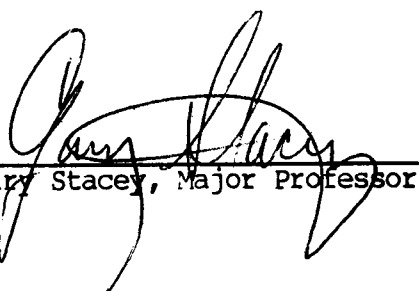
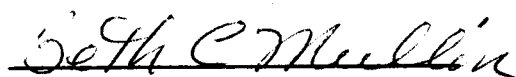
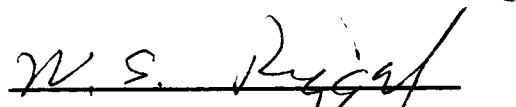
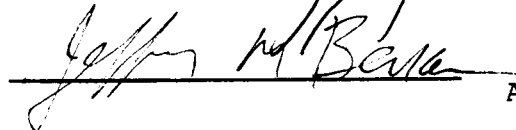


To The Graduate Council :

I am submitting herewith a dissertation written by Nirupama Arvind Deshmane entitled "Identification and Characterization of Nodulation Genes from Bradyrhizobium japonicum USDA 110." 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 Microbiology.


Gary Stacey, Major Professor

We have read this dissertation
and recommend its acceptance :

Accepted for council :


Vice Provost
and Dean of the Graduate Council

IDENTIFICATION AND CHARACTERIZATION OF NODULATION GENES

FROM BRADYRHIZOBIUM JAPONICUM USDA 110

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Nirupama Arvind Deshmane

August 1988

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This dissertation is dedicated to

my father, Mr. Arvind Deshmane, for his unflinching support
and encouragement
and

the memory of my mother, (Late) Mrs. Anuradha Deshmane,
who will always remain a source of inspiration.

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ABSTRACT

The Rhizobium - legume symbiosis is a complex association between two symbiotic partners that results in the formation of a root nodule. This process depends on the expression of a number of plant and bacterial genes that are considered critical for the maintenance of the symbiotic state. In order to identify some of the bacterial genes that are involved in nodulation, the DNA in and around the common nodulation genes of B. japonicum USDA 110 was analyzed. A number of novel symbiotic loci were identified by site-directed Tn5 mutagenesis. Specifically, four loci were identified that appear to play a role in host specific nodulation by Bradyrhizobium japonicum.

A DNA locus essential for the nodulation of Macroptilium atropurpureum (siratro) was identified. A locus essential for the nodulation of Glycine max (soybean) was also identified and encoded new nodulation gene(s) since none of the previously identified genes hybridized to it. A detailed restriction map of this DNA region was generated to study this new nodulation region at a molecular level. Analysis of Tn5 mutants in this region led to the definition of three loci: nodT, nodU and nodV. These loci were very well conserved among various Bradyrhizobium species, however, no homology was detected to the DNAs of any Rhizobium species examined indicating that these loci were unique to the genus,

Bradyrhizobium. The regulation of this region was studied with the aid of mini-Mu-lac insertion fusions. The nodT:lacZ and nodU:lacZ fusions were found to be induced by soybean seed exudate and isoflavones. The role of nodD in the induction of nodT was examined and it was found that induction of nodT required the nodD gene product.

A mutation in the nodT locus resulted in the loss of ability of the mutant to nodulate soybean. This mutant, NAD165, was also unable to nodulate several cultivars of soybean and other hosts of B. japonicum but could nodulate G. soja, suggesting that the nodT locus could possibly encode host specificity functions. Light microscopy revealed that the mutant could induce root hair curling but failed to induce cortical cell division.

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LIST OF SYMBOLS AND/OR ABBREVIATIONS

α	alpha
β	beta
δ	delta
γ	gamma
λ	lambda
$^{\circ}$	degree
%	percent
A_{600}	absorbance at 600 nm
amp^{r}	ampicillin resistant
Cm^{r}	chloramphenicol resistant
EDTA	ethylene diamine tetra acetic acid
g	gram
h	hour(s)
kb	kilo basepairs
kd	kilo dalton
kan^{r}	kanamycin resistant
kan^{s}	kanamycin sensitive
λ^{+}	lambda lysogen
m	meter
mm	millimeter
μm	micrometer
nm	nanometer
mg	milligram

ug	microgram
min	minute(s)
ml	milliliter
ul	microliter
M	molar
mM	millimolar
uM	micromolar
#	number
O.D	optical density
oz.	ounces
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
SSC	saline sodium citrate
str ^r	streptomycin resistant
tet ^r	tetracycline resistant
tet ^s	tetracycline sensitive
ts	temperature sensitive
X	times

CHAPTER I

INTRODUCTION

The gram negative, aerobic soil bacteria that can invade the roots of particular host plants and stimulate root nodules belong to the family Rhizobiaceae. Based on a variety of physiological traits, they are classified in the genus Rhizobium (fast-growing) or Bradyrhizobium (slow-growing) (see Table I). The bacteria reside in the nodule in a differentiated form, called the bacteroid and fix dinitrogen to ammonia which can be then assimilated by the plant. This process of nodulation and nitrogen fixation is a complex multi-step interaction between the bacterium and the plant and requires active participation of both partners. An interesting feature of this interaction is the specificity of the Rhizobium (or Bradyrhizobium) for a particular set of host plants. The bacterial strains which infect a certain plant are found also to infect, typically, a certain set of other host plants. This set is called the cross-inoculation group and has in fact been the basis of classification in Rhizobium/Bradyrhizobium taxonomy (Long, 1984.). For example, Bradyrhizobium japonicum can infect Glycine max (soybean), but also Vigna (cowpea), Glycine soja and Macroptilium; Rhizobium meliloti can infect Medicago, Melilotus and Trigonella; R. leguminosarum can infect Pisum, Vicia and Lathyrus. As can be seen, host range is not stringently limited, but there is still

TABLE I. General Phenotypic Differences between Fast-growing Rhizobium and slow-growing Bradyrhizobium Species

Phenotype	<u>Rhizobium</u>	<u>Bradyrhizobium</u>
Doubling time	2-4 h	7-20 h
Mannitol Medium	Acid production	Alkaline production
Disaccharide Utilization	(+)	(-)
Phosphogluconate Dehydrogenase	(+)	(-)
Chemoautotrophy	(-)	(+)
Antibiotic Sensitivity	5-50 ug/ml	100-1000 ug/ml
Free-living N ₂ fixation	(-)	(+)
Flagellation	peritrichous	subpolar
Guanine/Cytosine	50-63.1%	62.8-65.5%
Salt Tolerance	0.2-0.4 M	0.1 M

Adapted from Prakash and Atherly (1985)

some degree of species specificity in this interaction and it remains an intriguing aspect of nodulation. The series of events that lead to a nitrogen fixing nodule occur transiently in a few cells. This has made it difficult to delineate the different stages of nodulation. One of the most useful approaches in understanding this complex process has been the isolation of bacterial mutants that are defective in nodule formation.

