the nodules to ensure that they were only occupied by the applied bacterial inoculum (Somasegaren and Hoben, 1985).

Nodulation tests.

To determine the time course of nodulation, seedlings germinated as above were transferred to sterile plastic

growth pouches and inoculated with 10^6 cells of the appropriate bacterial culture. For each strain, 12-18 individual plants were used. After inoculation, the number of nodulated plants and the number of nodules per plant were recorded at regular time intervals.

Acetylene reduction assays

Nitrogen fixation activity was determined by the acetylene reduction assay as previously described (Wacek and Brill, 1976). Seedlings were transferred to sterile plastic growth pouches or sterile 60 ml serum vials containing vermiculite saturated with plant nutrient solution. Seedlings were inoculated with either 10^6 cells or 1ml of an early stationary phase (about 10^9) of the appropriate bacterial culture. After 18-21 days post-inoculation, whole nodulated roots were placed in a 60 ml vial which was then sealed with a serum stopper. Acetylene was injected to provide a final partial pressure of approximately 0.1 atm. After incubation at room temperature for one hour, the acetylene reduction activity was determined by injecting the gas sample (0.2 - 1 ml) from the vial into a gas chromatograph and measuring the amount of ethylene formed. A Shimadzu GC-8A gas chromatograph (Shimadzu Corp., Kyoto, Japan) with a flame ionization detector and fitted with a stainless steel column packed with Porapak-R was used to separate and measure the hydrocarbons. The operating

conditions were as followed; nitrogen carrier gas pressure, 6kg/cm^2; air pressure, 0.1 kg/cm^2; hydrogen gas pressure, 0.7 kg/cm^2; injector, detector, and column temperatures were maintained at 100^0C, 100^0C, and 75^0C, respectively.

Competition assays.

Twelve soybean plants were used for each strain in the competition assays. Competition experiments were designed to compare the mutants with wild-type strain USDA110 for nodule occupancy on soybean. Suspensions of two strains were adjusted to the same density (OD600 = 0.1) and mixed to the desired ratio 1:1, 1:10, 1:100, 10:1, and 50:1 (USDA110/Tn5 mutant) before inoculation. This suspension was diluted to a density of 2 x 10^5 cells per milliliter, and 1 ml/root was applied immediately to 3-day-old soybean seedlings in growth pouches (Vaughan's Seed Company, Downers Grove, IL). The actual cell concentration of each inoculum was checked by viable plate counts. After 19-20 days, nodules were collected, rinsed in 70% ethanol, washed twice in sterile distilled water, and immersed in 0.1% $HgCl_2$ in 0.06 N HCl for 5 min. Nodules were then washed five times in sterile distilled water. Following this pretreatment, the nodules were crushed and streaked on RDY agar with or without the appropriate antibiotics (i.e., kanamycin for the presence of Tn5). Plates were incubated at 30^0 C for 5-7 days, and the identity of strains was determined based on

the resistance to antibiotics.

Microscopy

Soybean and cowpea plants were infected with strains USDA110, NAD163 and AN218 and grown in sterile Leonard jar assemblies containing 50% sand and 50% vermiculite (Vincent, 1970). At 15 and 28 days after inoculation, nodule samples for microscopy were prepared as previously described (Roth and Stacey, 1989) and microscopy was performed by Gerry Sexton (The University of Tennessee). For light microscopy, a Zeiss photomicroscope was used with Nomarski interference optics. For electron microscopy, a Hitachi H600 microscope was used with either 75 or 100 kV beams.

CHAPTER III

RESULTS

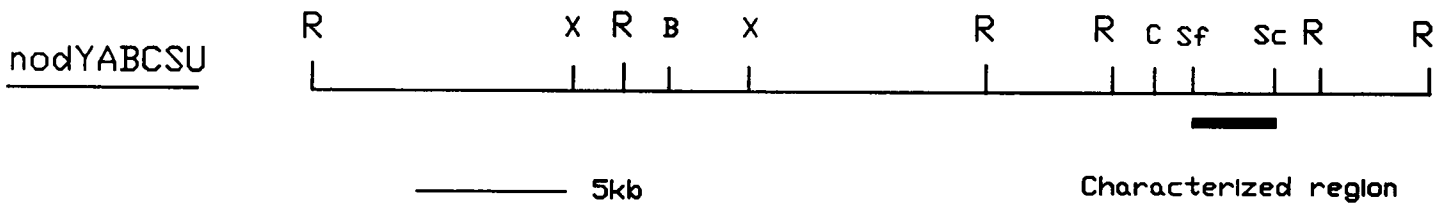
A. CLONING AND MOLECULAR CHARACTERIZATION OF A NEW SYMBIOTIC GENE INVOLVED IN NODULATION COMPETITIVENESS.

Nodulation efficiency of B. japonicum mutant NAD14.

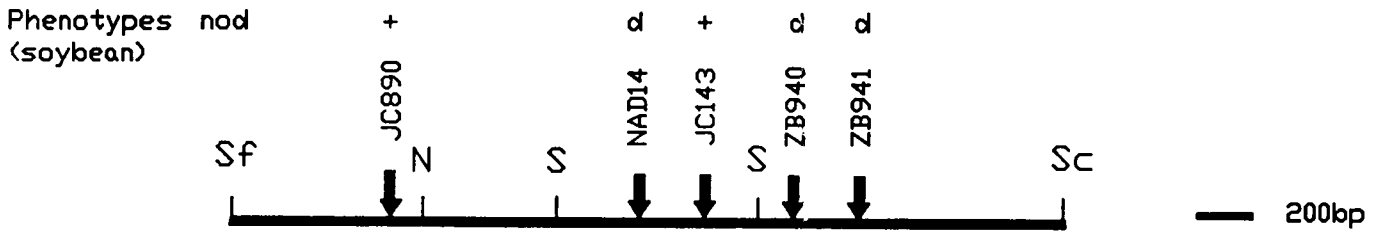
A delayed nodulation mutant NAD14 of B. japonicum, generated by site-directed Tn5 insertion mutagenesis, was previously described (Deshmane, 1988). Two additional B. japonicum mutants ZB940 and ZB941 were also isolated in this laboratory by site-directed Tn5::lacZ insertion mutagenesis and exhibited similar delayed nodulation phenotypes to that of NAD14. Restriction mapping showed that the NAD14, ZB940, and ZB941 mutations were clustered within 0.7 kb (Figure 3). In order to delimit the loci responsible for the symbiotic functions encoded in this region, a battery of Tn5-insertion mutants that had previously been obtained by random transposon mutagenesis of the cosmid clone pRjUT14 (Russell et al., 1985) was screened. Tn5 insertions were mapped by restriction endonuclease analysis, and two were found to flank the site of the Tn5 insertion in NAD14. These Tn5 insertions, ND143 and ZB890, were transferred by marker exchange into the B. japonicum genome generating strains JC143 and JC890, respectively. Strains JC143 and JC890 were tested for their ability to nodulate soybean

Figure 3. Restriction maps of plasmid pRjUT14 and characterized region. The vertical arrows indicate the position of Tn*5* or Tn*5*::*lacZ* insertions based on restriction analysis and sequencing data. The phenotypes of the inserts: +, wild-type; d, delayed nodulation on soybean.

A. pRjUT14



B. Characterized region



(i.e. cv Essex). Table 3 shows the symbiotic phenotypes of mutants NAD14, ZB940, ZB941, JC143 and JC890 on soybean. Mutants NAD14, ZB940, and ZB941 exhibited a 3 - 6 day delay in nodule formation as compared with the wild type. The average number and weight of nodules per plant induced by these mutants was similar to that of the wild type when the nodules were harvested 19-20 days after inoculation. Acetylene reduction assays indicated that the mutants were not affected in nitrogen fixation ability. JC143 and JC890 were indistinguishable from wild type. Therefore, these two mutants delimit the symbiotic locus identified by the NAD14 mutation (Figure 3).

Competition phenotype of mutant NAD14

Mutant NAD14 was further characterized for its ability to compete for nodule formation against the wild-type strain. Coinoculation experiments showed that NAD14 was less competitive relative to wild-type strain USDA110 (Table 4). When both strains were inoculated at a 10:1 or 50:1 ratio (USDA110/NAD14), 100% of nodules were occupied by the wild-type strain. Mutant NAD14 occupied less than 12% of nodules when coinoculated with the wildtype at a 1:1 ratio while at a 1:10 or 1:100 ratio (USDA110/NAD14), the mutant formed 47% and 89% of the nodules, respectively. These results indicate that the mutant NAD14 has approximately a 10-fold decrease in competitive ability when compared to

Table 3: Symbiotic phenotypes of B. japonicum mutants on soybean (Glycine max cv. Essex) plants

Strains	Fix phenotype[a]	Delay of nodulation on G. max (days)	Avg. nodule number per plant[b]	Avg. weight of nodules per plant[c] (mg)
Wild type	+	0	12 ± 2	53 ± 14
NAD14	+	3-6	11 ± 2	50 ± 8
ZB940	+	3-5	11 ± 2	55 ± 17
ZB941	+	2-4	9 ± 2	44 ± 18
JC143	+	0	10 ± 1	57 ± 10
JC890	+	0	12 ± 1	52 ± 8

[a], + = wild-type level of acetylene reduction activity.

[b] and [c], The numbers represent the mean of two experiments ± standard error. Each experiment used at least 10 plants per strain.

Table 4: Results of mixed-infection experiments with *B. japonicum* strain USDA110 and mutants

Ratio of strains within coinoculated mixture			% Nodule occupied by the following strain[a]			
			USDA110	NAD14	USDA110	JC143
A		B	A	B	A	B
USDA110			100	0	ND	ND[b]
		NAD14	0	100	ND	ND
1	:	1	88 ± 5	12 ± 5	49	51
1	:	10	53 ± 3	47 ± 3	4	96
1	:	100	11 ± 1	89 ± 1	0	100
10	:	1	100	0	ND	ND
50	:	1	100	0	ND	ND

[a] Thirty-two to fifty nodules were used for nodule occupancy determination. The numbers represent the means ± standard error of three experiments.

[b] ND, not determined.

Table 5: Results of mixed-infection experiments with *B. japonicum* wild-type strain USDA110 and mutant strains ZB940 and ZB941

Ratio of strains within coinoculated mixture			% Nodule occupied by the following strain[a]			
			USDA110	ZB940	USDA110	ZB941
A		B	A	B	A	B
1	:	1	85	15	73	27
1	:	10	36	64	24	76
1	:	100	3	97	3	97

[a] Thirty-two to fifty nodules were used for nodule occupancy determination.

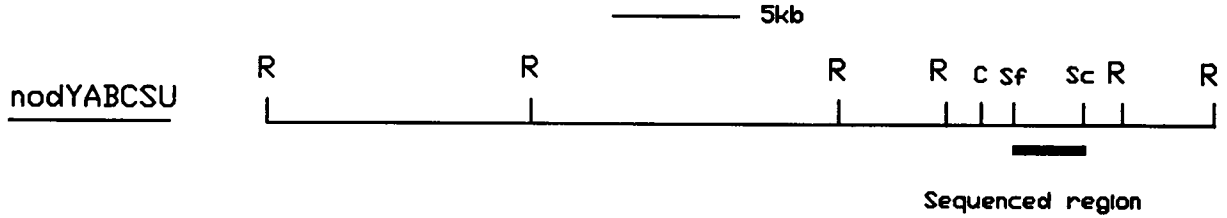
USDA110. In addition, mutants ZB940 and ZB941 were also tested for their competitiveness in nodule formation against the wild type. Both mutants were less competitive relative to the wild type (Table 5). To test whether this phenotype was a non-specific effect caused by the insertion of Tn*5* or Tn*5*::lacZ in the mutants, a Tn*5*-induced mutant JC143 which has a wild-type nodulation phenotype was used as a control strain in competition experiments. JC143 retained its competitive ability under the same assay conditions (Table 3), indicating that the reduction of competition observed with mutant NAD14 was likely due to the disruption of a gene important to nodulation. Bhagwat et al. (1991) reported that strains of *B. japonicum* with a delayed nodulation phenotype retained their competitive ability. Therefore, these data suggest that the DNA region mutated in these mutants contains a gene(s) involved in interstrain competition for nodulation.

Nucleotide sequencing and identification of *nfeC*.

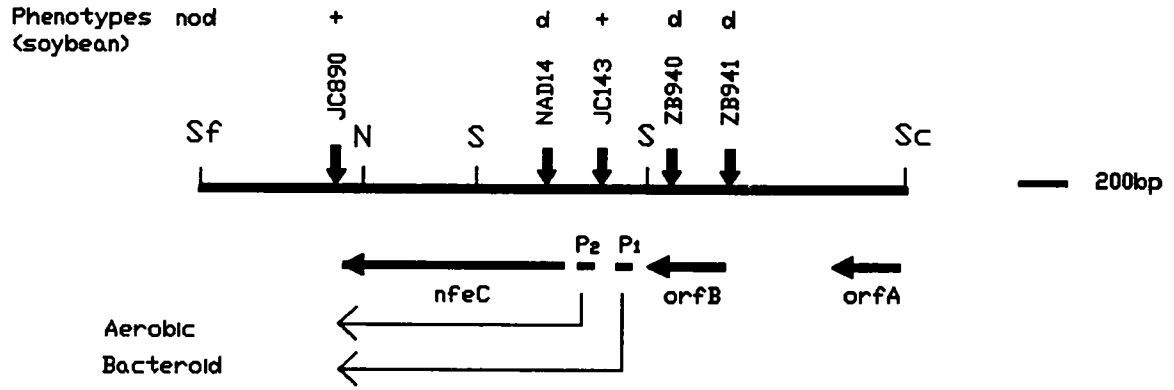
To identify the gene(s) mutated by the NAD14, ZB940, and ZB941 insertions, a 2.2kb DNA region from the wild-type *B. japonicum* strain USDA110 was sequenced. This region encompasses the location of the Tn*5* or Tn*5*::*lacZ* insertions NAD14, JC143, JC890, ZB940, and ZB941. The sequencing strategy is shown in Figure 4. The resulting DNA sequence, shown in Figure 5, was analyzed for open reading

Figure 4. Physical map of the B. japonicum nfeC gene. **A.** The nfeC gene is located on the right about 43 kb from the nodYABCSU operon. **B.** The vertical arrows indicate the position of Tn5 insertions based on sequencing data. The phenotypes of the Tn5 inserts: +, Wild-type; d, delayed nodulation on soybean. The transcription of the nfeC gene is from right to left. Promoter regions: P1, bacteroid-specific RpoN-type; P2, aerobic-specific E. coli consensus type. Restriction endonuclease sites: R, EcoRI; C, ClaI; Sf, SfuI; Sc, SacI; S, SalI. **C.** The arrows indicate the sequencing strategy. The tail end of the arrow represents the end of a deletion, and the length of the arrow represents the sequence determined from that deletion.