Nodule Formation

The interaction between the bacterium and its host plant is based on an intricate signal exchange between the two partners. The infection of legumes probably begins by a response of the bacterium to nutrients and signal compounds exuded by the root (Bauer, 1981). The response is characterized by the induction of early nodulation genes and the probable appearance of cell surface receptors which bind to plant--derived lectins (Innes et al., 1986; Rossen et al., 1985; Mulligan et al., 1985; Kosslak et al., 1988). Rhizobial cells have been observed to attach rapidly end-on to the root surface, in a polar orientation. The lectin hypothesis, as first proposed by Bohloul and Schmidt (1974) suggested that the host plant lectins interacted selectively with compatible rhizobial polysaccharides on the outer surface and determined the host specificity of a particular species of Rhizobium (Bauer, 1981). Dazzo and his colleagues have subsequently described several studies supporting the lectin model in the Rhizobium trifolii--

Trifolium repens (clover) symbiosis (reviewed in Dazzo and Truchet, 1983; Dazzo and Gardiol, 1984). The binding of R. trifolii to white clover appeared to be a biphasic process (Dazzo et al., 1984). Initial adsorption was reversible; bound rhizobia subsequently elaborated microfibrils that irreversibly anchored the bacteria. Lectins have also been implicated in symbioses involving Pisum sativum (pea) (Kato et al., 1981), Glycine max (soybean) (Halverson and Stacey, 1986), Medicago sativa (alfalfa) (Paa et al., 1981) and Lotononis (lotus) (Law and Stridjom, 1984). However, several reports have challenged the lectin hypothesis. Some Rhizobium species bound lectins isolated from plants which they did not infect or nodulate (Dazzo and Hubbel, 1976; Wong, 1980). Furthermore, several legumes are known to be infected and nodulated by more than one species of Rhizobium (Long, 1984). Lectin pretreatment of B. japonicum did not enhance its attachment to soybean roots (Pueppke, 1984) and finally, mutant plants have been isolated that lacked seed lectin and yet were nodulated by Rhizobium (Pull et al., 1978; Su et al., 1980). These plants have subsequently be shown to produce root lectin which appears to function in the symbiosis (Halverson and Stacey, 1986, Sengupta-Gopalan et al., 1984). Therefore, the idea that host plant lectins and bacterial surface polysaccharides are the sole determinants of host specificity is probably too simplistic. It is more likely that these events are just a part of a complex sequence of signal

and response exchanges between the two symbionts and each step contributes to the overall biological specificity (reviewed in Bauer, 1981, Halverson and Stacey, 1986).

Following attachment, infective rhizobia are thought to release compounds that induce root hair curling. The rhizobia become entrapped in the curl of the root hair, a step which appears important for the initiation of infection process (Nutman, 1959; Fahreus, 1957). The entrapped bacteria penetrate the cell wall and the host deposits new cell wall material internal to the point at which the rhizobia are entrapped. The new cell wall material is used to form a tubular structure called the infection thread (Callahan and Torrey, 1981). The bacteria are carried single file into the infection thread which grows as it follows the nucleus to the base of the root hair (Fahreus, 57.; Nutman, 1959). The bacteria within the infection thread divide as they are carried towards the cortex. Shortly before or concurrent with the initiation of infection thread synthesis, cortical cells divide, probably due to the stimulation by diffusible substances released by the bacterium (Libbenga and Harkes, 1973.; Bauer et al., 1985). These substances may be cytokinins, which have been reported to be synthesized by rhizobia (Bauer et al., 1985). Studies by Bauer et al. (1985) suggest that induction of cortical cell division could be a likely determinant of host specificity and this specificity resides in the inducer substances secreted by rhizobia.

The growing infection thread passes through the cell wall of

the root and branches into adjacent newly divided cortical cells. The bacteria are released from the tip of the infection thread into the cytoplasm of cortical cells and are enclosed by a membrane of plant origin, called the peribacteroid membrane (Bergerson and Briggs, 1958). The bacteria divide, enlarge and differentiate to form pleiomorphic bacteroids which fill the infected cell. The inducer(s) of bacteroid development in the nodule is not known. The formation of bacteroids is characterized by frequent polymorphism, disappearance of polyhydroxybutyrate, less condensed nucleoids and alterations in DNA content, heme content, ribosome number, cell wall composition and osmotic sensitivity (Bergerson, 1955; Dart and Mercer, 1963; Newcomb, 1976; Vance et al., 1980; Sutton et al., 1981).

The host cells also undergo several modifications. The infected host cells increase in volume and are often polyploid due to endoreduplication (Mitchell, 1965; Libbenga et al., 1973; Libbenga and Torrey, 1973). The cytoplasm becomes dense and filled with ribosomes (Newcomb et al., 1979). Changes also occur in the uninfected cells of the nodule which are intermingled with the infected cells (Dart, 1977; Newcomb, 1981). It has been suggested that the uninfected cells may become specialized in metabolic pathways that are related to nitrogen fixation (Robertson and Fernden, 1980; Newcomb and Tandon, 1981).

Thus, the host and symbiont undergo extensive differentiation

to form a highly specialized structure, the root nodule, which is the site of nitrogen fixation. Clearly, several sets of genes in both the host plant and the bacterial symbiont are involved in this process. What are these genes? How and when are they expressed? What are the factors regulating their expression? These are some of the questions that have intrigued researchers in recent years. However, the work on the bacterial symbionts has been more rewarding, mainly because they are relatively less recalcitrant to physiological and genetic manipulations.

Molecular Genetics of Rhizobium

Identification of Symbiotic Genes

Our knowledge of the bacterial genes involved in nodulation and nitrogen fixation has mainly come from studies involving several species of the fast growing Rhizobium such as R. meliloti, R. leguminosarum and R. trifolii (Long, 1984, Stacey, 1987). These strains are readily amenable to genetic techniques such as conjugation and transduction and to an extent, transformation. In contrast, the slow growing rhizobia, assigned to the genus Bradyrhizobium (Jordan, 1982), have proved to be recalcitrant to routine genetic manipulations.

In Rhizobium, symbiotic (sym) genes are those that are necessary for the formation of a functional symbiosis with the host plant. The sym genes can be classified into three groups: nif, fix

and nod. The nif genes include those that encode the polypeptides of nitrogenase and all other genes yet to be identified that are analogous to nif genes of Klebsiella pneumoniae. The fix genes are those that are necessary for nitrogen fixation, but are not homologous to any of the known K. pneumoniae nif genes. It is likely that some fix genes will be renamed nif genes, once their function is known. Genes involved in the development of a nodule structure are called nod genes (reviewed by Hodgson and Stacey, 1986, Stacey, 1987).

The identification and characterization of sym genes was facilitated by using a mutational approach to the analysis of symbiosis. Traditionally, symbiotic mutants were acquired using mutagenizing agents such as nitrosoguanidine (NTG) and ultra violet light (Stacey et al., 1982; Noel et al., 1982; Forrai et al., 1983), as well as by isolating spontaneously generated antibiotic and phage resistant mutants (Rolfe et al., 1980; Raleigh and Signer, 1982). The isolation of such mutants helped in the phenotypic characterization of the steps involved in symbiosis. However, the isolation and characterization of the genes involved progressed rapidly when better methods of mutagenesis were devised. In recent years, Tn5 mutagenesis has been the method of choice for the isolation of symbiotic mutants. The advantages of Tn5 as a mutagenizing agent are two-fold: the mutants obtained are generally single, random mutants and, moreover, the use of Tn5 as a genetic and physical marker allows the molecular cloning of the DNA region

that is mutated by the Tn5 insertion via selection for kanamycin and/or streptomycin (Ruvkun and Ausubel, 1980, deBruijn and Lupski, 1984). The construction of genomic libraries in broad host range vectors and subsequent en masse conjugation into Sym mutants has allowed, by complementation, the isolation of symbiotic genes (Long et al., 1982). Another approach used in the identification of sym genes in R. meliloti was by the generation of R-primes which carried the symbiotic genes from strains that contained Tn5-induced symbiotic mutations (Banfalvi et al., 1983). Finally by using cloned K. pneumoniae nif genes as hybridization probes, analogous nif sequences have been identified in several rhizobia (Hodgson and Stacey, 1986, Stacey, 1987). In a similar strategy, by using cloned nod genes from R. meliloti and R. leguminosarum as hybridization probes, the analogous genes from other rhizobia have been isolated (Russel et al., 1985; Marvel et al., 1986; Noti et al., 1985; Lamb and Hennecke, 1986, Nieuwkoop et al., 1987).

Organization of Nod Genes

In several fast-growing Rhizobium species, the symbiotic genes were located on large indigenous plasmids called sym plasmids (Prakash et al., 1980). These plasmids ranged in size from 100 Mda (as in R. leguminosarum) (Hirsh et al., 1980, Hooykaas et al., 1981) to 1000 Mda or larger (Banfalvi et al., 1981) in R. meliloti (which contains two of these "megaplasms") (Hirsch et al., 1980). In the genus Rhizobium, most of the nif and nod genes

were located on one large sym plasmid. In addition to the plasmid-borne sym genes, chromosomally determined functions were also shown to be involved in the establishment of a nitrogen fixing nodule (Finan et al. 1985, Rolfe and Shine, 1983).

The first nodulation genes to be identified were those that encode root hair curling ability, (hac) (Long et al., 1982). Complementation of a R. meliloti nodulation defective (Nod⁻) mutant by a genomic cosmid library led to the isolation of a cosmid, pRmSL26 (Long et al., 1982). Saturation transposon Tn5 mutagenesis, complementation analysis and DNA sequencing led to the definition of four genes, nodD and nodABC, located on a 8.7 kb EcoRI fragment of pRmSL26 (Jacobs et al., 1985). The same genes were located on a 14 kb and 10 kb EcoRI fragments in R. trifolii (Djordjevic et al., 1985) and R. leguminosarum (Downie et al., 1985), respectively, and a 3.9 kb HindIII fragment in B. japonicum (Russel et al., 1985; Nieuwkoop et al., 1987). Hybridization analysis and a comparison of DNA sequences revealed that these genes were well conserved in all Rhizobium and Bradyrhizobium species examined (Kondorosi et al., 1984; Torok et al., 1984; Egelhoff et al., 1985; Scott, 1986). The biochemical role of the nodABC gene products is unknown. The nodC protein was shown to have a hydrophobic carboxyterminal end and was found to be associated with the bacterial outer membrane (John et al., 1985; Jacobs et al., 1985; Torok et al., 1985; Rossen et al., 1984).

John et al. (1988) have recently demonstrated that in R. meliloti, the nodC protein exists in the membrane, probably as a dimer. The domain structure of Nod C protein shows striking similarity with cell surface receptors. The nodABC genes were found to be contiguous and in one transcriptional unit, which in R. leguminosarum also contained two additional genes, nodI and nodJ (Evans and Downie, 1986). The predicted amino acid sequence of the nodI protein showed marked homology to that of a group of bacterial ATP binding proteins involved in active transport (Evans and Downie, 1986). The nodJ gene appears to encode a very hydrophobic integral membrane protein that may function in conjunction with nodI protein in the uptake of some plant metabolite(s) (Evans and Downie, 1986). DNA sequences homologous to nodIJ have been identified in R. meliloti, B. japonicum and other rhizobia (Nieuwkoop et al., 1987; Evans and Downie, 1986). In B. japonicum and B. parasponia, an open reading frame (ORF) upstream of nodA, located on the same operon has been identified by DNA sequence analysis (Nieuwkoop et al., 1987; Scott, 1986). The ORF in B. japonicum (also termed nodL) shared less than 30% homology with the ORF from B. parasponia, (termed nodK) found in a similar position upstream of nodA. Hybridization analysis has indicated that nodL is conserved in all Rhizobium and Bradyrhizobium species tested (Banfalvi et al., submitted).

Based on their phenotypic properties, the Nod mutants can be grouped into two broad categories: (i) those that completely lose

the ability to induce root hair curling; these mutants map to the nodABC genes and totally block nodulation ability and (ii) those that retain nodulation ability, the level of which may vary on different host plants. Nod⁻ mutants defective in root hair curling could be complemented for nodulation on their normal host plant with the analogous genes from heterologous Rhizobium strains, suggesting that the highly conserved nodABC genes (often referred to as common nod genes) may not be involved in host recognition. Nodulation genes involved in host range recognition have been implicated on the basis that mutations in these genes were not complemented by genes from heterologous species of Rhizobium (Downie and Johnston, 1986). Two of those genes, nodE and nodF (also called hsnA and hsnB for host specific nodulation) were homologous among R. meliloti, R. leguminosarum and R. trifolii (Horvath et al., 1986; Shearman et al., 1986.; Djordjevic et al., 1985). Interestingly, a strain of R. trifolii which had a mutation in nodE was shown to acquire the ability to nodulate peas, albeit at a very low level, but could no longer nodulate clover (Djordjevic et al., 1985). The predicted amino acid sequence of the nodF gene resembled that of acyl carrier proteins from bacteria, plants and animals (Shearman et al., 1986). The conservation was the strongest around the pantothenate binding site, and in the light of this resemblance, it is possible that nodF protein acts as an acyl carrier protein in the acylation or

synthesis of lipopolysaccharide (LPS), exopolysaccharide (EPS) or cyclic B-2 glucan. Two other host specific nodulation genes, nodG and nodH (also called as hsnC and hsnD, respectively) have been identified in R. meliloti (Horvath et al., 1986). The nodH gene determined the host specific hair curling event and nodH mutants of R. meliloti had an altered host range (Horvath et al., 1986). Mutations within R. meliloti nodeFG (hsnABC) genes affected infection thread development and nodulation of their natural plant hosts to different extents (Horvath et al., 1986; Djordjevic et al., 1985). The putative nodG polypeptide of R. meliloti shows homology to ribitol dehydrogenase of K. pneumoniae and could therefore be involved in polyol metabolism (Debelle et al., 1987). In R. leguminosarum and R. trifolii, a set of genes, nodLMN have been implicated in host specific nodulation (Surin and Downie, 1988). In R. meliloti, a region essential for efficient nodulation, termed efn, was found to be closely linked to the hac and hsn genes (Putnoky et al., 1987) and contained the second copy of nodD, nodD₂, as well as additional genes. The biochemical function of this region is not known.