A. pRjUT14



B. Sequenced region



C. Sequencing strategy

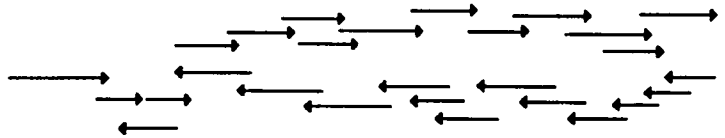
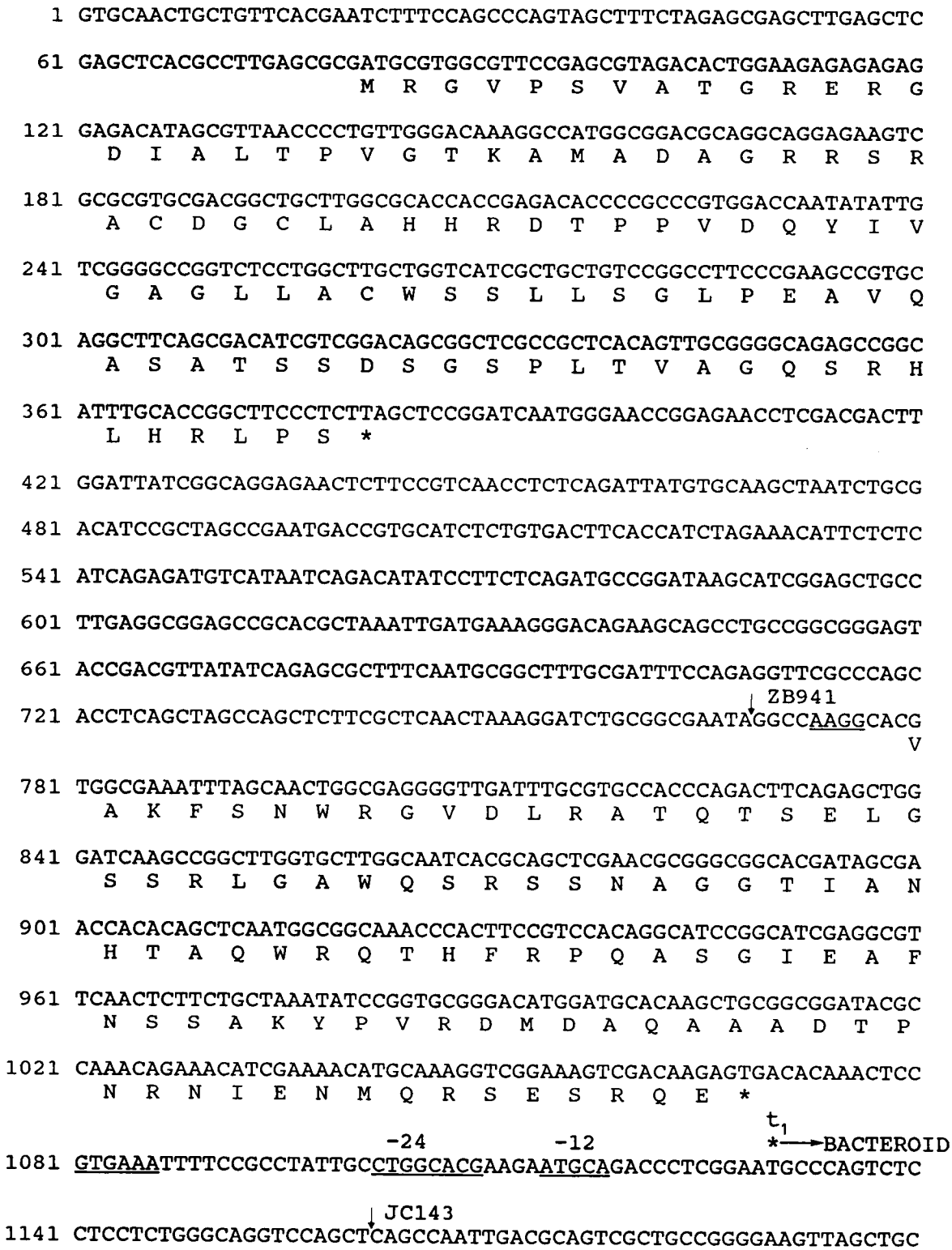


Figure 5. Nucleotide sequence of the nfeC gene with predicted amino acid sequence. The presumptive ribosome-binding site (SD) is indicated and the sites of the Tn5 insertion in the mutants, NAD14, JC143, and JC890 are marked by arrows. The two transcriptional start sites are designated by asterisks. The RpoN consensus promoter upstream of t_1 (-12/-24) (corresponding to P1) and similarity to the E. coli consensus promoter upstream of t_2 (-10/-35) (corresponding to P2) are underlined.


1 GTGCAACTGCTGTTCACGAATCTTTCCAGCCCAGTAGCTTTCTAGAGCGAGCTTGAGCTC
61 GAGCTCACGCCTTGAGCGCGATGCGTGGCGTTCCGAGCGTAGACACTGGAAGAGAGAGAG
M R G V P S V A T G R E R G
121 GAGACATAGCGTTAACCCCTGTTGGGACAAAGGCCATGGCGGACGCAGGCAGGAGAAGTC
D I A L T P V G T K A M A D A G R R S R
181 GCGCGTGCGACGGCTGCTTGGCGCACCACCGAGACACCCCGCCCGTGGACCAATATATTG
A C D G C L A H H R D T P P V D Q Y I V
241 TCGGGGCCGGTCTCCTGGCTTGCTGGTCATCGCTGCTGTCCGGCCTTCCCGAAGCCGTGC
G A G L L A C W S S L L S G L P E A V Q
301 AGGCTTCAGCGACATCGTCGGACAGCGGCTCGCCGCTCACAGTTGCGGGGCAGAGCCGGC
A S A T S S D S G S P L T V A G Q S R H
361 ATTTGCACCGGCTTCCCTCTTAGCTCCGGATCAATGGGAACCGGAGAACCTCGACGACTT
L H R L P S *
421 GGATTATCGGCAGGAGAACTCTTCCGTCAACCTCTCAGATTATGTGCAAGCTAATCTGCG
481 ACATCCGCTAGCCGAATGACCGTGCATCTCTGTGACTTCACCATCTAGAAACATTCTCTC
541 ATCAGAGATGTCATAATCAGACATATCCTTCTCAGATGCCGGATAAGCATCGGAGCTGCC
601 TTGAGGCGGAGCCGCACGCTAAATTGATGAAAGGGACAGAAGCAGCCTGCCGGCGGGAGT
661 ACCGACGTTATATCAGAGCGCTTTCAATGCGGCTTTGCGATTTCCAGAGGTTCGCCCAGC
ZB941
721 ACCTCAGCTAGCCAGCTCTTCGCTCAACTAAAGGATCTGCGGCGAATAGGCCAAGGCACG
V
781 TGGCGAAATTTAGCAACTGGCGAGGGGTTGATTTGCGTGCCACCCAGACTTCAGAGCTGG
A K F S N W R G V D L R A T Q T S E L G
841 GATCAAGCCGGCTTGGTGCTTGGCAATCACGCAGCTCGAACGCGGGCGGCACGATAGCGA
S S R L G A W Q S R S S N A G G T I A N
901 ACCACACAGCTCAATGGCGGCAAACCCACTTCCGTCCACAGGCATCCGGCATCGAGGCGT
H T A Q W R Q T H F R P Q A S G I E A F
961 TCAACTCTTCTGCTAAATATCCGGTGCGGGACATGGATGCACAAGCTGCGGCGGATACGC
N S S A K Y P V R D M D A Q A A A D T P
1021 CAAACAGAAACATCGAAAACATGCAAAGGTCGGAAAGTCGACAAGAGTGACACAAACTCC
N R N I E N M Q R S E S R Q E *
t1
-24 -12 *→BACTEROID
1081 GTGAAATTTTCCGCCTATTGCCTGGCACGAAGAATGCAGACCCTCGGAATGCCCAGTCTC
JC143
1141 CTCCTCTGGGCAGGTCCAGCTCAGCCAATTGACGCAGTCGCTGCCGGGGAAGTTAGCTGC

```
                                                  -35                  -10
1201 GCATGACATAGAGGGGAGCGCCTGCTCGCGCGGGACAGCTAGCGGGTGCATCCCCGTAAA
                      t2
                     *------>AEROBIC                    SD
1261 CTTCTTGCGATGGCATAGCAGGTTCGAGGTTGCCTGCTTCACAAGATATTTGGATGAAAC
                                                          M  K  R
                                                       NAD14
1321 GCGATAGGCCGAGGGTTATGAAGAGAATGCCTGGAAGACCCGCGCTATGGCTTAGCGTGG
       D  R  P  R  V  M  K  R  M  P  G  R  P  A  L  W  L  S  V  V

1381 TCGGATTTTTAATCTGCCTCGCCTGGCCGGCTGCCATGAGCAATAATATTCGGGCCAGCG
       G  F  L  I  C  L  A  W  P  A  A  M  S  N  N  I  R  A  S  D

1441 ACAGTCCCCTTGTCGAACGACTGAAGTCAACTTGGCGAGCGCAAGATGGTGAGACGATAG
       S  P  L  V  E  R  L  K  S  T  W  R  A  Q  D  G  E  T  I  E

1501 AACAAATTATCTCCAATGTCTCGAAGGTGGCACAGTTCGTGCCTCAAATGTGGGGCGTTG
       Q  I  I  S  N  V  S  K  V  A  Q  F  V  P  Q  M  W  G  V  V

1561 TTGAACTTGGCCAAAACGACTTTATTTTGGTTTCGTGGACCAGACACCGGGACAACAAAT
       E  L  G  Q  N  D  F  I  L  V  S  W  T  R  H  R  D  N  K  S

1621 CGGACGAACAGTACGTTATCGCCTGGAAGATCGCCCTCGACGGCACGCTTGAACTTGCGT
       D  E  Q  Y  V  I  A  W  K  I  A  L  D  G  T  L  E  L  A  S

1681 CGACTTATGCGAAGCCGATGGAATTGGGCTGGCGCGCCCTGGCGCTCTCCCTAATCGCCA
       T  Y  A  K  P  M  E  L  G  W  R  A  L  A  L  S  L  I  A  S

1741 GTGAAGTCGCAGATGGCGAAAGGGACGCAAATCTTCGCTTTCTGCATGATCCGGCCAACT
       E  V  A  D  G  E  R  D  A  N  L  R  F  L  H  D  P  A  N  F

1801 TCAACTTTGTGACTACCCCGCAAGGCCGGCTCGGCGATCTTCTGCGGCAGGGCCGCTGCA
       N  F  V  T  T  P  Q  G  R  L  G  D  L  L  R  Q  G  R  C  T

1861 CTATCATTGAACCAGTTGGAGTGGACTACTTACCGAAGCGGAATGATAAACCGGCCGAGA
       I  I  E  P  V  G  V  D  Y  L  P  K  R  N  D  K  P  A  E  K

1921 AGGGTGGCGTGTGGCGCGTTCTGCTCTTGGTGAATTGTAGTATCCAAGGACCACGTTATT
       G  G  V  W  R  V  L  L  L  V  N  C  S  I  Q  G  P  R  Y  F

1981 TTACACACAACGGGGTCATCACTTTCGAGAAGAGAGAAGGACAGGATTGGGAGCCACAAT
       T  H  N  G  V  I  T  F  E  K  R  E  G  Q  D  W  E  P  Q  S

2041 CCTCCTTTGCCAAACGCATCGCGAAATTTAAAAATGGCTCCTGGTTCCAACGCACCGAGC
       S  F  A  K  R  I  A  K  F  K  N  G  S  W  F  Q  R  T  E  P
                                                    JC890
2101 CAAAAGAAGAGGAAGACTTAGGAGAGCCAAGGCCTGAGTAAACCGCCGCAGAGCAACATC
       K  E  E  E  D  L  G  E  P  R  P  E  *

2161 TTGCCGCAGTGGCGAGCGCCTCGAACCTATTGCAAGACATACGCGCTCTACCCTTGCTTT

2221 GTTTTAGCATCCGCAACGGTCCGGGTGCCT
```

frames (ORFs). Three putative ORFs defined by methionine initiation and transcription termination codons were present, starting at nucleotides 81, 780, and 1254, respectively. These ORFs were designated ORFA, ORFB and nfeC, and encode predicted proteins of 100, 96, and 275 amino acids with deduced molecular weights of 10291, 10,652, and 31,352, respectively.

The exact transposon insertion sites of all the Tn5 or Tn5::lacZ mutants were determined by sequencing BamHI fragments of the mutant DNAs which contain the kanamycin resistance gene (IS50L) of Tn5 plus the flanking region of B. japonicum DNA (Figure 5). The insertion point for transposon NAD14 lies within the nfeC coding sequence. By contrast, strains JC143 and JC890, which display no altered symbiotic phenotype, have the Tn5 inserted 152 bp upstream from the start codon of nfeC and 4 bp downstream from the end of nfeC, respectively. Thus, as indicated by restriction mapping, these mutations bracket the NAD14 insertion and identify nfeC as important to soybean nodulation. The nfeC gene has a putative ribosome binding sequence at 8 base pairs (bps) 5' of the translational start and also a putative -24/-12 promoter consensus sequence 196 bps upstream of the most likely ATG start codon.

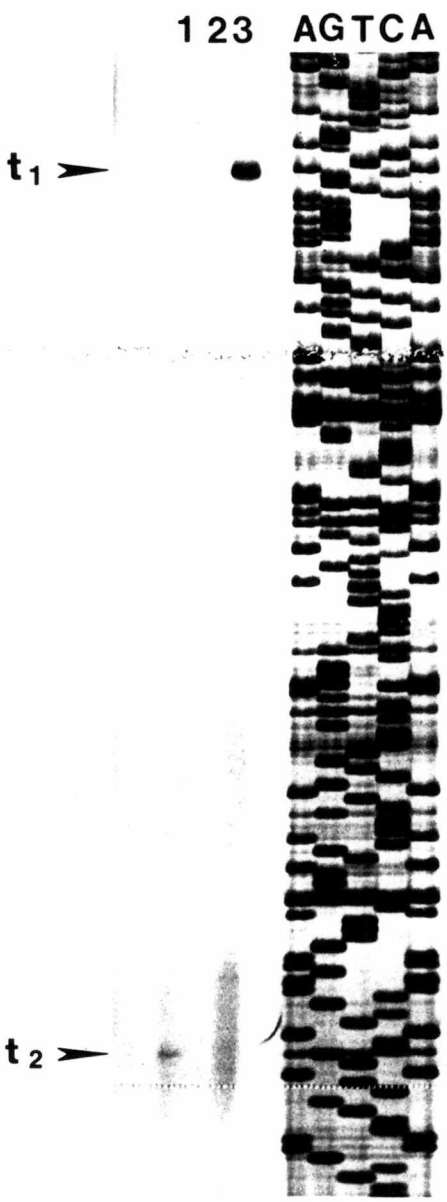
The insertion sites of mutations ZB941 and ZB940 fall

within the ORFB region. The ZB941 Tn5 insert lies 11 bp upstream from the start codon of ORFB, just before a putative ribosomal binding sequence. Therefore, this insertion may interrupt the expression of ORFB. Although the exact position of the insert ZB940 was not determined, restriction analysis of the original Tn5::lacZ cosmid (pZB886) used in the construction of this mutant, and direct sequencing of this cosmid using the Tn5 primer indicated that the ZB940 insert lies between nucleotides 955 and 965. Both mutants exhibited a delayed nodulation phenotype and were less competitive for soybean nodulation when compared to the wildtype. These phenotypes indicate that ORFB might also be involved in the nodulation competition. However, it cannot be eliminated that ZB940 and ZB941 somehow affect the expression of the downstream nfeC gene. A mutation in ORFA was not found and, therefore, no conclusions can be drawn regarding its function in nodulation. The predicted proteins of ORFA, ORFB and nfeC were analyzed using the MicroGenie package (see Material and Methods). This analysis did not suggest any membrane spanning domains or DNA binding motifs. Comparison of the DNA or amino acid sequence of ORFA, ORFB, and nfeC with sequences in the GenBank, EMBL, and Swissprot databases showed no significant similarities.