In B. japonicum, a combination of random and site directed transposon Tn5 mutagenesis led to the identification of several genes involved in nodulation (Nieuwkoop et al., 1987; Rostas et al., 1985.; So et al., 1987; Sadowsky et al., 1986). The nod genes, nodLABCIJ and nodD have been identified and characterized by Tn5 mutagenesis and hybridization analysis using probes from other

rhizobia and by DNA sequencing. Nieuwkoop et al. (1987) have defined a locus in B. japonicum which determined the ability to nodulate Macroptilium atropurpureum and Marvel et al. (1987) defined a locus in Bradyrhizobium. sp. (Parasponia) Rp501 that encoded the ability to nodulate Parasponia, a non legume. Both loci were found to be closely linked to the common nod genes.

Regulation of Nod Gene Expression

The regulation of nod gene expression has been analyzed in different Rhizobium species by placing the Escherichia coli lacZ gene under control of the nod gene promoters. The nodD gene was found to be expressed constitutively in all Rhizobium species tested (Rossen et al., 1986; Mulligan and Long., 1986 Innes et al., 1986). On the other hand, it was found to be induced by the soybean seed exudate in B. japonicum (Banfalvi et al., submitted). There was little or no constitutive expression of other nod genes (nodABC) in Rhizobium or Bradyrhizobium, however, induction was observed when the bacteria were exposed to plant secreted factors (specifically, flavanoids) (Rossen et al., 1986; Mulligan and Long., 1986 Innes et al., 1986). The plant factors that regulate nod genes (stimulators and inhibitors) have also been identified using gene fusions to lacZ. The inducing molecules, which activated nod genes at very low concentrations included flavanoids such as luteolin (from alfalfa) (Peters et al., 1986) , 7, 4' - dihydroxy flavone (from clover) (Redmond et al., 1986) and

naringenin (from pea) (Firmin et al., 1986) and Vicia sativa (Zaat et al., 1987). Research done in our laboratory has indicated that genistein (an isoflavone), was the optimal inducer of B. japonicum nod genes (Banfalvi et al., submitted, Kossiak et al., 1988). This induction did not occur in strains mutated in nodD, suggesting that the nodD gene (in conjunction with plant factors) regulated the induction of other nod gene transcriptional units (Rossen et al., 1986; Mulligan and Long et al., 1986). Moreover, in R. leguminosarum, the nodD gene was found to be autoregulatory since multiple copies of nodD inhibited the expression of a nodD:lacZ fusion (Rossen et al., 1986). In R. meliloti, 3 copies of nodD have been detected on the sym plasmid, 2 of which (nodD₁ and nodD₂) were necessary for efficient nodulation of alfalfa (Gottfert et al., 1987; Gyorgypal et al., 1988; Homma and Ausubel, 1987). This may account for the fact that in R. meliloti, nodD was not autoregulatory and Tn5 insertions in nodD₁ have only a slight effect on nodulation (Gottfert et al., 1987). A comparison of DNA sequences of nodD from several Rhizobium species showed an overall nucleotide sequence homology of 50%, with the aminoterminal end being the most conserved (Horvath et al., 1987). This conserved domain exhibited significant homology to the E. coli regulatory protein lysR (a DNA binding protein) (Applebaum et al., 1988; Horvath et al., 1987). Interestingly, a nodD gene from Rhizobium strain MPIK3030 could not only suppress nodD mutations in R.

meliloti, but could also extend the host range of R. meliloti transconjugants to include siratro (Horvath et al., 1987). Furthermore, MPIK3030 nodD was able to interact with plant factors from its native host, siratro, as well as those from alfalfa. By using chimeric nodD constructs, it was shown that the C terminal portion of the nodD protein was involved in the recognition of the plant factors (Horvath et al., 1987). These results showed that the nodD protein host specifically mediated the induction of nod genes, and therefore could no longer be termed a common nodulation gene. In R. leguminosarum, nodF and nodE (Shearman et al., 1986) as well as nodLMN (Surin and Downie, 1988) were also inducible by plant exudates. These results suggested that plant inducible promoters may be conserved. Sequence analysis and a comparison of all inducible nod operons indicated that there was a highly conserved DNA sequence of about 40 nucleotides--the "nod box" (Rostas et al., 1986; Spaink et al., 1987). In B. japonicum, the region 5' to the plant inducible nodD and nodABC shared homology with the nod box (Banfalvi et al., submitted). Deletion analysis indicated that this region was essential for nodABC gene expression in R. meliloti (Rostas et al., 1986; Spaink et al., 1986) and has been implicated in nodD mediated transcriptional activation of these genes. Hong et al., (1987) have demonstrated that fragments of DNA which contained the nod boxes preceding nodA (nodA nod box) and nodF (nodF nod box) from R. leguminosarum bound to protein(s) obtained from cell free extracts of Rhizobium strains containing a

copy of nodD. The protein-DNA complexes were identified by retardation of DNA fragments during polyacrylamide gel electrophoresis. Furthermore, extracts from Rhizobium strains containing mutant forms of nodD which were specifically affected in autoregulation or flavanoid dependant activation formed either no protein DNA-complexes or formed a complex with altered mobility compared to that obtained with extracts from wild type strains. Fisher et al., (1988) have shown that antibody to nodD from R. meliloti removed the specific DNA binding activity from the cell free extracts. A synthetic double-stranded oligonucleotide identical to the 3' half of nodA nod box blocked the formation of protein-DNA complexes providing evidence that nodD interacts with the nod box, presumably as a transcriptional activator.