Transcriptional regulation of the B. japonicum nfeC gene.

The transcription start sites of the nfeC gene were determined by primer extension. Total RNA was isolated from B. japonicum strain USDA110 grown under free-living aerobic and anaerobic conditions or from bacteroids isolated from soybean root nodules. Two synthetic oligonucleotide primers complementary to the coding region of nfeC (see Materials and methods) were used in independent primer extension reactions using plasmid pJY105 as template. These experiments revealed two major extension products beginning at positions 184 (t_1) and 40 (t_2) bp upstream of the putative translational start codon (Figure 6). The mapped transcription start site (t1) coincided with the presence of a putative -24/-12 promoter consensus sequence (i.e., RpoN/NtrA binding site; Morett and Buck, 1989; Thöny and Hennecke, 1989). The conserved GC doublet of this sequence lies 13 bp upstream of the transcriptional start site. The transcript, t_1, was observed only with bacteroid RNA as template, suggesting that the t_1 promoter requires a bacteroid-specific factor(s) for its expression. By contrast, t_2 was obtained only with RNA from aerobically grown B. japonicum cells. Several reasons support the assertion that the transcript, t_2, is not an artifact caused by non-specific extension, hairpin secondary structure or the degradation of the primary transcript. First, there are no obvious regions of secondary structure between t_1 and the putative start codon. Second, if the extension product was

Figure 6. Determination of the transcriptional initiation sites of the nfeC transcripts. Primer extension with 20 μg of total B. japonicum RNA extracted from three different growth conditions; lane 1, free-living aerobic; lane 2, free-living anaerobic; lane 3, bacteroids (bacteria isolated from soybean nodules) compared to a DNA ladder. Transcripts t_1 and t_2 are indicated.


1 2 3 AGTCA
t1
t2

an artifact, the product should be observed with RNA from all three growth conditions. Third, two different primers produced the same extension products in the mRNA from the aerobic growth conditions and bacteroids. In addition, the DNA region upstream of the t_2 start has significant similarity to the consensus for constitutively expressed E. coli promoters (Hawley and McClure 1983; Harley and Reynolds 1987). The transcription start sites are indicated in the DNA sequence shown in Figure 3.

The bacteroid specific expression from t_1 is intriguing in light of the presence of a putative RpoN-binding sequence. All other such B. japonicum promoters are expressed both symbiotically and under anaerobic conditions (Hennecke et al. 1988; Thöny et al. 1989). Therefore, in order to confirm that the growth conditions used were indeed anaerobic, we hybridized a 1.4kb EcoRI fragment from pJY400, carrying parts of nifD and nifK, to the mRNA used in the primer extension experiments. Positive hybridization was found to mRNA isolated from anaerobically grown cells and bacteroids, but not from cells grown aerobically (Figure 7).

As shown in Figure 5, the position of Tn5 in JC143 lies between the t_1 and t_2 start sites but JC143 has a wild-type nodulation phenotype. This is likely due to the documented nonpolarity of Tn5 caused by the presence of a outward reading promoter within Tn5 (Corbin et al., 1983; Mulligan

and Long, 1985; Horvath et al., 1986; Fisher et al., 1987). If this was the case, one would expect higher constitutive expression of nfeC in strain JC143. As predicted, slot blot hybridization using the internal fragment (0.4kb SalI/NruI) of nfeC as a probe showed that this fragment hybridized more strongly to mRNA from strain JC143 when compared to the wild type or strain NAD14 (Figure 8).

In summary, a new symbiotic gene (nfeC) from B. japonicum USDA110 was identified. Mutations in this gene affected the ability of the strain to compete with the wildtype for soybean nodulation. Two promoters for nfeC were identified that are differentially regulated in response to growth conditions. One promoter was activated in bacteroids, but not under aerobic or anaerobic free-living conditions. The other promoter was activated only under aerobic conditions.

B. Identification and characterization of a novel B. japonicum gene involved in host-specific nitrogen fixation.

Phenotypes of NAD14 and other deletion mutants NAD163 and NAD164 on different symbiotic host plants.

The B. japonicum mutants NAD163 and NAD164 were previously constructed by deletion of all internal XhoI

Figure 7. Verification of anaerobic growth conditions of B. japonicum strains. **A.** a 1.4 kb EcoRI fragment of pJY400, carrying parts of nifD and nifK, was used as a probe to the mRNA of B. japonicum used in the primer extension experiments. 1, aerobically grown strain USDA110; 2, anaerobically grown strain USDA110; 3, anaerobically grown strain Bj2101 (nifA mutant); 4, USDA110 bacteroids. **B.** Same filter hybridized with a 23S rRNA gene probe as a control for RNA quantity on the filter. Numbers on left indicate quantity of RNA spotted in μg.

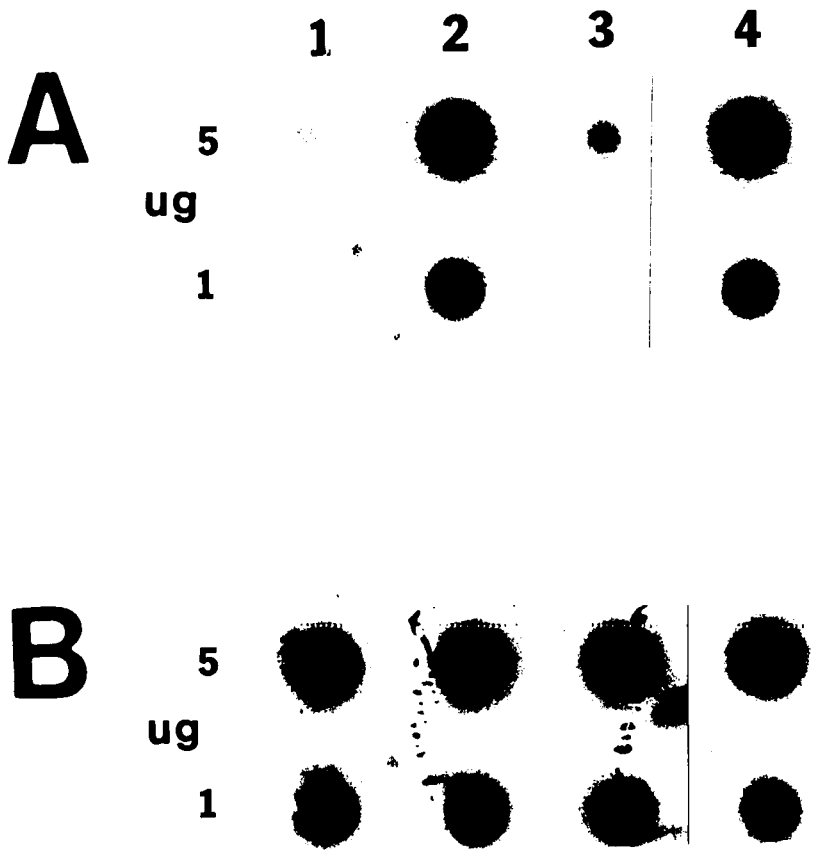
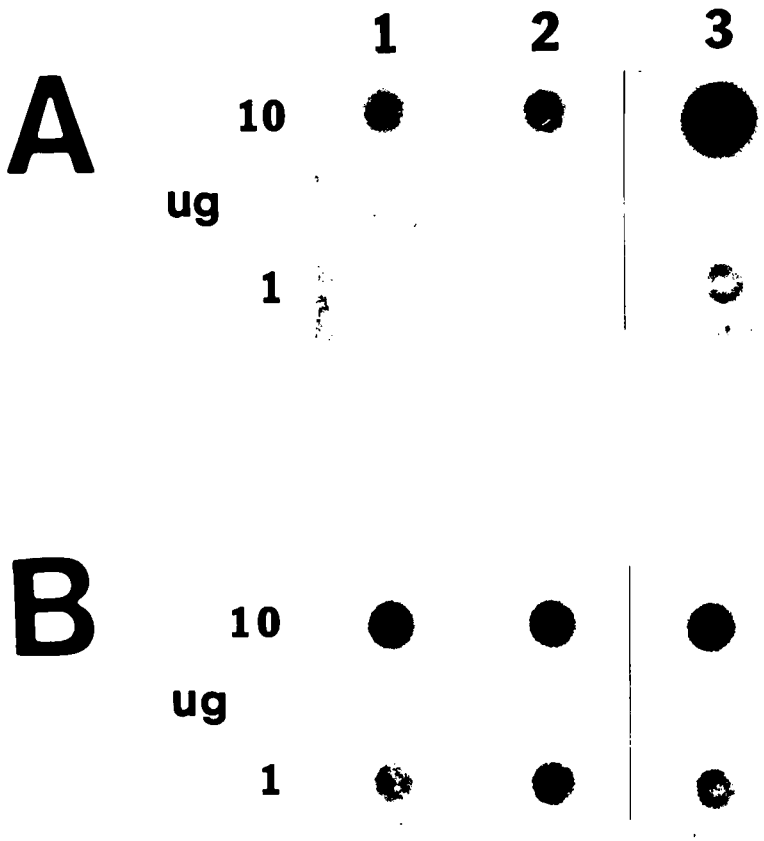


Figure 8. Analysis of nfeC transcription in the Tn5-induced mutant JC143 via RNA dot blots. **A.** An internal (0.4kb SalI/NruI) fragment of nfeC was used as a probe to the mRNA of B. japonicum wild type and mutants. 1, wild-type USDA110; 2, NAD14; 3, JC143. **B.** Same filter hybridized with a 23S rRNA gene probe as a control for RNA quantity on the filter. Numbers on left indicate quantity of RNA spotted in μg.



fragments (about 30kb) from pRjUT14 and pRjUT24, respectively, and replacement with a kanamycin resistance cassette. These mutations were subsequently marker exchanged into the genome of B. japonicum strain USDA110 (Deshmane, 1988) (Figure 9). The phenotypes (Nod^d, Fix^+) of the deletion mutants NAD163 and NAD164 on soybean plants were identical to that of strain NAD14, an insertion in nfeC as previously described (Table 6). Since there are several reports that some B. japonicum mutations result in an alteration of host range (e.g., Heron et al., 1989; Göttfert et al., 1990; Sadowsky et al., 1991; Stacey et al., 1994), mutants NAD14, NAD163 and NAD164 were examined for their ability to nodulate and fix nitrogen on four host plants; e.g., Glycine max cv. Essex, G. soja PI468397, siratro, and cowpea cv. Purple Hull. Mutant strains NAD14 and NAD164 induced effective nodules on all four hosts based on their acetylene reduction activities. However, strain NAD163 induced ineffective nodules on cowpea but effective nitrogen fixing nodules on G. max, G. soja and siratro, indicating that the deleted region in mutant NAD163 contains a host-specific fixation locus. In addition, mutant NAD163 induced yellow chlorotic leaves on 30 day old cowpea plants and formed tiny nodules (Figure 10), a phenotype characteristic of a number of nif or fix mutants (e.g., Ma et al., 1982; Hirsch and Smith, 1987). To define the stage in nodule development at which mutant NAD163 was

Figure 9. Physical map of the deleted regions in B. japonicum mutants NAD163 and NAD164. The phenotypes of the mutants: +, wild-type; d, delayed nodulation on soybean; -, no nitrogen fixation in cowpea nodules. E, EcoRI; X, XhoI.

Bradyrhizobium japonicum (USDA110)

Cluster II

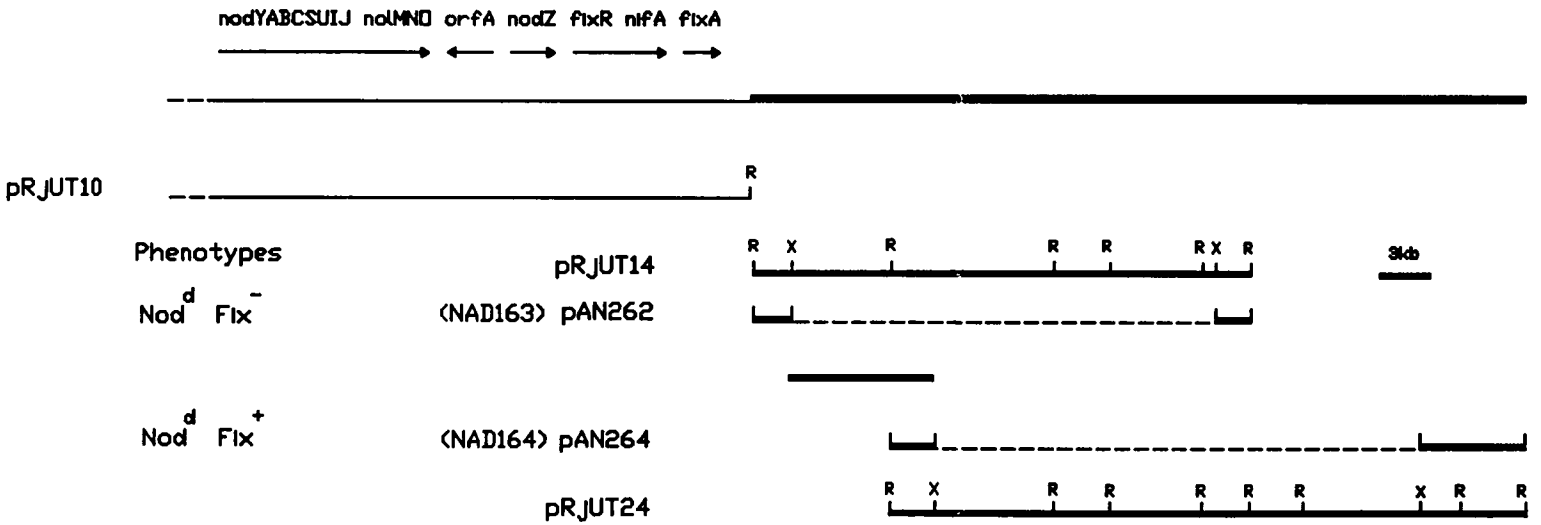


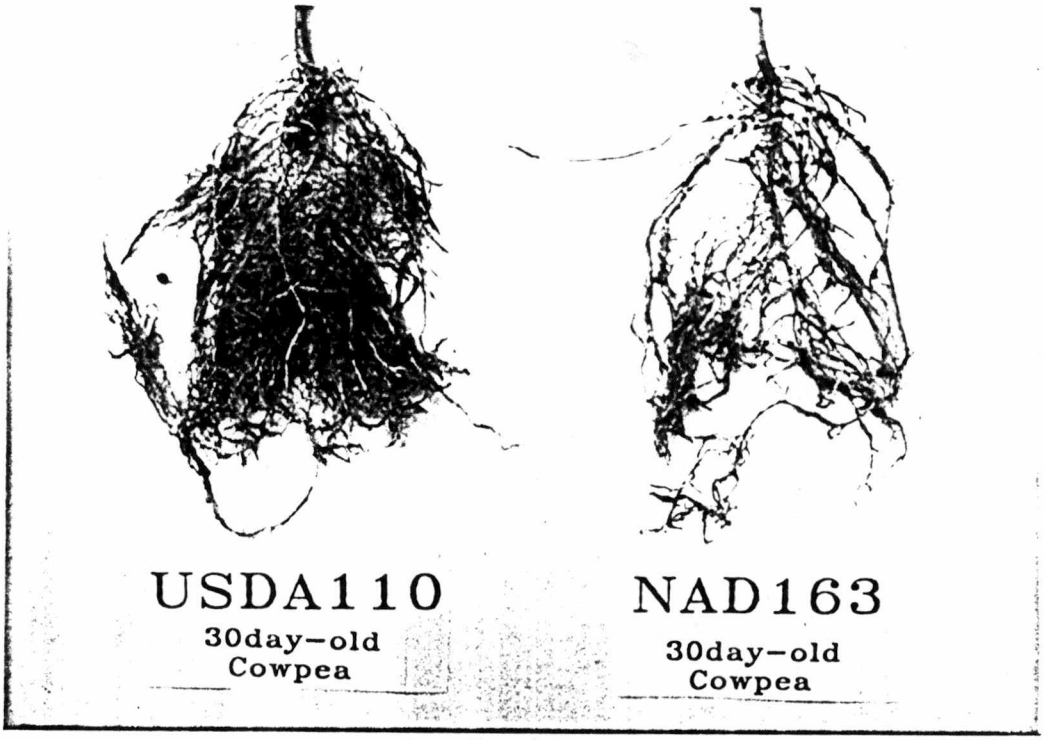
Table 6: Symbiotic phenotypes of *B. japonicum* mutants on soybean (*Glycine max* cv. Essex) plants

Strain	Fix phenotype[a]	Delay of nodulation on *G. max* (days)	Avg. nodule numbers per plant[b]	Avg. weight of nodules per plant[c] (mg)
Wild type	+	0	11 ± 1	49 ± 1
NAD14	+	3-6	9 ± 2	47 ± 10
NAD163	+	3-4	10 ± 1	33 ± 4
NAD164	+	2-4	10 ± 1	43 ± 7

[a] + = wild-type level of acetylene reduction activity.

[b] and [c] The numbers represent the mean of two experiments ± standard error. Each experiment used at least 10 plants per strain.

Figure 10. Plant tests of a deletion mutant NAD163 on cowpea. These plants are 30-day-old cowpea infected by wild type or mutant. Uninoculated plants were used as a control. The plants were grown with N-free plant nutrition solution.

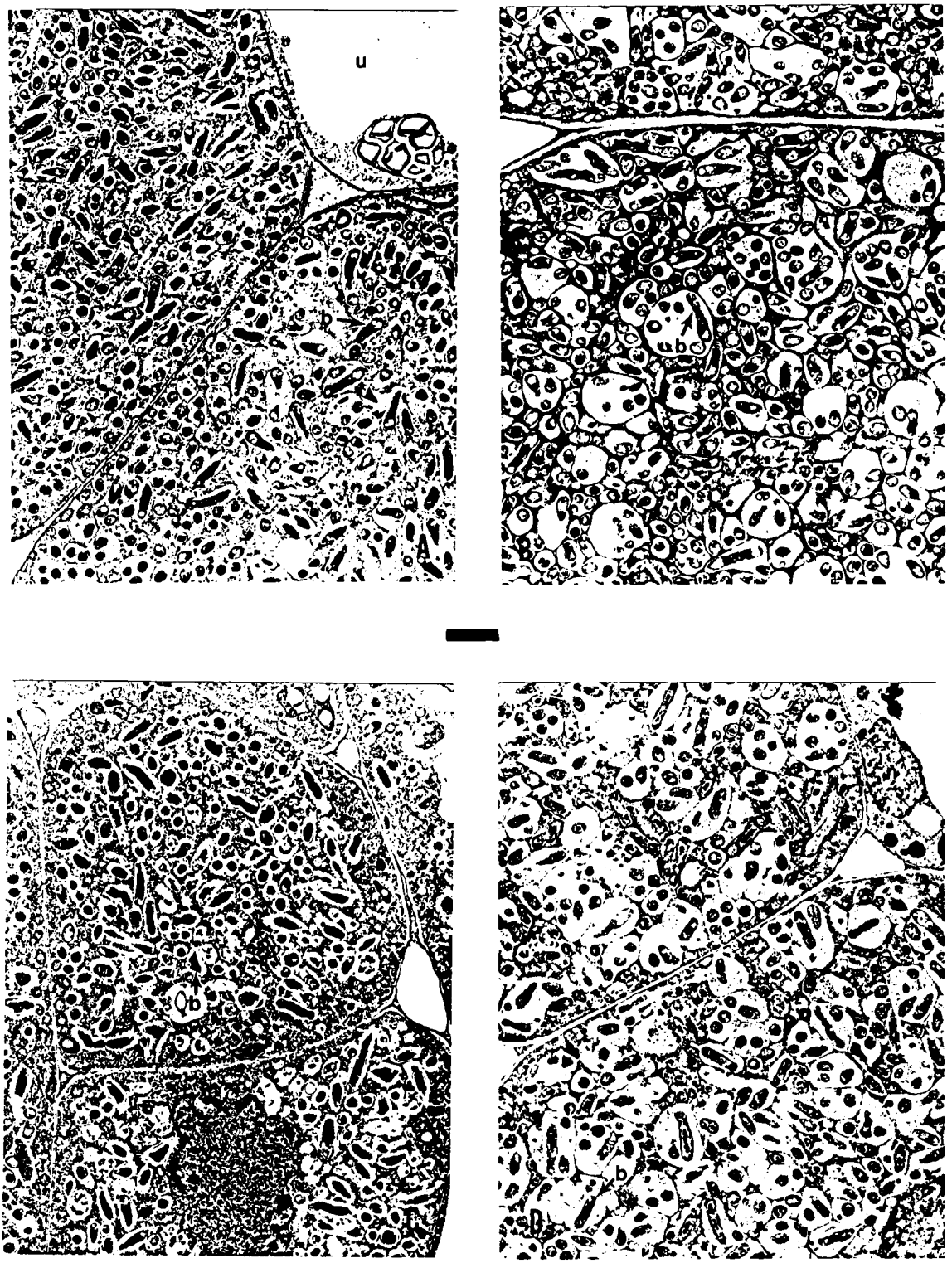


USDA110
30day-old
Cowpea

NAD163
30day-old
Cowpea

blocked, root nodules formed on cowpea and soybean were examined by light and electron microscopy. On cowpea, infected cells had few bacteroids and contained large vacuoles (Figure 11). Similar observations have been reported from respiration mutants of B. japonicum in which the soybean nodule cells infected by these mutants contained only few bacteroids and large vacuoles (Ramseier et al., 1989; O'Brian et al., 1987). These mutants are defective in cytochrome aa_3 and c-type cytochromes (O'Brian et al., 1987; Ramseier et al., 1991). In comparison, several hundred bacteroids per sectioned cell were seen in wild-type nodules. On soybean, nodule cells infected by this strain differentiate bacteroids similar to wild-type infected cells (Figure 11). However, the infected cells have larger symbiosomes which contain more bacteroids and more symbiosome space compared to the wild type, but the form of the symbiosomes seems to be less stable. These results suggested that the mutation in NAD163 which had a severe effect on cowpea might also affect to a lesser extent the development of bacteroids in soybean. However, unlike cowpea, the mutant clearly produces nitrogen fixing nodules on soybean. Based on the maps of the deleted regions in NAD163 and NAD164, the specific locus responsible for the phenotype seen should be present on that part of the NAD163 deletion which does not overlap with the NAD164 deletion (Figure 9).

Figure 11. Electron micrographs of sections from 15-day-old and 28-day-old nodules formed on soybean Glycine max and cowpea by the deletion mutant strain NAD163 of B. japonicum. **A** and **B** are soybean nodules; **A**, 15-day-old nodules; **B**, 28-day-old nodules. **C** and **D** are cowpea nodules; **C**, 15-day-old nodules; **D**, 28-day-old nodules. Abbreviations: b, bacteroid; v, vacuole; n, nucleus; u, uninfected cell. Bar represents 2 μm. Microscopy by Gerry Sexton.


u
b
ab
Qb
b

Isolation and characterization of B. japonicum mutant AN218.

It is well known that legume nodulation by rhizobia exhibits a high degree of host specificity. Indeed, a number of bacterial genes have been identified that influence host specificity during the early steps of infection and nodulation (see Barbour et al.,1992; Fisher and Long, 1992; Kondorosi, 1992, for review). However, little is known about genetic determinants that influence the host specificity of late symbiotic events (e.g., nitrogen fixation).

To understand the genetic mechanism of host specificity in the interaction between rhizobia and their hosts, it is important to identify the genes that influence both early and late steps in symbiotic development. Therefore, we sought to delimit the host specific fixation locus deleted in strain NAD163. A large number of Tn5-insertion mutants of B. japonicum was screened for insertions within the region deleted in strain NAD163 but present in strain NAD164. All of the Tn5- or Tn5::lacZ-induced mutants examined were Fix$^+$ on cowpea based on their acetylene reduction activity (Table 7). The acetylene reduction assay is a very sensitive method and the level of activity of the mutant-induced nodules was slightly different between each strain depending on the condition of the plants. Therefore, the acetylene reduction activity is defined as (Fix$^+$) when

Table 7: Acetylene reduction activity of *B. japonicum* symbiotic mutants on cowpea

No	Strain	Fragment[a]	C_2H_2 reduction activity (n moles/h/plant)[b]	Relative N fixed (%)
1	None		0	0
2	USDA110	wild type	2680 ± 700	100
3	**NAD163**	**deletion**	**208 ± 41**	**8**
4	AN218	12.2	1574 ± 407	59
5	NAD31	12.2	1770 ± 497	66
6	NAD65	12.2	1574 ± 303	59
7	NAD150	12.2	1919 ± 1193	72
8	NAD17	12.2	2207 ± 532	82
9	NAD170	12.2	2230 ± 519	83
10	NAD68	12.2	2269 ± 1157	85
11	NAD130	12.2	2230 ± 439	83
12	NAD128	12.2	2530 ± 407	94
13	NAD124	12.2	1770 ± 442	66
14	AN161	12.2	1585 ± 420	59
15	NAD122	12.2	1470 ± 282	55
16	AN230	12.2	1309 ± 511	49
17	AN165	10.5	2000 ± 519	75
18	NAD154	10.5	2058 ± 575	77
19	NAD156	10.5	3141 ± 1145	117
20	NAD63	10.5	3624 ± 1068	135
21	NAD162	10.5	2300 ± 851	86
22	NAD32	10.5	1470 ± 305	55
23	AN222	10.5	2426 ± 708	91
24	AN174	10.5	3394 ± 929	127
25	NAD158	4.2	2369 ± 1179	88
26	AN224	4.2	1975 ± 701	74
27	NAD148	4.2	3175 ± 1050	119
28	AN236	4.2	2230 ± 1213	83
29	NAD134	4.2	2115 ± 1214	79
30	ZB940	7.0	1793 ± 539	67
31	ZB941	7.0	1493 ± 334	56

[a] The numbers represent the size of the *Eco*RI fragments of pRjUT14 containing the insertion. Tn*5* or Tn*5*::*lacZ* insertions were generated by site-directed mutagenesis with plasmid pRjUT14 and localized by restriction analysis.

[b] The numbers represent the means ± standard error from five 18-day-old cowpea plants per strain.

the average activity over all experiments was greater than 30% of the wild-type activity and (Fix$^-$) when the average activity was less than 10% of the wild type.

One Tn5-induced mutant strain AN218 exhibited a 2-3 day delay with regard to the change of internal nodule color from white to pink when compared to the wild type or other Tn5 mutants. Since leghemoglobin (Lb) confers the pink color on the nodules and is detectable just before the development of nitrogenase activity (e.g., Verma et al., 1979; Bisseling et al., 1980), Strain AN218 may be defective in some function which is correlated with its N_2-fixation ability. To examine the delayed Lb accumulation in nodules infected by AN218, the nodules formed on cowpea and soybean were examined by light and electron microscopy. As shown in Figure 12, the development of bacteroids in cowpea nodule cells infected by AN218 was significantly delayed when compared to the wild type. For example, the infected cowpea nodule cells at 28 days after infection by strain AN218 were similar to that at 15-day-old nodules infected by the wild type. These results are consistent with the delay in Lb accumulation in AN218-induced nodules. On soybean, significant differences were not observed in bacteroid development between AN218- and wild type infected nodule cells (Figure 13).

Strain AN218 was further tested for the ability to

Figure 12. Electron micrographs of sections from 15-day-old and 28-day-old nodules formed on cowpea by wild-type strain and the Tn5-induced mutant AN218. **A** and **B** are nodules infected by the wild type; **A**, 15-day-old nodules; **B**, 28-day-old nodules. **C** and **D** are the nodules infected by a mutant AN218; **C**, 15-day-old nodules; **D**, 28-day-old nodules. Abbreviations: b, bacteroid; v, vacuole; n, nucleus; u, uninfected cell; cw, cell wall. Bar represents 2 μm. Microscopy by Gerry Sexton.