In R. trifolii, transcriptional activation of nod gene promoters by 7, 4'-dihydroxyflavone was found to be growth phase dependent and maximum stimulation was found in early log phase cells (Djordjevic et al., 1987). This growth phase dependence was found to be also true for the constitutive nodD as well. Inhibitory compounds present in white clover root exudate were identified as umbelliferone (a coumarin) and formononetin (an isoflavone) (Djordjevic et al., 1987). An interesting observation, that all strong stimulatory and inhibitory compounds possessed a 7 --hydroxy moiety suggested that there was one binding site for these compounds. When the inhibitors were tested at a 10-fold

molar excess over the stimulatory compounds, a complete inhibition of nod gene induction was observed (Djordjevic et al., 1987). An intriguing feature of the regulatory molecules was that an inhibitor of nod genes in one Rhizobium strain could act as a stimulator of nod genes in other Rhizobium strains. Daidzein, a repressor of nod genes in R. leguminosarum (Firmin et al., 1986) and R. trifolii (Djordjevic et al., 1987) is found to be a stimulator in B. japonicum (Banfalvi et al., submitted) as well as another set of bacterial genes in R. fredii (Olson et al., 1985; Sadowsky et al., 1988). As the response to regulatory molecules is very rapid, it is believed that the regulators traverse the bacterial cell wall or perhaps there is some involvement of an active membrane bound uptake or transmembrane signalling system (Djordjevic et al., 1987). It is also possible that the phenolic compounds act at a common membrane site and that a secondary signal is responsible for the induction of nod genes, either in combination with the nodD product or perhaps by activating the nodD product itself. Stimulatory compounds may mediate the conversion of nodD product to a positive regulator while the inhibitory compounds may compete for and occupy the binding site, resulting in poor or no activation of nodD product (Djordjevic et al., 1987). The activated nodD may subsequently stimulate transcription from the nod box sequence which is upstream of other nod genes.

Polysaccharides in Nodulation

The genes encoding products involved in exopolysaccharide biosynthesis appear to play an important role in nodulation. In R. meliloti, mutations in the exo loci, some of which are located on the second megaplasmid, result in the formation of nodules which lack infection threads (Inf⁻) and fail to fix nitrogen (Finan et al., 1985; Leigh et al., 1986). The Exo⁻ mutants elicit nodules on alfalfa, that have few bacteria, which occur in superficial intercellular spaces rather than within nodule cells (Finan et al., 1985). In Agrobacterium tumefaciens, the chvA and chvB genes are known to be essential for oncogenic transformation of dicotyledons and attachment to plant cells (Douglas et al., 1985). The chvB gene has been implicated in the production of cyclic B - (1 -2) - glucan (Puvanesarajah et al., 1986), a unusual exopolysaccharide found only in members of the Rhizobiaceae (Dell et al., 1983, Gorin et al., 1961, Zenvenhuizen et al., 1979). An analogous locus (called ndv) has been studied in R. meliloti and mutations in this locus have been shown to be important for nodule development (Dylan et al., 1985). Borthakur et al. (1985) identified a gene, psi (for polysaccharide inhibition), located close to the nod genes on the sym plasmid of R. phaseoli. This gene product, which is possibly membrane associated, inhibited EPS synthesis and nodulation ability when cloned in R. phaseoli on a multi-copy plasmid. A second gene, psr (polysaccharide restoration), also located on the sym plasmid

restored the synthesis of EPS and nodulation. Using lacZ fusions, it has been further demonstrated that transcription of psi was inhibited by psr (Borthakur et al., 1987). It is hypothesized that psi may be involved in the repression of EPS synthesis by bacteroids as a reduced amount of EPS is found in nodules.

Rhizobium - Legume Communication

It is clear from the literature reviewed so far, that the plant and its symbiont communicate with each other in several ways to form a nitrogen fixing nodule. The initial communication between the bacterium and the plant appears to be the induction of Rhizobium nod genes by compounds secreted from the roots of the legumes. In response to this induction, the bacteria appear to synthesize low molecular weight factors that induce root hair deformation (Bhuvaneswari and Solheim, 1985) and in case of a small seeded legume, Vicia sativa subspecies nigra, inhibit over all root growth (van Brussel et al., 1986). These factors could also be of plant origin that are modified by nodABC gene products (Downie et al., 1986). Such signal molecules could possibly be involved in the maintenance of infection threads and induction of cortical cell divisions (Downie and Johnston, 1986; Bauer et al., 1985). The induced host specificity gene products may initiate a modification of the bacterial outer surface to form a new coat (possibly involving exopolysaccharide, liposaccharide and/or cyclic B-2

glucan) which is recognized by the host plant lectins (Downie and Johnson, 1986). The binding of the plant lectin to the modified bacterium may then initiate a cascade of events culminating in a nitrogen fixing nodule.

Rationale for Present Work

In order to define and understand the series of events in nodulation, one direct approach would be to isolate a range of mutants blocked in different stages of the symbiosis. Characterization of such mutants, and the genes involved, may allow the dissection of the legume-Rhizobium symbiosis at a molecular level. Although several genes involved in nodulation have been identified in the genus Rhizobium, relatively little progress has been made in the genus Bradyrhizobium. In the genus Rhizobium, nod genes have been shown to be clustered in a small DNA region on the sym plasmid. Therefore, it is reasonable to examine the DNA region around the common nod genes in B. japonicum for the presence of other nod genes. Four genomic cosmid clones which together encompassed about a 100 kb DNA region were used as targets for site-directed Tn5 mutagenesis and/or deletion mutagenesis. The mutants generated by this method were analyzed for their symbiotic phenotype on Glycine max. DNA regions with mutations that resulted in symbiotically defective phenotype were further characterized at

a molecular and phenotypic level. As a result, three unique loci essential for the nodulation of soybean by B. japonicum USDA 110 were identified.

CHAPTER II

MATERIAL AND METHODS

Bacterial Strains, Phages, Media and Growth Conditions

All bacterial strains used in this study are described in Table II. Bradyrhizobium japonicum cultures were grown at 30°C in Yeast Extract Salts (YS) medium or in Rhizobium defined yeast extract medium (RDY) containing (per liter) 5 g gluconic acid, 1 g glutamic acid and 1 g yeast extract, in a mineral salts base (5g CaCl₂, 0.145g Boric acid, 0.125g FeSO₄, 0.075g CuSO₄, 0.0043g MnSO₄, 0.108g ZnSO₄, 0.0125g NaMoO₄, 7g nitrilo acetate, pH7.5) (Bishop et al., 1974). Rhizobium spp. strain MPIK3030 and R. trifolii were grown in TY (Beringer, 1974) medium at 30°C. Rhizobium meliloti, Rhizobium leguminosarum, and Rhizobium fredii were grown in TA (Orosz et al., 1973) medium at 30°C. E. coli cultures were grown in LB medium (Maniatis et al., 1982). All lysogenic cultures were grown in LB medium at 30°C; others at 37°C. Antibiotics were added to the medium at the following concentrations: For the selection of B. japonicum, 150 ug/ml kanamycin (kan), 150 ug/ml streptomycin (str), 150 ug/ml tetracycline (tet) and 200 ug/ml chloramphenicol (Cm) were used. For the selection of E. coli, 25 ug/ml ampicillin (amp), 25 ug/ml kanamycin, 20 ug/ml tetracycline, 30 ug/ml chloramphenicol, and 30 ug/ml rifampicin (rif) were used.