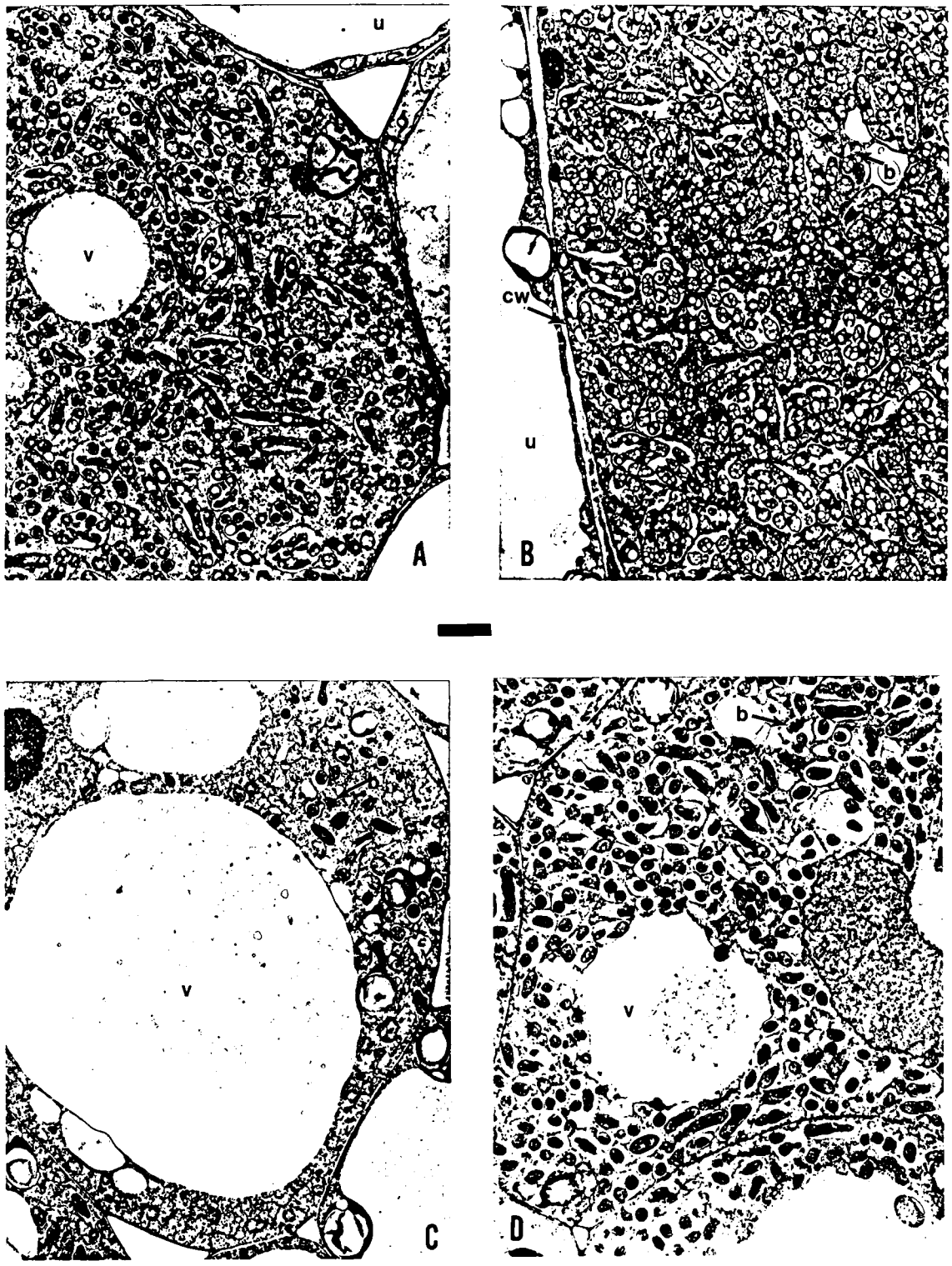
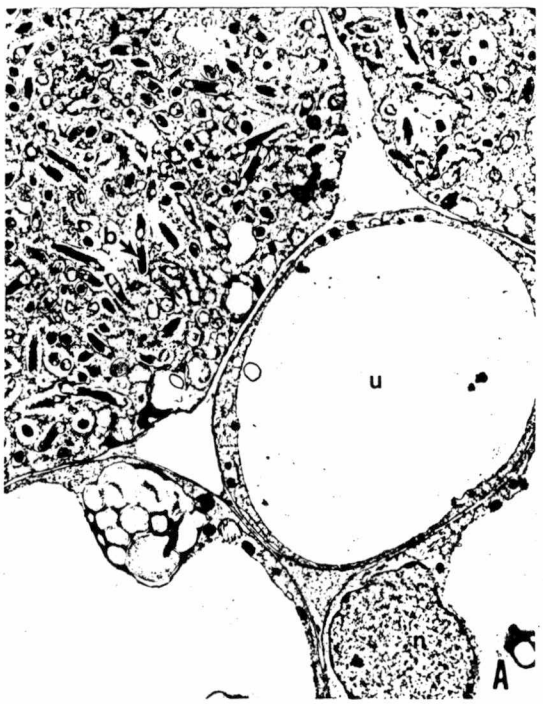
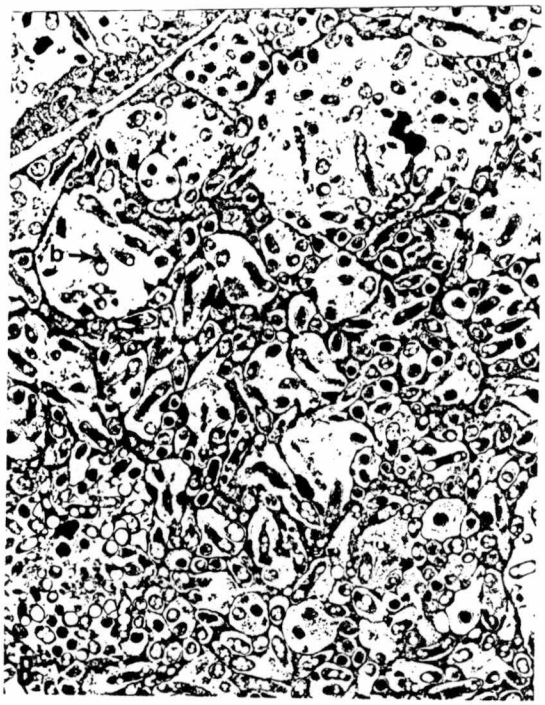
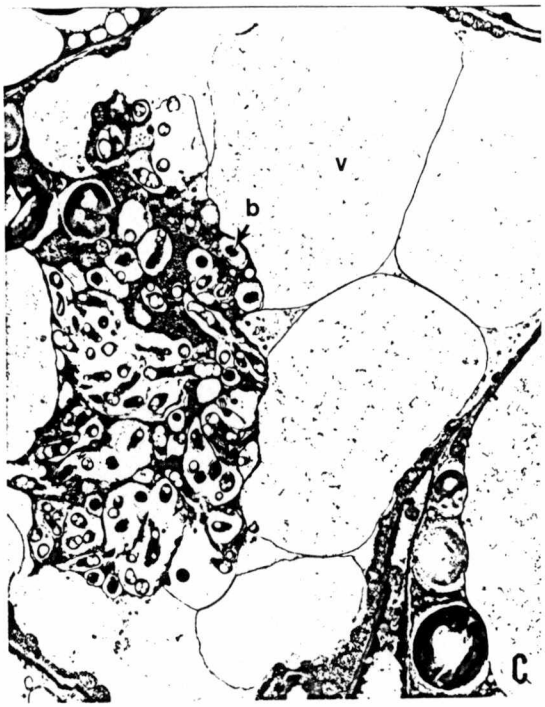

u
v
A
b
cw
u
B
v
C
b
v
D

Figure 13. Electron micrographs of sections from 15-day-old and 28-day-old nodules formed on soybean (Glycine max cv. Essex) by the wild type and a Tn5-induced mutant AN218. **A** and **B** are the nodules infected by the wild type; **A**, 15-day-old nodules; **B**, 28-day-old nodules. **C** and **D** are the nodules infected by mutant AN218; **C**, 15-day-old nodules; **D**, 28-day-old nodules. Abbreviations: b, bacteroid; v, vacuole; n, nucleus; u, uninfected cell. Bar represents 2 μm. Microscopy by Gerry Sexton.

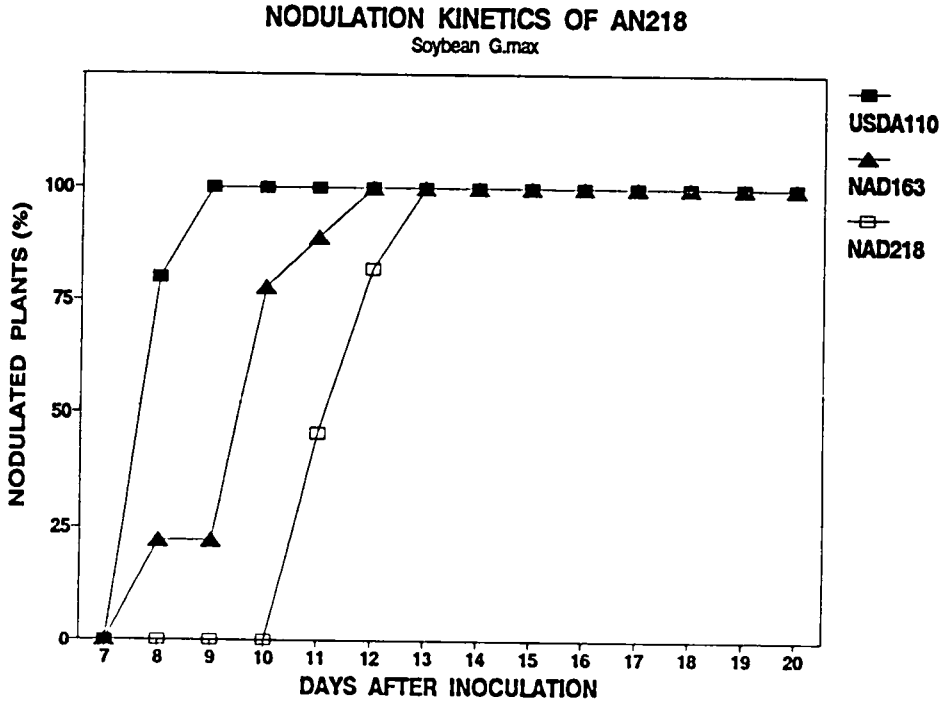

b
u
n
A

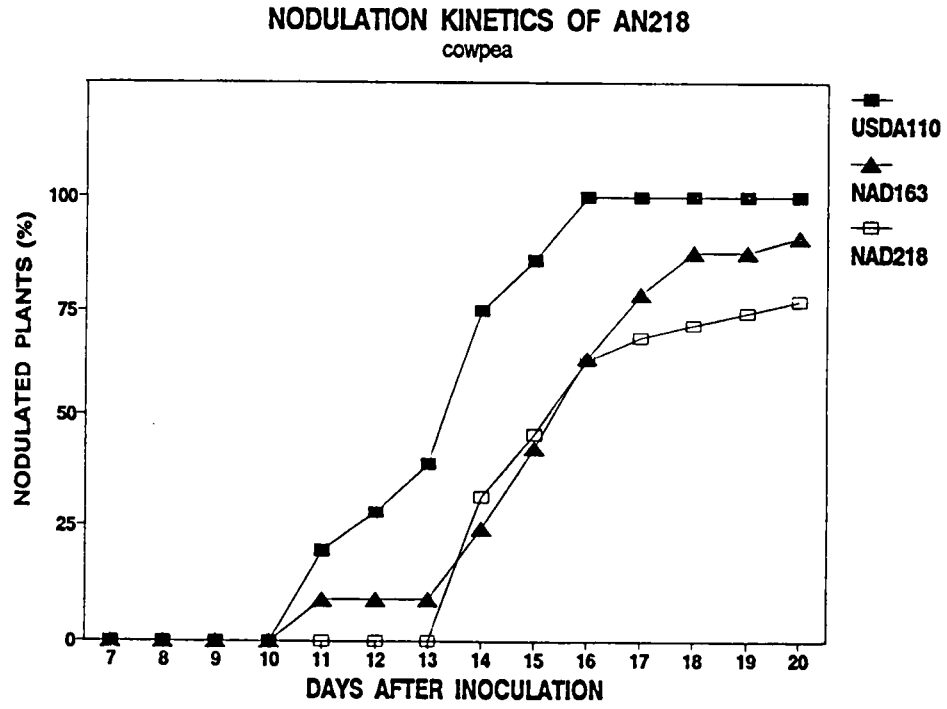

b


v
b
C


b
D

Figure 14. Nodulation kinetics of wild type B. japonicum USDA110 and mutants NAD163 and NAD218 on soybean Glycine max cv. Essex and cowpea cv. Purple Hull.





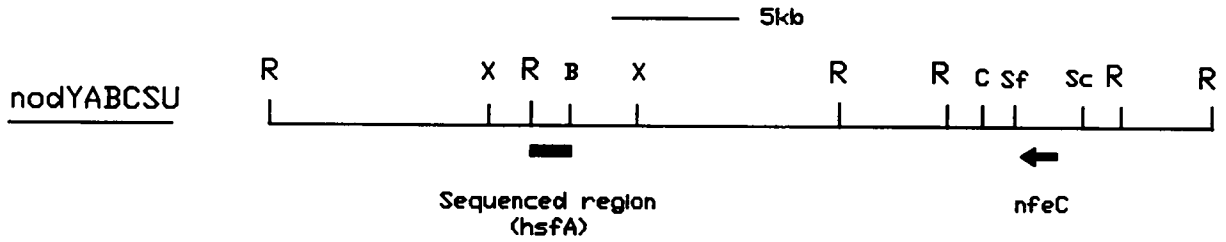
nodulate soybean and cowpea. Figure 14 shows the nodulation kinetics of NAD163 and AN218. Both mutants exhibited a 3 - 4 day delay in nodule formation on both host plants as compared with the wild-type strain. These data for NAD163 and AN218 suggested that the mutations in these strains affected the efficiency of nodule formation on soybean as well as cowpea.

Construction of a deletion mutant (BjjC208) B. japonicum and its symbiotic phenotype.

To confirm that the region identified by AN218 was responsible for the host specific phenotype of NAD163 on cowpea, a wild-type 1.6kb EcoRI/BamHI fragment encompassing the region of the AN218 mutation was deleted and replaced with a spectinomycin/streptomycin (Sm/Sp) resistance cassette and then mobilized into B. japonicum USDA110 (see Materials and Methods) (for a map, see Figure 15). The resulting B. japonicum mutant was called BjjC208. Verification that the 1.6 kb EcoRI/BamHI fragment was deleted from this mutant was obtained by genomic Southern hybridization analysis (Figure 16). When a 5.8 kb XhoI fragment of pJY200, which contains the 1.6kb EcoRI/BamHI fragment, was used as a probe to EcoRI and BamHI digested genomic DNA from wild-type USDA110 and mutant BjjC208, the wild type showed two hybridizing junction fragments and the expected 1.6kb EcoRI/BamHI band. However, the mutant showed

Figure 15. Physical map of the B. japonicum hsfA gene region. **A.** The hsfA gene is located about 27 kb from the nodYABCSU operon. **B.** The vertical arrows indicate the position of Tn5 insertions based on sequencing data. The phenotypes of the Tn5 inserts: +, Wild type; d, delayed nodulation on soybean; -, no nitrogen fixation in cowpea nodules. The transcription of the hsfA gene is from right to left. orfB is in the opposite orientation to hsfA. P, bacteroid-specific RpoN-type promoter. Restriction endonuclease sites: R, EcoRI; B, BamHI; H, HindIII; Sc, SacI; S, SalI; X, XhoI; C, ClaI; Sf, SfuI.

A. pRJUT14



B. Sequenced region (hsfA)

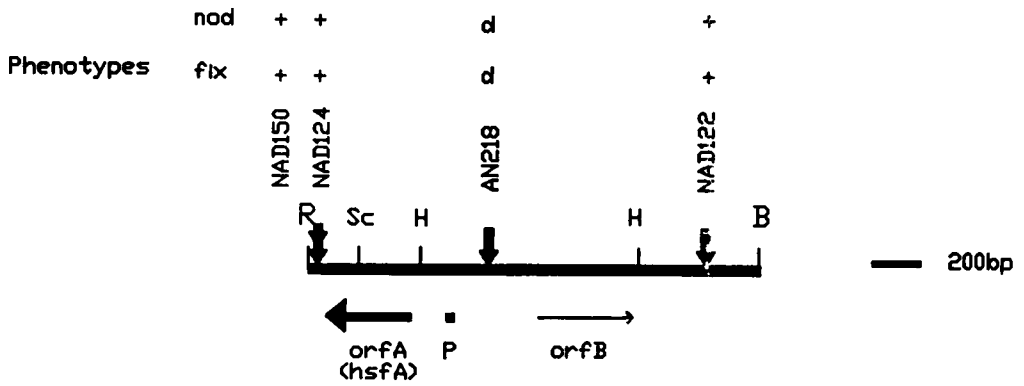
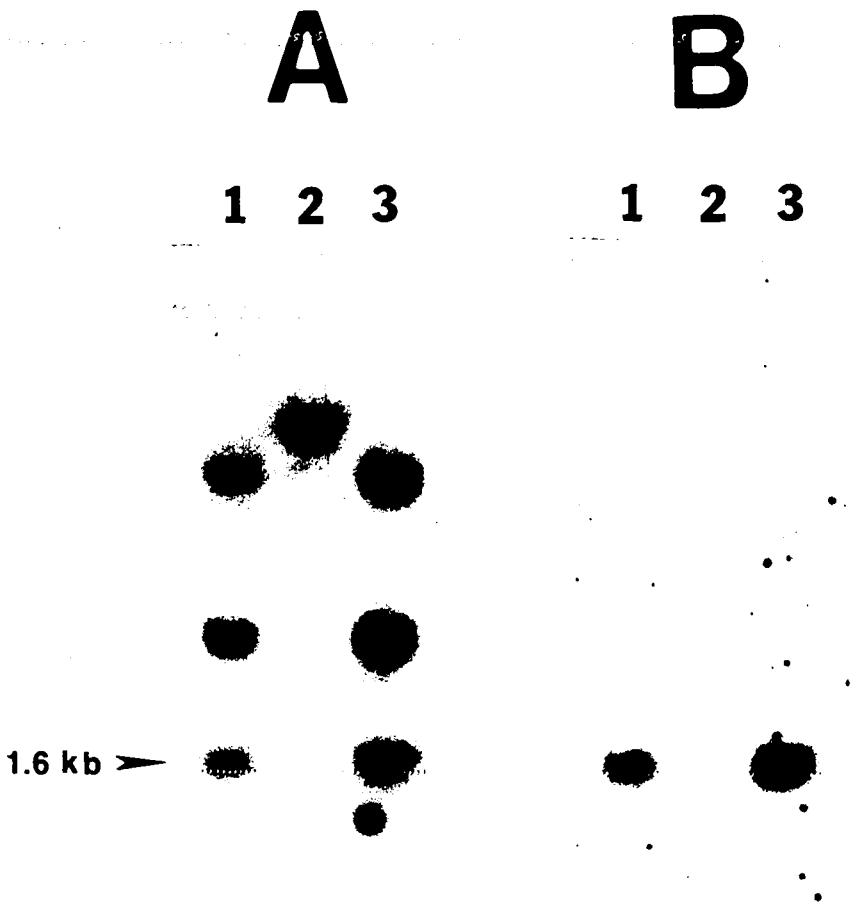


Figure 16. Verification of the deletion in the mutant BjjC208. Hybridization of Southern blots of EcoRI and BamHI digested genomic DNAs from wild-type USDA110 (lane 1 and 3) and mutant BjjC208 (lane 2). **A.** A 5.8kb XhoI fragment of pJY200 was used as a probe. **B.** After the filter was stripped, the same filter was reprobed with a 1.6kb EcoRI/BamHI fragment within the 5.8 kb region.



a larger hybridizing fragment. Since the EcoRI and BamHI sites of the 1.6kb EcoRI/BamHI fragment was blunt-ended during the construction of mutant BjjC208, the subsequently ligation resulted in fusion of this fragment to the junction fragments and Sm/Sp cassette; thereby, explaining the larger hybridizing band seen with the mutant. However, to confirm that this larger hybridizing fragment did not contain the 1.6kb fragment, the 1.6 kb EcoRI/BamHI fragment was isolated, labeled and used to reprobe the same filter. In the mutant, the larger fragment, which hybridized with the 5.8 kb XhoI probe, did not hybridize to the 1.6 kb probe, indicating that the 1.6kb fragment was indeed deleted in mutant BjjC208.

Mutant BjjC208 was tested for its symbiotic ability on cowpea. Table 8 shows the symbiotic phenotypes of BjjC208 and NAD163. Similar to the uninoculated control plants, the mutant exhibited no acetylene reduction activity. In this experiment, plants inoculated with NAD163 had about 6% of the wild-type acetylene reduction activity. Both mutants showed similar nodule morphology and produced small and white nodules. Therefore, it seems likely that the region mutagenized by AN218 is responsible for the phenotype seen with the larger NAD163 deletion mutant.

Nucleotide sequencing and sequence analysis

Table 8: Symbiotic phenotypes of BjjC208 on cowpea

Strain	Nodule morphology		C_2H_2 reduction activity (%)[b]
	size	color inside[a]	(n moles/h/plant)
None	-	-	0
USDA110	normal	pink	1054 ± 175 (100)
NAD163	small	white	41 ± 12 (4)
BjjC208	small	white	44 ± 44 (4)

[a] Pink, all nodules had an internal color of pink, indicating the presence of leghemoglobin; white, most nodules were white but some nodules were very pink.

[b] The numbers represent the means ± standard error of two experiments; each experiment used five 19 day-old plants per strain. The values in parenthesis are the percent of acetylene reduction activity relative to the wild-type activity.

A 1.5kb DNA region from the wild-type strain USDA110 was sequenced on both strands to identify the gene(s) mutated by the AN218 insertion. This region encompasses the location of the Tn5 insertions AN218, NAD150, NAD124, and NAD122 (Figure 15). Since the flanking insertions NAD150, NAD124, and NAD122 showed no altered symbiotic phenotype on cowpea, these mutants delimit the symbiotic locus identified by the AN218 mutation. Two major open reading frames oriented in opposite directions were identified within the DNA sequence (Figure 17). The exact position of the Tn5 insertions in the various mutants was determined by sequencing the kanamycin resistance gene (IS50L) of Tn5 plus the flanking region of B. japonicum DNA. The position of the AN218 Tn5 insertion was found to lie between the two open reading frames. Recall that this mutant exhibits a 2-3 day delay in Lb accumulation compared to the wild type. The location of the AN218 Tn5 insertion offers an explanation for these results in that the mutation may not completely block expression of the adjacent genes. The predicted ORFA and ORFB proteins were analysed using the MicroGenie Sequencing analysis program. This computer analysis did not suggest any membrane spanning domains or DNA binding motifs. Comparing ORFA and ORFB DNA or amino acid sequences to sequences in the Genebank, EMBL, or Swissprot databases did not reveal any significant similarities.

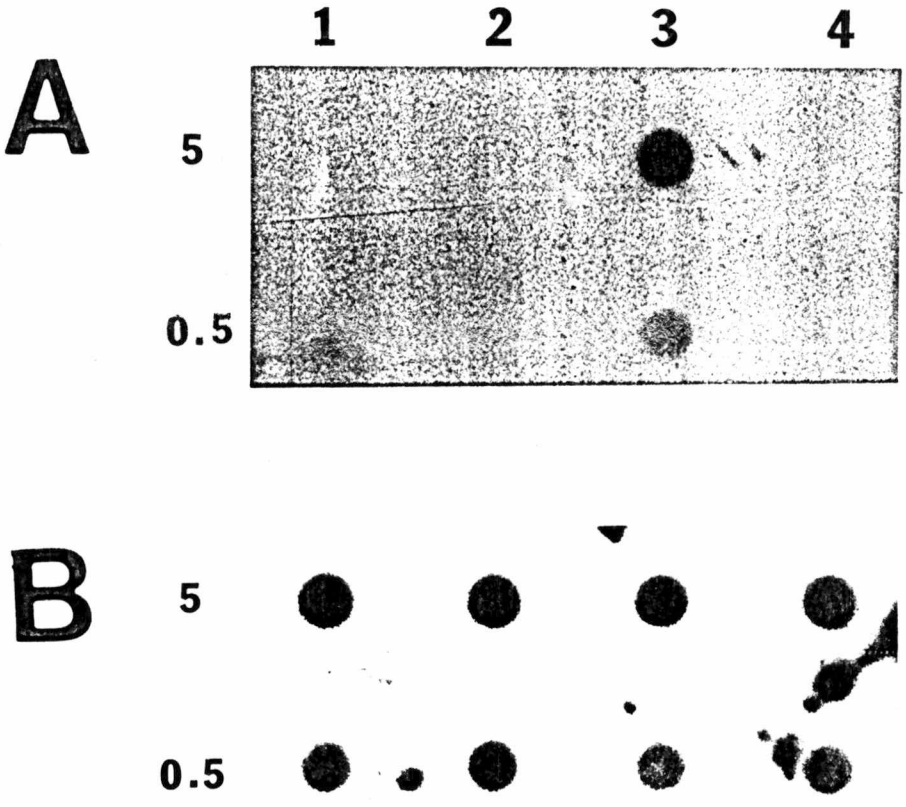
Figure 17. Nucleotide sequence of the hsfA gene with predicted amino acid sequence. The presumptive ribosome-binding site (SD) is indicated and the sites of the Tn5 insertion in the mutants, NAD122, AN218, NAD150 and NAD124 are marked by arrows. The transcriptional start site is designated by an asterisk. The RpoN consensus promoter -12/-24 is underlined. OrfB starts at nucleotide 784 and terminates at 437.

```
     BamHI
   1 GGATCCAGTCAGCTTTCGTGTGTCGATAGACCGCGCGCATCAAGAGTTCATGATCGATCG
  61 TGTCGAAAAAGCTCTTGTTGTCGAGATCTACGACCCAGTCGTGCTGCCAGCACGCTTACG
 121 CGCCTGCTCTATGCCTGATGGGCCGATTTTCCGGGCCTGTAGCCGTAGGAGTCCGGATCA
                                                    ↓ NAD122
 181 AATTCTCCATCCAACACCGGCTCCAGACACCGTTTGACCACCATCTGGGCGATACGGTCC
 241 GCGACCGTCCGGTATGCCAAGAGGACGAATCCCACCGCCGGCCTTAGGAATCTCGACCCG
 301 CCGCACTGGTGGCGGGAAGTAACTTCCTGACACCAACCGATTCCACAGCCGGTAGCGGTG
 361 GTTCTCAAGATCGCCGGCAAAGTCTTCGAGACTCTGGCCATCGACGCCCGCCGCCTTTCC
 421 CTTCTTCACAAGCTTATAGGCTTCCCACACCATCCGCTTGGTGATGGGCAACGGTTTGCC
 481 GGACTTCACCGTGCTCCTCCCGGTCTTCCCGGTTGGCGCAGTGACGCCGGCAGATAACGC
 541 AACCCCTTTGCTCCACGGCCATTACAGCCGCTTCCTCACTATTACGGGTTGCTCCGTAGC
 601 ATTGCGTCAGGTTCCGACGTTCCGTGTTGAAGCCTGTGACGAGATCATGCCGCCTATGCA
 661 CCGGCTGCCGCGTGGGCCGTAAACAGGCTCCGCCCACACTCCTCCGAGCGCTACTACCCC
 721 ACGCCCGTCTTGACAGCATCTTGAAACCATTTCGATACGTCATCGGCGGTTCGCTTGCAC
 781 TCATCTTCTTGTCACTTACCTGACGGTTTCATTCACCGCCTTTTTCCTCATCGCTCACCA
 841 CGCGCCTTTTGAACGCGGCAGCATGAGGTGGTTTGAACGCCTGCCCCTGAAGGCCGGCTC

 901 CGGGAGGCCAAACTCCCATCTTCAACACAGCATCACCGGCGTCGCCGCCGGTGTTCGTTG

     ↓ AN218
 961 CACACGGTAATGCGGGTTTTGTTTCCGACAGGAATGTCGCGACTCCGACGTCGCGCAGCG

                                                        -24
1021 GCTCCGTTTGACCAACCCATCAAATGAAAGGCGCTGCCGACAGTTCTACCTTCTGGCATG

          -12              *———►BACTEROID
1081 TAGCTTGCTGCGTGGGCCGAACGCGACCTTGCCGTTCCGAAGATACACGAGCAATCAATC

1141 GGCCGAGTGGTCGCGACGCAACGAACATCGTTTCTCCTACCGCCCTATGAAGAGAGGACT

                          SD          Primer
1201 GCTAGCTTATGAGTGATAGGAGGCATGCAAAGCTTGGCATATTTCTGTTGGCCGGCGCTC
                               M  Q  S  L  A  Y  F  C  W  P  A  L

1261 AGCGCAATGAGACGTACATTCGCGCGCAACAAAGCCCCCAATGATCAAGCATTTCGGCGC
     S  A  M  R  R  T  F  A  R  N  K  A  P  N  D  Q  A  F  R  R
                                                  SacI
1321 CGATCGTCCCTCCTTGCAACCGCAGAGAAGGTGGAACTGGGAGCTCCTCGTCGCACTTCT
     R  S  S  L  L  A  T  A  E  K  V  E  L  G  A  P  R  R  T  S

1381 CATAAATGCGGTGCTTTGGGCTGGAATCTACTTGGTCATCACAGCCTTCTTCTGACAGCC
     H  K  C  G  A  L  G  W  N  L  L  G  H  H  S  L  L  L  T  A

1441 TCCCGTGCATGGCGACCGCGCGAGGCTTCTAGGGCCTATCGCATGCTGCAGCCGCCACGG
     S  R  A  W  R  P  R  E  A  S  R  A  Y  R  M  L  Q  P  P  R
                                                NAD124 ——┬—— NAD150
1501 CTGCGAATAACCTGATGCCGGCGCGCAATTCGCGCTGCGACCTGGATTTAGAGTGCGCTG
     L  R  I  T  *
                         EcoRI
1561 TTCCGGCGACCGCAATAGGAATTC
```

RNA slot blot hybridization

Since the AN218 mutation lies between the two putative ORFs, it was not possible to determine which gene was responsible for the phenotype observed on cowpea. Therefore, the expression of both ORFs was examined by RNA slot blot hybridization using the internal DNA fragments of both ORFs as probes. Total RNA of wild-type B. japonicum strain USDA110 was isolated from free-living growth conditions, aerobic and anaerobic, or from bacteroids isolated from soybean root nodules. Plasmid pJY202 was digested with PstI-HindII or NruI-HindIII and a 0.26 kb PstI-HindIII internal DNA fragment of ORFA and a 0.57 kb NruI-HindIII DNA fragment from ORFB were isolated. These DNA fragments were used as probes in the RNA slot blot hybridization. The ORFA probe hybridized only to bacteroid RNA (Figure 18), indicating that ORFA is expressed only in planta, similar to the results for nfeC gene expression from the t1 promoter. In contrast, the ORFB probe did not hybridize to RNA isolated from free-living or bacteroid growth conditions, suggesting that ORFB may not be transcribed. Since one would assume that the gene responsible for host-specific nitrogen fixation must be expressed in the bacteroids, these results strongly suggested that ORFA is the gene essential for nitrogen fixation in cowpea nodules. For this reason, we chose to term ORFA as hsfA (host specific fixation).

Figure 18. Analysis of hsfA transcription regulation via RNA dot blots. **A.** an internal (0.26kb PstI/HindIII) fragment of hsfA was used as a probe to the mRNA of B. japonicum USDA110 grown under three different conditions: lane1, aerobically; lane 2, anaerobically; and lane3, bacteroids. A deletion mutant NAD163 (aerobic) was used as a control (lane 4). **B.** Same filter hybridized with a 23S rRNA gene probe as a control for RNA quantity on the filter. Numbers on left indicate quantity of RNA spotted in μg.