Table II Bacterial Strains, Phages and Plasmids

Strains	Phages	Plasmids	Relevant characteristics	Reference or Source
<u>Bacteria</u>				
<u>Bradyrhizobium japonicum</u>				
USDA 110			wild type, colony type 1110	Kuykendall
USDA 46			wild type	D. Kuykendall
USDA 76			wild type	D. Kuykendall
USDA 112			wild type	D. Kuykendall
USDA 122			wild type	D. Kuykendall
USDA 123			wild type	D. Kuykendall
USDA 6			wild type	D. Kuykendall
61A101C			wild type	Nitragin Company
SR			wild type	D. Kuykendall
73RS			wild type	D. Kuykendall
AN6			wild type	D. Kuykendall
AN 314			USDA 110 (nod ⁺ :Tn5)	this study
NAD 90			USDAll0 (fixA:Tn5)	this study
NAD 138			USDAll0 (hsn:Tn5)	"
NAD 27			USDA 110 (5.8 kb EcoRI:Tn5)	"
NAD 14			USDA 110 (7.7 kb EcoRI:Tn5)	"
NAD 163			USDA 110 (Δ prJUT14)	"
NAD 164			USDA 110 (Δ prJUT24)	"
NAD 165			USDA 110 (nod ⁺ :Tn5)	"
NAD 167			USDA 110 (nod ⁺ :Tn5)	"
NAD 168			USDA 110 (nod ⁺ :Tn5)	"
NAD 172			USDA 110 (nod ⁺ :Tn5)	"
NAD 173			USDA 110 (nod ⁺ :Tn5)	"
NAD 174			USDA 110 (nod ⁺ :Tn5)	"
NAD 180			USDA 110 (nod ⁺ :Tn5)	"
NAD 181			USDA 110 (nod ⁺ :Tn5)	"
NAD 201			USDA 110 (nod ⁺ :lacZ)	"

Table II (continued)

Strains, Phages, Plasmids	Relevant Characteristics	Reference or Source
NAD 202	USDA 110 (nodU:lacZ)	"
NAD 203	AN 314 (nodF:lacZ)	"
<i>R. meliloti</i>	wild type	A. Kondorosi
<i>Rhizobium</i> spp. MPIK3030	wild type	A. Kondorosi
<i>Rhizobium</i> spp. NGR234	wild type	B. Rolfe
<i>R. trifolii</i>	wild type	B. Rolfe
<i>R. leguminosarum</i>	wild type	N. Brewin
<i>R. fredii</i>		
USDA 191	wild type	A. Atherly
USDA 193	wild type	H. Keyser
USDA 194	wild type	H. Keyser
<i>Escherichia coli</i>		
DH1	F ⁺ , recA1, endA1, gyrA96, thi ⁻ 1, hsdR 17, supE ⁺ 44, λ ⁻ F ⁺ , hsd20, recA 13, ara-14, proA2, lact1 galK2 rpsL20	Low, 1968
HB101	recA, trpE, λ ⁺ hsdR, lacY, galK, rpsL 594	Bolivar, 1978
DBCS31-1	araD 139, (argF-lac)U169,	D. Berg
EG47	rpsL 150, relA 1, flbB 301,	Goldberg et al, 1974
MC4100	ptsF 25, deoC 1	Ratet and Richard 1986
(Mu cts)	araD 139, rpsL, Mu	Casadaban, 1975
M8820 (Mu)	arg-lac (IPOZYA)XIII	
MC4100 (Mu cts)	mini-mu-lac insertion vehicle PR48	Ratet et al., 1988
MudIIPR48	MudIIPR48 (pND1013)	this study
ND527		

TABLE II (continued)

Strains Phages Plasmids	Relevant Characteristics	Reference or Source
Phages		
λ Tn5	kan ^r	D. Berg
Pl Tn5	kan ^r	Quinto and Bender, 1984
Plclrl00cm	Cm ^r	D. Kaiser
fFR nodC	M13mp8 with insertion of 0.7 kb <u>nodC</u> fragment of <u>R. meliloti</u>	F. R. Quinones
fNGnodD	M13mp18 with insertion of 275 bp <u>nodD</u> fragment of <u>R. meliloti</u>	F. R. Quinones
FBH hsnA	M13mp18 with insertion of 0.5 kb <u>hsnA</u> fragment of <u>R. meliloti</u>	B. Horvath
FBH hsnB	M13 mp18 with insertion of 0.5 kb <u>hsnB</u> fragment in <u>R. meliloti</u> .	B. Horvath
FBH hsnC	M13mp18 with insertion of 1.6 kb <u>hsnC</u> fragment in <u>R. meliloti</u>	B. Horvath
fFR hsnD	M13mp18 with insertion of 320-bp <u>hsnD</u> fragment in <u>R. meliloti</u>	F. R. Quinones
Plasmids		
pRJUT10, pRJUT2, pRJUT14, pRJUT24	pHC79 clones of <u>b. japonicum</u> Trimethoprim resistant, <u>Tra</u> ⁺	Russel et al, 1985
pRK2073	5.3 kb <u>EcoRI</u> fragment cloned into pBR329	Leong et al, 1982 this study
pGSE3	5.3 kb <u>EcoRI</u> fragment cloned into pBR322	this study
pND1013	mini- μ -lac insertion in <u>nodT</u> located on pND1013	this study
pND79	mini- μ -lac insertion in <u>nodU</u> located on pND1013	this study
pND228	mini- μ -lac insertion in <u>nodU</u> located on pND1013	this study
pBR329	Amp ^r , tet ^r , Cm ^r	Boyer and Roulland-Dussoix, 1969
pBR322	Amp ^r , tet ^r	

Enzymes and Isotopes

DNA restriction endonucleases and modifying enzymes were purchased from New England Biolabs, Boehringer Mannheim, or Bethesda Research Laboratories. Radioactive isotopes were purchased from New England Nuclear Corporation.

Procedures

Isolation of Genomic and Plasmid DNA

Chromosomal DNAs from Rhizobium/Bradyrhizobium cultures were isolated on a small scale as follows. A 3 ml stationary phase culture was pelleted in a 1.5 ml microcentrifuge tube and washed with TNE buffer (10mM Tris - HCl pH 7.4, 10 mM NaCl, 0.1 mM EDTA) containing 0.1% sarcosyl. The pellet was resuspended in 450 ul of TE buffer (10 mM Tris - HCl, 0.5 mM EDTA, pH 7.4), 150 ul of 5% SDS in TNE buffer and 150 ul of proteinase K (Sigma Chemical Co., St. Louis, MO.) (2.5 mg/ml TNE). The suspension was then incubated at 37°C for 1 h (or more) or until the lysate was clear. The cell lysate was extracted 3 times with phenol : chloroform : isoamyl alcohol (25:24:1) followed by 4 extractions with chloroform. The aqueous layer was precipitated with 2.5 volumes of ethanol, the DNA pellet was dried and resuspended in 1X TE buffer.