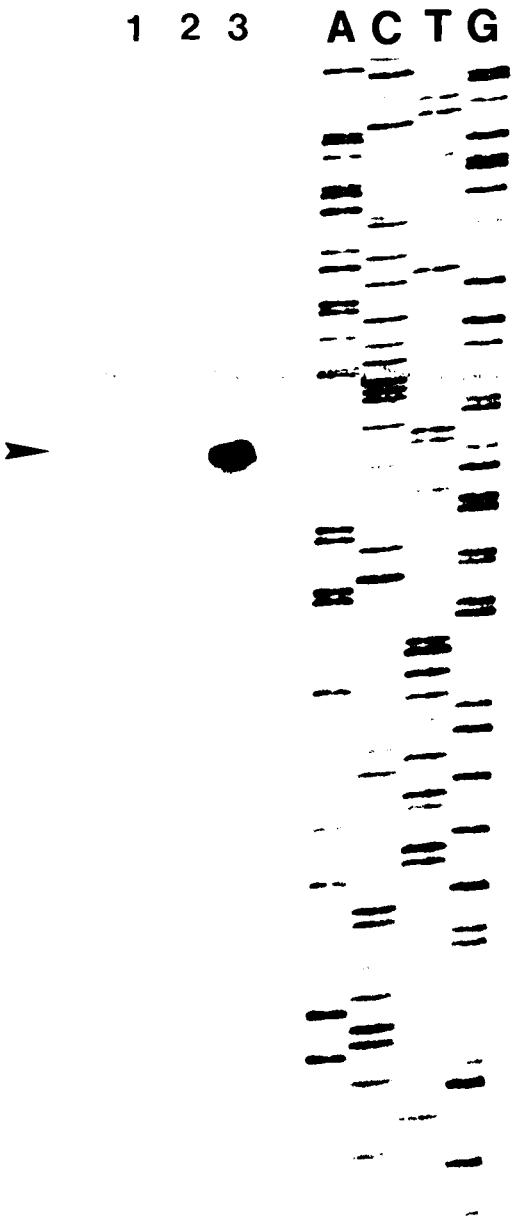
Transcriptional regulation of hsfA

The transcription start site of hsfA was determined by primer extension. A synthetic oligonucleotide primer complementary to the coding region of hsfA (see Materials and methods) was used in primer extension reactions with the same RNA used in the above slot blot hybridization. These experiments revealed one major extension product and only when bacteroid RNA was used as template (Figure 19). These results were consistent with the RNA slot blot hybridization results in that the internal fragment of hsfA hybridized only to bacteroid RNA. The mapped transcription start site coincided with the presence of a putative -24/-12 promoter consensus sequence.

Construction of a hsfA mutant BjjC211 by site-directed mutagenesis and its symbiotic phenotype

To confirm that the hsfA gene is important for host-specific nitrogen fixation, a hsfA mutant was constructed by site-directed mutagenesis. A streptomycin/spectinomycin (Sm/Sp) cassette was inserted into the coding region of the hsfA gene in plasmid pJY211. Restriction analysis of the mutated plasmid pJY211 confirmed that the Sm/Sp cassette was inserted within the hsfA coding region within a 1.6 kb EcoRI/BamHI fragment. This mutation was marker exchanged into the genome of B. japonicum USDA110. The resulting three transconjugants (called BjjC211-1,-2,-3) were verified

Figure 19. Determination of the transcriptional initiation site of the hsfA transcript. Primer extension with 20ug of total B. japonicum RNA extracted from three different growth conditions; lane 1, free-living aerobic; lane 2, free-living anaerobic; lane 3, bacteroids compared to a DNA ladder. Arrow indicates the hsfA transcript.


1 2 3
A C T G

by Southern hybridization analysis. Genomic DNA from the wild-type USDA110 and mutant were digested with EcoRI and BamHI and probed with a 1.6kb EcoRI/BamHI fragment containing the hsfA gene. This probe hybridized to the 1.6 kb EcoRI/BamHI fragment of the wild type. However, the three transconjugants (BjjC211-1,-2,-3) showed two shifted fragments hybridizing to this probe but not the 1.6 kb fragment lost due to insertion of the Sm/Sp cassette (Figure 20).

To determine whether the insertion mutation in hsfA exhibited a defect in host-specific nitrogen fixation, all three transconjugants (BjjC211-1,-2,-3) were tested for their symbiotic ability on cowpea and soybean. Table 9 shows the symbiotic phenotypes of BjjC211 and other mutants. BjjC211 transconjugants were consistent with BjjC208 and NAD163 in their acetylene reduction activity. All mutants except AN218 were Fix^- (less than 10% of the wild-type) on cowpea but Fix^+ on soybean based on their acetylene reduction activities. AN218 showed 22% less activity than wild type. In addition, the newly constructed mutants BjjC211 and BjjC208 showed the same phenotypes of NAD163 that they induced yellow chlorotic leaves on 28-day-old cowpea plants and formed tiny nodules (Figure 21). These results confirm the hypothesis that the cowpea-specific nitrogen fixation phenotype of mutants NAD163 and BjjC208

Figure 20. Verification of the insertion of a Sm/Sp cassette in the transconjugants BjjC211-1, -2, -3. Hybridization of Southern blots of EcoRI and BamHI digested genomic DNAs from wild-type USDA110 (lane 1) and transconjugants BjjC211-1 (lane 2), -2 (lane 3), and -3 (lane 4) using a 1.6kb EcoRI/BamHI fragment containing the hsfA gene as a probe.

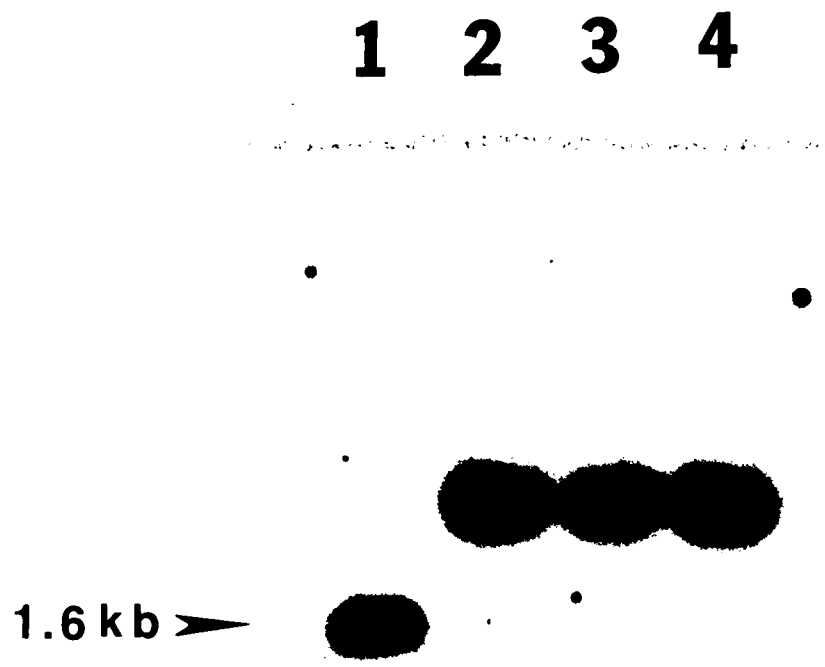


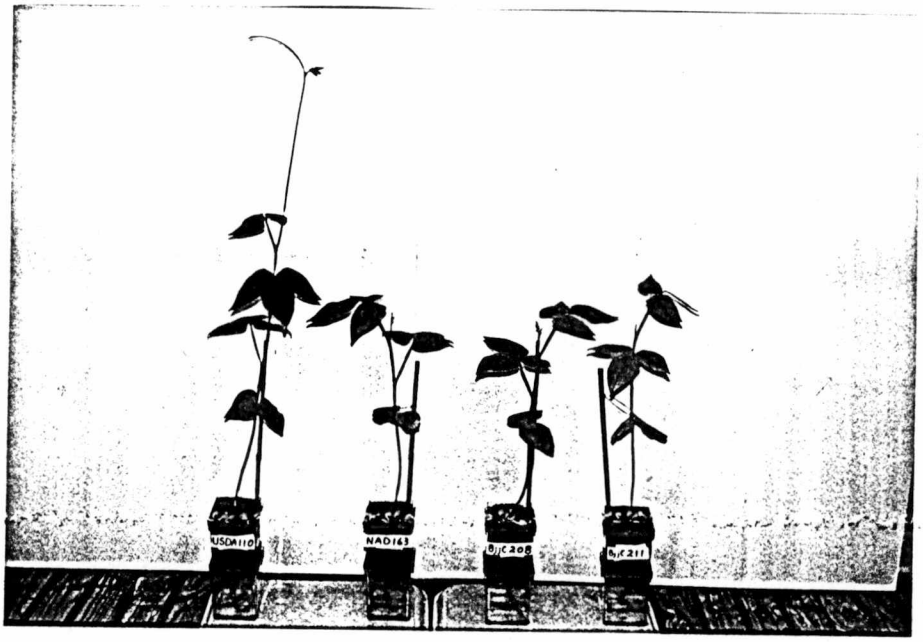
Table 9: Symbiotic phenotypes of B. japonicum mutant BjjC211 on cowpea and soybean (Glycine max cv. Essex)

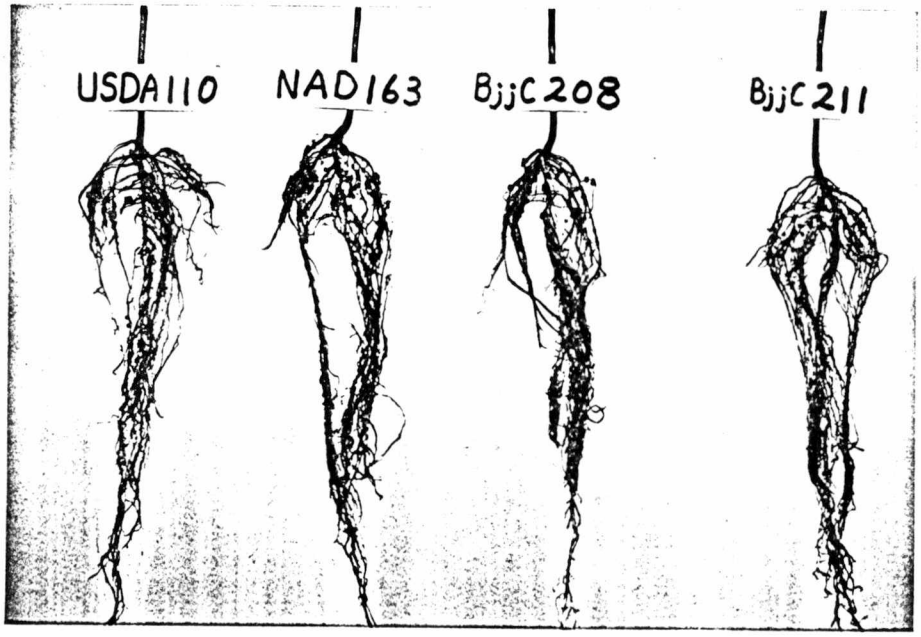
Strains	Nodule color inside[a]		C_2H_2 reduction activity[b]	
	cowpea	soybean	cowpea (%)	soybean (%)
None	-	-	0	0
USDA110	pink	red	1229 ± 63 (100)	265 ± 59 (100)
AN218	pink	red	853 ± 48 (69)	207 ± 49 (78)
NAD163	white	red	29 ± 10 (2)	315 ± 55 (119)
BjjC208	white	red	87 ± 43 (7)	324 ± 30 (122)
BjjC211-1	white	red	99 ± 24 (8)	256 ± 34 (97)
BjjC211-2	white	red	80 ± 39 (7)	327 ± 51 (123)
BjjC211-3	white	red	109 ± 36 (9)	311 ± 47 (117)

[a] Pink or red, all nodules had an internal color of pink or red, indicating the presence of leghemoglobin; White, most of nodules were white but some nodules were very light pink.

[b] The numbers repesent the means and standard deviations of the means from five 19-day-old plants per strain (n moles/h/plant). The value in parenthesis is the percent of acetylene reduction activity relative to the wild-type activity.

Figure 21. Plant tests of a insertion mutant BjjC211 on cowpea. These plants are 28-day-old cowpea infected by the wild-type or mutants. Uninoculated plants were used as a control.


USDA110
NAD163
BjjC208
BjjC211


USDA110
NAD163
BjjC208
BjjC211

is caused by a mutation in the hsfA gene.

CHAPTER IV

DISCUSSION

A. CLONING AND MOLECULAR CHARACTERIZATION OF A NEW SYMBIOTIC GENE INVOLVED IN NODULATION COMPETITIVENESS.

Previously, a Tn*5*-induced delayed nodulation mutant NAD14 of *B. japonicum* was isolated in our laboratory and linked to the known nodulation gene cluster (i.e., *nodYABC*; Deshmane, 1988). The present work provides a more thorough molecular and phenotypic description of this mutant. The gene identified by the NAD14 mutation appears to be important for the ability of *B. japonicum* to efficiently nodulate soybean. A mutation in this gene does not affect nitrogen fixation but causes a delay in nodule formation. More importantly, the mutant is significantly reduced in its competitive ability for nodulation when compared to the wild type.

Other mutants in nodulation efficiency and competitive ability have been reported from *R. meliloti* (i.e., *nfe1*, *nfe2*; Sanjuan and Olivares, 1989), *R. fredii* (McLoughlin et al., 1987), and *B. japonicum* (Bhagwat et al., 1991). Nucleotide sequence information is available only for the *nfe1* and *nfe2* genes from *R. meliloti* (Sanjuan and Olivares,

1989; Soto et al., 1993). The coding sequence of both of these genes is preceded by a RpoN-type promoter. Similar to nfeC, the nfe1 gene is expressed from two different promoters, only one of which is a RpoN-type promoter. Expression of the nfe genes (nfe1 and nfe2) was activated in microaerobically grown free-living cells and in alfalfa nodules, but not in aerobically grown cells.

Transcription of nfeC is initiated from two closely spaced but independently regulated promoters. One promoter, P1 (corresponding to the t_1 start), is expressed only in bacteroids. The other promoter, P2 (corresponding to the t_2 start), is expressed only under aerobic conditions. The P1 promoter is preceded by a RpoN-regulated promoter consensus sequence (Thöny and Hennecke 1989). In contrast, the sequence upstream of the P2 promoter has similarity to an E. coli σ^{70} consensus promoter (Hawley and McClure, 1983; Harley and Reynolds, 1987). Therefore, the regulation of the nfeC gene is likely different from that of the nfe1 and nfe2 genes identified in R. meliloti. Indeed, the nfeC gene of B. japonicum and the nfe genes of R. meliloti may have unrelated functions. However, nfeC has many features in common with the nfe1 and nfe2 genes with respect to mutant phenotypes and promoters of the genes; hence, the designation of this gene as nfeC. B. japonicum has two functionally interchangeable rpoN genes (Kullik et al.,

1991). The $\underline{rpoN}_{1/2}$ double mutant (N50-97) induced nodules, but these contained fewer bacteroids and lacked nitrogen fixation activity. Since the nfeC promoter (P1) is active only in bacteroids and the $\underline{rpoN}_{1/2}$ mutant is defective in bacteroid formation, it is difficult to prove directly whether the bacteroid-specific promoter (P1) of nfeC is regulated by RpoN.

It should be noted that the existence of two promoters in highly regulated prokaryotic operons is not uncommon. For example, the tandem promoters for the glnB gene from B. japonicum (Martin et al., 1989) and the glnA gene from K. pneumoniae (Dixon, 1984) are differentially regulated. In both cases, one promoter resembles a consensus nitrogen fixation gene promoter while the other promoter is E. coli-like . The nfeC tandem promoters resembled those of the glnB and glnA genes in that they all contain a putative RpoN-type promoter and a E. coli σ^{70} consensus promoter.

A common feature of RpoN-dependent promoters is that they are usually activated by binding of an additional regulatory protein upstream from the promoter (Ames and Nikaido, 1985; Buck et al., 1986; Gussin et al., 1986; Johnson et al., 1986; Inouye et al., 1987). The RpoN-type promoter of the nfeC gene is expressed only in bacteroids but not under free-living conditions. These results suggest

the presence of an upstream activator responsible for bacteroid-specific expression. When the free-living bacteria undergo conversion to bacteroids, massive changes in the cellular protein composition take place (Werner, 1992), indicating specific regulation of gene expression in bacteroids. Therefore, it is not surprising to find a bacteroid-specific promoter. However, the nfeC promoter identified in this study is different from other known active genes in bacteroids (Gussin et al., 1986; Murphy et al., 1988; Morett and Buck, 1989). For example, nif gene expression is high in bacteroids and requires RpoN, but these genes are also expressed under anaerobic growth conditions (Hennecke et al., 1988; Thöny et al., 1989). Thus, the P1 promoter of nfeC is the first known bacteroid-specific promoter in B. japonicum, that does not appear to be under oxygen control. Further study of this gene should add to our understanding of the regulation of bacteroid-specific genes. Scott-Craig et al. (1992) have identified two promoters activated at high levels in B. japonicum bacteroids. However, expression of both promoters was also detected in microaerobically grown cells. Sequence analysis of the region upstream of the transcription start sites revealed no similarity between these two promoters and nfeC.

Since little is known about genes repressed under

anaerobic conditions in B. japonicum, no canonical promoter sequence for such genes is known. The oxygen-dependent promoter (P2) of the nfeC gene has an E. coli consensus-like sequence, but it is unknown how this promoter is regulated during the transition from aerobic growth conditions to anaerobic conditions. Recently, Gabel et al.(1993) reported that the cytochrome $aa_{3\ gene}$ in B. japonicum has a promoter repressed under low levels of oxygen. However, this gene was also expressed at reduced levels in bacteroids. Thus, this promoter is likely different from the P2 promoter of nfeC. Again, there is no apparent similarity between the promoter regions of the cytochrome aa_3 gene and nfeC. Further investigation of nfeC expression should provide interesting insights into bacteroid-specific gene expression in B. japonicum.

B. Identification and characterization of a novel B. japonicum gene involved in host specific nitrogen fixation.

This work was initially started with a deletion mutant of B. japonicum, NAD163, which exhibited a delayed nodulation phenotype on soybean. This work was part of a continuing effort to identify additional symbiotic genes in B. japonicum. The deleted region in NAD163 is about 30 kb downstream of nifA/fixA and includes the nfeC gene. This mutant was able to induce effective nodules on G. max, G.

soja, and siratro but formed ineffective nodules on cowpea. Therefore, mutant NAD163 exhibited a host-specific phenotype for nitrogen fixation. Such bacterial host-specific fixation mutants have been reported from several other Rhizobium species and Bradyrhizobium strains (Stacey et al., 1982; Chen et al., 1985; Wilson et al., 1987; Ward et al., 1989; Hotter and Scott, 1991).

Nucleotide sequence information is available for only one gene of R. loti NZP2037 required for effective nodulation of L. pedunculatus but not for L. corniculatus (Ward et al., 1989). However, no significant similarity was detected between the hsfA gene sequence and this R. loti gene. R. loti mutants were isolated based on symptoms of nitrogen starvation by measuring the dry weights of the tops of both inoculated and uninoculated plants. Plants forming ineffective nodules showed no increase in dry weight over the uninoculated controls. Microscopy analysis of these mutant-induced nodules indicated that the rhizobia released from the infection threads failed to develop into mature bacteroids. These observations are similar to those made in this study with cowpea nodules infected with the B. japonicum deletion mutant NAD163. Microscopic examination of these cowpea nodules revealed that, while some bacteroids were found in the infected cell cytoplasm, they appeared to be readily broken down and usually failed to develop into

mature bacteroids. Consistent with this earlier work, mutant NAD163 induced yellow chlorotic leaves on 30-day-old cowpea plants and formed tiny nodules, a phenotype characteristic of a number of fix mutants (e.g., Regensburger et al., 1986; Ramseier et al., 1989; Rossbach et al., 1989). In contrast, soybean nodule cells infected by NAD163 developed nitrogen fixing bacteroids similarly to wild-type infected cells. However, the mutant infected cells usually contained bigger symbiosomes with more bacteroids per symbiosome when compared to the wildtype. However, these symbiosomes are not tight like that of wild-type infected cells with more symbiosome space. These differences between the mutant and wild-type infected cells were not reflected in the measurements of acetylene reduction activity. These results suggest that the mutation in NAD163 differentially affects bacteroid development depending on the particular host plant infected.

The location of the specific locus responsible for the phenotype of mutant strain NAD163 was determined by screening Tn5-induced mutants. One Tn5 mutant AN218 was isolated and exhibited a 2-3 day delay in leghemoglobin accumulation relative to the wild type. Sequencing of the region mutated by AN218 led to the identification of the hsfA gene which is responsible for the host-specific nitrogen fixation. The position of the AN218 Tn5 insertion

was found upstream of the hsfA gene. In this position, the mutation may not directly block gene expression but may partially reduce the level of hsfA transcription. Like the nfeC gene, the hsfA gene has a putative RpoN-type promoter (-24/-12), which is expressed only in bacteroids and not under free-living conditions. Therefore, this gene may also have an upstream activator responsible for bacteroid-specific expression as was proposed for the nfeC gene. Therefore, it is possible that the AN218 Tn5 insert might disrupt the action of this activator resulting in reduced hsfA expression. Sequence analysis of the regions upstream of nfeC and hsfA did not show any apparent repeated or inverted sequences that might identify a common activator sequences between these promoters.

Similar to mutant AN218, a delayed nitrogen fixation mutant Δ2330Ω has been reported with the mutation located 5' to the B. japonicum glyA gene and only partially affecting gene expression (Rossbach and Hennecke, 1991). The glyA gene encodes for serine hydroxymethytransferase (SHMT) which catalyzes the biosynthesis of glycine from serine and the transfer of a one-carbon unit to tetrahydrofolate. Disruption of glyA by mutation results in a phenotype in which infection thread formation and bacteroid development are blocked. However, a mutation about 300 base pairs (bp) 5' of the glyA coding region reduced SHMT activity by 60%

compared to the wildtype and delayed onset of nitrogen fixation by one week.

Symbiotic nitrogen fixation depends not only on genes directly concerned with the establishment of an active nitrogenase but also, indirectly, genes concerned with essential metabolic functions in bacteroids. Therefore, mutations in the latter genes can result in a defect in symbiotic nitrogen fixation (Fix^-) because the bacteroids do not fully proliferate in the nodules. In addition, the missing function may create a state unfavorable to the reduction of nitrogen while bacteroid development remains unaffected. For example, B. japonicum mutants lacking the high affinity molybdate uptake system are Fix^- because nitrogenase suffers from an inadequate supply of Mo (Maier et al., 1987). Respiration defective mutants are also Fix^- because respiration supplies the energy required for nitrogenase activity (O'Brian et al., 1987; Thöny-Meyer et al., 1989; Bott et al., 1990; Bott et al., 1991). In R. meliloti, R. leguminosarum bv. viciae, and B. japonicum, C4-dicarboxylate transport mutants formed ineffective (Fix^-) nodules (Roson et al., 1981; Bolton et al., 1986; Humbeck and Werner, 1987) presumably due to the inability of the bacteroids to transport the needed carbon source. In most host-specific fixation mutants that have been reported, nitrogen fixation ability is significantly reduced when

compared to the wild type, but not completely blocked (Stacey et al., 1982; Chua et al., 1985; Wilson et al., 1987; Ward et al., 1989; Hotter and Scott, 1991; Crank et al., 1993). These results suggest that the host-specific failure is due to some postinfection breakdown in the interaction between the plant and bacterium and not directly related to the nitrogen fixation enzymatic machinery. Although the biochemical function of the hsfA gene product is not known, microscopy analyses suggest that it plays some role in mature bacteroid development. It has been reported that mutations in B. japonicum genes that are directly involved in nitrogenase function (e.g., nifD, nifK, nifH, nifB) do not severely affect bacteroid development and persistence (Hahn et al., 1984; Fuhrmann et al., 1987). In contrast to these, mutations in the nitrogen fixation regulatory genes of B. japonicum such as nifA, $rpoN_{1/2}$, fixLJ, and mutations in other genes such as respiration genes lead to pronounced, premature bacteroid degradation. These data suggest that the hsfA gene product may be involved in the regulation of symbiotic N_2 fixation genes or metabolic functions essential for bacteroid development.

There are several possible explanations for the host-specific N_2 fixation phenotype of hsfA mutants. Upon invasion of the plant, nitrogen fixation mutants appear to be treated like plant pathogens, i.e., the host plant mounts

an active defense against the invader (Vance 1983). The biosynthesis of glyceollin, a phytoalexin, is induced after soybean is infected by pathogenic microorganisms (Ebel, 1979) but not after being infected with wild-type B. japonicum strain (Wingender et al., 1989). However, Werner et al. (1985) reported that a Fix$^-$ mutant of B. japonicum did induce glyceollin accumulation in soybean nodules. These infected nodules differed from wild type by an early disintegration of the symbiosome membrane (SM). Chalcone synthase (CHS) is a key enzyme in the plant defense response to pathogens in legumes (Collinge and Slusarenko, 1987; Ryder et al., 1987; Habereder et al., 1989). Therefore, induction of CHS can be used as an assay for the induction of the plant defense response. Fix$^-$ mutants of R. meliloti and B. japonicum were found to induce higher levels of CHS mRNA than wild-type controls when inoculated onto host plants (Grosskopf et al., 1993; Estabrook and Sengupta-Gopalan, 1991). These results suggest that Fix$^-$ mutants are recognized as a pathogens and induce plant defence reactions which prevent the bacteroid development. Thus, it would appear that some B. japonicum genes encode functions that enable the bacteria, directly or indirectly, to avoid eliciting this plant defense response or may be involved in overcoming the plant's defense reactions.

Bauer et al. (1985) tested the ability of cowpea and

soybean plants to be nodulated by the same strains of B. japonicum. Cowpea appeared to require 100-fold higher inoculum levels to initiate early nodulation responses. However, when this higher threshold was exceeded the response of the cowpea plants was much greater. These results suggest that cowpea may have different response mechanisms for symbiotic development in comparison to soybean. Microscopy analysis of nodules induced by the hsfA mutant suggests a different response in soybean and cowpea. Fifteen day old cowpea nodule cells infected by NAD163 possessed a few bacteroids but these were not apparent in 28 day old cowpea nodule cells. These data suggest that hsfA plays a role in maintaining the integrity of B. japonicum in cowpea nodules. Such a role may involve protection from host defence cell reaction. Therefore, if the hsfA gene product is involved in avoiding a plant defense response, the relative importance of hsfA function may vary among host plant species depending on their own sensitivity to rhizobial infection.

Another possibility is that the hsfA gene product may play a role in the biosynthesis of a bacterial cell surface component critical to symbiotic development in cowpea plants. Chen et al. (1985) reported that Rhizobium strain NGR234 mutants altered in exopolysaccharide (EPS) production exhibited a host-specific nitrogen fixation phenotype.

These symbiotic EPS mutants were tested on five hosts, Macroptilium atropurpureum, Desmodium intortum, Desmodium uncinatum, Lablab purpureus, and Leucaena leucocepnala and found to be Fix^+ on some but Fix^- on others. R. loti EPS mutants also exhibited a host-specific nitrogen fixation phenotype. Such mutants were fully effective on Lotus pedunculatus, but were ineffective on Leucaena leucocephala (Hotter and Scott, 1991). Bacterial cell surface components have been implicated in various stages of symbiotic development (Gray et al., 1991).

A host-specific phenotype arises from a defect in a specific signalling interaction or in a specific pathway of bacteroid differentiation. For example, different host plants may supply different carbon substrates to the bacteroids and the hsfA gene product might be involved in the utilization of such carbon sources. Bacteroids in AN218 infected cells have lower amount of poly-β-hydroxybutyrate (PHB) on both soybean and cowpea when compared to the wildtype. PHB is a carbon storage product that allows bacterial cells to respond more favorably to starvation and other stress conditions (McDermott et al., 1989; Tombolini and Nuti, 1989). In B. japonicum, bacteroids accumulate carbon as PHB, under conditions in which energy for nitrogenase is thought to be limiting (McDermott et al., 1989). Therefore, the lack of PHB

accumulation in AN218 bacteroids suggests that hsfA may also affect carbon metabolism. However, it was difficult to directly compare PHB accumulation ability in NAD163 (Fix^- on cowpea) and wild-type bacteroids because the NAD163 infected cells have fewer bacteroids. It has also been shown that a reduction in the levels of exported, fixed nitrogen can lead to a failure by the plant to induce the enzymes necessary for nitrogen assimilation leading to early senescence of the nodules (Atkins et al., 1984a; 1984b). The degradation of cowpea nodules infected by hsfA mutants is somewhat reminiscent of early nodule senescence.

Cowpea nodule cells infected by NAD163 contain few bacteroids and relatively larger symbiosomes than those produced in wild-type nodules. These results are similar to those of soybean nodule cells infected by cytochrome defective mutants of B. japonicum (O'Brian et al., 1987; Ramseier et al., 1989; 1991). Therefore, the mutation in NAD163 might affect, directly or indirectly, B. japonicum respiration. It is known that rhizobia have at least two respiratory pathways (Appleby, 1984; O'Brian and Maier, 1989; Bergersen and Turner, 1990; Hennecke, 1993). Since a low oxygen concentration is required for nitrogenase activity, it was proposed that one pathway must have a bacteroid-specific high affinity terminal oxidase to adapt to low oxygen conditions (Appleby, 1984; O'Brian and Maier,

1989; Bergersen and Turner, 1990; Hennecke, 1993). For example, several studies reported that bacteroids have several bacteroid- specific cytochromes including CO-reactive cytochromes C-552, C-554, C-550, C-555 and cytochromes P-450 and P-420 (Appleby, 1969; Kretovich et al., 1973; Daniel and Appleby, 1976; Keister and Marsh, 1990). Therefore, the hsfA gene product may be involved in bacteroid-specific respiration which may be differentially regulated under different host nodule conditions.

Clearly, several hypotheses concerning HsfA function can be posed and must now be tested. Further analysis of biochemical function of the hsfA gene product may enable us to identify additional steps between the initial induction of root nodule formation and the final establishment and maintenance of a fully effective, nitrogen fixing symbiosis.

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