Plasmid DNA was isolated on a small scale by the alkaline lysis method as described by Maniatis et al. (1982). A 3 ml culture grown overnight was pelleted in an 1.5 ml Eppendorf tube,

resuspended in 100 ul of solution I (50 mM glucose, 25mM Tris pH 8, 10 mM EDTA pH 7.5 and 10 mg/ml lysozyme) and incubated for five minutes. This was followed by addition of 200 ul of solution II (0.2 N NaOH, 1% SDS), incubated for 5 minutes on ice and finally followed by addition of 150 ul of precooled solution III (3 M Potassium acetate, pH 6). The cell debris was removed by centrifugation and the lysate transferred to another tube. The lysate was extracted with phenol : chloroform : isoamyl alcohol (25:24:1), then chloroform. The DNA in the aqueous layer was precipitated by 2.5 volumes of ethanol and used for further manipulations.

Large scale isolation of plasmid DNA was performed by the Triton-lysozyme lysis procedure as described by Maniatis et al. (1982). A one liter culture of E. coli containing the plasmid of interest was grown overnight in LB broth containing appropriate antibiotics and harvested using a Sorvall GSA rotor at 8000 rpm for 10 min. The cell pellet was washed with 1X TE buffer and the pellet was resuspended in Solution A (15% sucrose, 50 mM Tris pH 8.5, 0.05 M Na₂EDTA) containing lysozyme and incubated at 37°C until lysis occurred. Addition of 0.1 ml of 20% SDS optimized the lysis. After the cells lysed, 14.5 ml of Solution B (0.1% Triton X - 100, 0.05 M Tris pH 8.5, 0.05 M Na₂EDTA) was added and incubated for 30 min. The lysate was centrifuged to remove cell debris and the clear lysate was poured in a 100 ml graduated cylinder, that

had been baked overnight in a dry oven at 400°C. The total volume of the solution was adjusted to 28.9 ml with TE buffer. CsCl (26.13 g) was added to the solution and dissolved completely. Ethidium bromide (13.9 mg) was added to each solution and mixed well. The suspension was then poured into Ti 60 tubes and overlayed with mineral oil to balance, and centrifuged in a Ti60 rotor at 40,000 rpm at 20°C for 48 h. After 48 h, the DNA was observed with the aid of a long wave ultraviolet lamp (UVP Inc.; San Gabriel, CA) and extracted with a 18 gauge needle, then the ethidium bromide was removed by successive extractions with secondary butanol. The aqueous layer was then precipitated with 2.5 volumes of ethanol.

DNA Manipulations

Restriction endonuclease digestions and modification reactions of DNA samples were carried out according to the manufacturer's recommendations. Ligation reactions were performed at room temperature. All DNAs were analyzed on either a 0.7% or 1% agarose gels using 1X TAE (Tris 1M, Na. acetate 0.125M, EDTA.2H₂O 0.025M pH 7.8) as buffer. The sizes of DNA fragments were estimated using bacteriophage lambda DNA digested with HindIII or a 1 kb DNA ladder (obtained from BRL) as size markers. The DNA in the gels was stained with ethidium bromide and visualized using an ultraviolet transilluminator (Fotodyne, Fotosystem, New Berlin, WI). The gels were photographed by a Polaroid camera using a type 55

positive/negative film or type 667 high speed film (Polaroid Corp.; Cambridge, MA) and processed as recommended by the manufacturers.

Electroelution

DNA fragments were recovered from agarose gels as described by Davis et al. (1986). The gel piece containing the fragment was excised and placed in a dialysis bag containing 1X TAE buffer. The dialysis bag containing the gel piece was then electrophoresed causing the DNA to migrate out of the gel into the dialysis bag buffer. The fragment was then recovered from the buffer and purified by extracting with phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated with ethanol. The dialysis tubing (13 mm diameter, Spectrapor from Spectrum) was prepared for electroelution by boiling in 2% NaHCO_3 with 1 mM EDTA for 1 min, followed by cooling and rinsing three times in water.

Southern Blots and Hybridizations

Transfer of electrophoresed DNA to nitrocellulose filter paper (Scheicher and Schuell Inc.) was done as described by Southern (1975). The agarose gel was denatured in 0.5 M NaOH, 1.5 M NaCl for 30 minutes followed by neutralization in 1 M Tris - Cl, 0.5 M NaCl for 40-60 minutes. The DNA was transferred overnight using 20X SSC (3 M NaCl, 0.3 M Na. citrate) as transfer buffer and immobilized onto the support by baking at 60°C for 2 hours.

The DNA filters were prehybridized for 4 hours in plastic bags at 37°C in 3X SSC buffer containing 50% formamide, 0.2% Ficoll,

0.2% bovine serum albumin, 0.2% polyvinyl pyrrolidone and 50 ug of sheared and denatured salmon sperm DNA. ^{32}P labelled radioactive probes were prepared by nick translations using a ^{32}P -dCTP (3,000 Ci/mmol; New England Nuclear, Boston, MA) as recommended by the manufacturers. Approximately 10^8 counts were added to each bag. Hybridizations were carried out at 37°C with continuous shaking for 12-16 hours. For high stringency washes, the filters were washed for 30 minutes, 3 times, at 65°C with 1X SSC, 0.1% SDS followed by one wash for 30 minutes at 65°C with 1X SSC. For low stringency conditions, filters were washed, 3 times at 37°C with 2X SSC, 0.1% SDS for 30 minutes each, followed by one wash with 1X SSC. The filters were air dried and autoradiographed with Kodak X - Omat AR film or Kodak X - K film at -70°C , with or without intensifying screens.

If the filters were reused for hybridizations, they were first washed (2X) with boiling water for 10 minutes to remove the existing probe and air-dried. The filters were autoradiographed as before to ensure the removal of preexisting radioactivity. The filters were then reused in hybridization experiments with other radioactive probes.

Colony Hybridizations

Colony hybridizations of B. japonicum were performed following the procedure of Hahn and Hennecke (1984). The colonies were transferred to nitrocellulose filters and soaked in 0.5 N NaOH for

7 min, followed by neutralization with 1M Tris pH 7.5 for 2X, 1 minute. The blots were then soaked in 1.5M NaCl, 0.5M Tris HCl (pH 7.5) for 4 min and air dried for 1 h followed by baking at 60°C for 2 h. Hybridization conditions were as described before.

Bacterial Transformation

Bacterial transformations were performed essentially as described by Maniatis (1982). E. coli strains were grown overnight in 5 ml of LB medium (with or without antibiotics) at 30°C or 37°C as required. The culture was diluted 100-fold in 50 ml of LB medium (with or without antibiotics) and grown at 30°C or 37°C to an $A_{600} = 0.2$. Cells were harvested by centrifugation at 6000 rpm in a Beckman JA-17 rotor for 5 minutes at 4°C. The cell pellet was washed with 10mls of precooled 20 mM NaCl, recentrifuged and the cell pellet was washed with 10 mls of 30 mM $CaCl_2$. The cell pellet was resuspended in 2 mls of 30mM $CaCl_2$ and made competent by incubating on ice for 20 minutes. A 100 ng of DNA in 100ul of 30 mM $CaCl_2$ was mixed with 200 ul of competent cells and incubated on ice for 1 hour. After heat shocking the suspension (42°C, 2 min), 2 mls of LB broth was added and incubated for 1 hour at 30°C or 37°C before plating 0.1 - 0.2 mls on appropriate selection plates, followed by overnight incubation at 30°C or 37°C.

Packaging of Cosmid DNA and Transduction

Cosmid DNA was packaged into lambda phage using packaging

extracts (Amersham Corporation, Arlington Heights, IL.) according to the manufacturer's recommendation. The packaged lambda were then used to infect E. coli HB101 or DH1 cells. A 10 ml overnight culture was grown in LB broth supplemented with 0.2% maltose (which induces phage receptors on the E. coli cell surface). The cells were harvested by centrifugation at 6000 rpm, in a Beckman JA-17 rotor for 5 minutes at 4°C and resuspended in 10 mM MgSO₄ (for phage adsorption). A 100 ul of the cells were mixed with 50 ul of the packaged lambda and incubated at 37°C for 15 minutes to allow adsorption of the phage to the E. coli cells. Two ml of LB broth was added to each tube and incubated at 37°C for 1-2 h before plating 0.2 ml portions onto appropriate selection plates, followed by incubation overnight at 37°C.

Bacterial Conjugations

Bacterial conjugations were done by the triparental mating system as described by Ditta et al. (1980). E. coli (pRK2073), a helper strain, and E. coli donor strains were grown overnight at 37°C in 5 ml LB broth containing the appropriate antibiotics. The recipient, Bradyrhizobium japonicum, was grown in YS medium to an early stationary phase at 30°C. For the mating, 0.2 ml of B. japonicum (ca 10⁸ cells), 0.1 ml of the helper strain, and 0.1 ml of the donor strain (ca 10⁸ cells) were mixed and plated on YS agar which was incubated at 30°C for 2-4 days. The resulting colonies were resuspended with 3 ml of YS salts (YS broth without yeast

extract) containing 0.01% Tween 80. A 0.3 ml aliquot of the suspension was plated onto RDY medium containing appropriate antibiotics and incubated at 30°C. The transconjugants were first observed after an incubation period of 8-10 days.

Site-Directed Tn5 Mutagenesis

Tn5 mutagenesis was performed on three target cosmids using two different Tn5 donor vehicles, λ : Tn5 (de Bruijn and Lupski, 1984) or Pl: Tn5 (Quinto and Bender, 1984). The amp^r, tet^r target cosmids of interest (pRjUT2, pRjUT10, pRjUT14) were transformed into E. coli strains DBCS 31-1 (λ^+) and their presence in the resulting amp^r, tet^r transformants was verified by plasmid isolation. The plasmid bearing cells were grown overnight at 30°C in LB broth containing amp and tet. The culture was diluted 100-fold and grown at 30°C in LB containing amp and tet to an A₆₀₀ of 0.8 (ca. 10⁹ cells/ml). The cells were then mixed with λ : Tn5 at an m. o. i. of 1-10 and incubated at 30°C for 2 h. Aliquots (0.2 ml) of the infected cells were then plated on LB agar plates containing kan, amp and tet (20 ug/ml each) to select for the presence of Tn5 in the plasmid bearing cells. The plates were incubated at 30°C for 48 h. The resulting amp^r, tet^r, kan^r colonies could be due to the transposition of Tn5 onto the chromosome or the target cosmid. These colonies were collected from the plates with 5 ml of LB broth, centrifuged and resuspended in 20 ml of LB broth. Cosmid DNA was isolated by the alkaline

lysis method and transformed into competent cells of E. coli DH1 or packaged into lambda phage and transduced into E. coli in order to screen for the phenotype of the Tn5 mutated cosmid (amp^r, tet^r, kan^r). The Tn5 mutated cosmids were isolated from the transformants by the small scale isolation procedure. The Tn5 insertions were mapped on the cosmids with respect to the previously mapped restriction endonuclease sites on Tn5 and the cosmid.

In case of Pl:Tn5 mutagenesis, the target cosmids were transformed into a PlCm lysogenic strain, E. coli EG47 (Quinto and Bender, 1984) and superinfected with Pl:Tn5. The selection was maintained for both the chloramphenicol resistance encoded by the resident PlCm prophage and the kanamycin resistance encoded by the Tn5 element. A large class of survivors was a result of transposition of the Tn5 from the superinfecting phage on to the chromosome or the cosmid. Cosmid DNA was isolated from the Cm^r tet^r Kan^r amp^r transductants as before and transformed into competent cells of E. coli DH1.

E. coli DH1 transformants or transductants containing the Tn5 mutated cosmids were used as conjugal donors, pRK2073 was the helper and B. japonicum USDA 110, the recipient, in the triparental mating system described above. Since the Tn5 expresses both kanamycin and streptomycin resistance in B. japonicum (Putnoky et al., 1983), transconjugants were isolated on RDY complex medium containing streptomycin and kanamycin each at 150 ug/ml. The

background level of resistance for both these antibiotics was 50 ug/ml. To counterselect the donor E. coli, chloramphenicol was added to the medium at 30 ug/ml, a concentration at which B. japonicum USDA 110 was found to be resistant to the antibiotic. In all mating experiments, the donor, helper and recipient cells were plated individually on the selective medium and no colonies were seen even after 14 days. The str^r , kan^r transconjugants could have resulted either due to the cointegration of the plasmid into the genome (homologous recombination due to a single crossover event) or true replacement of wild type DNA with mutated DNA and a loss of the vector DNA termed "marker exchange" (homologous recombination due to a double crossover event) (see Figure 1). A transconjugant containing a true replacement of wild type DNA with Tn5 containing DNA, also termed as a "homogenote", would be resistant to kan and str, but it would be sensitive to the antibiotic marker encoded by the vector. To distinguish between these two events, the resulting kan and str resistant transconjugants were replica plated on RDY containing kan and str and RDY containing kan, str and tet. After incubation at 30°C for 4 - 5 days, true homogenotes resulting from double crossover events were identified by their sensitivity to tetracycline. The presence of the Tn5 was further confirmed by positive hybridization of EcoRI digested genomic DNAs isolated from the putative homogenotes with a radiolabelled Tn5 probe.

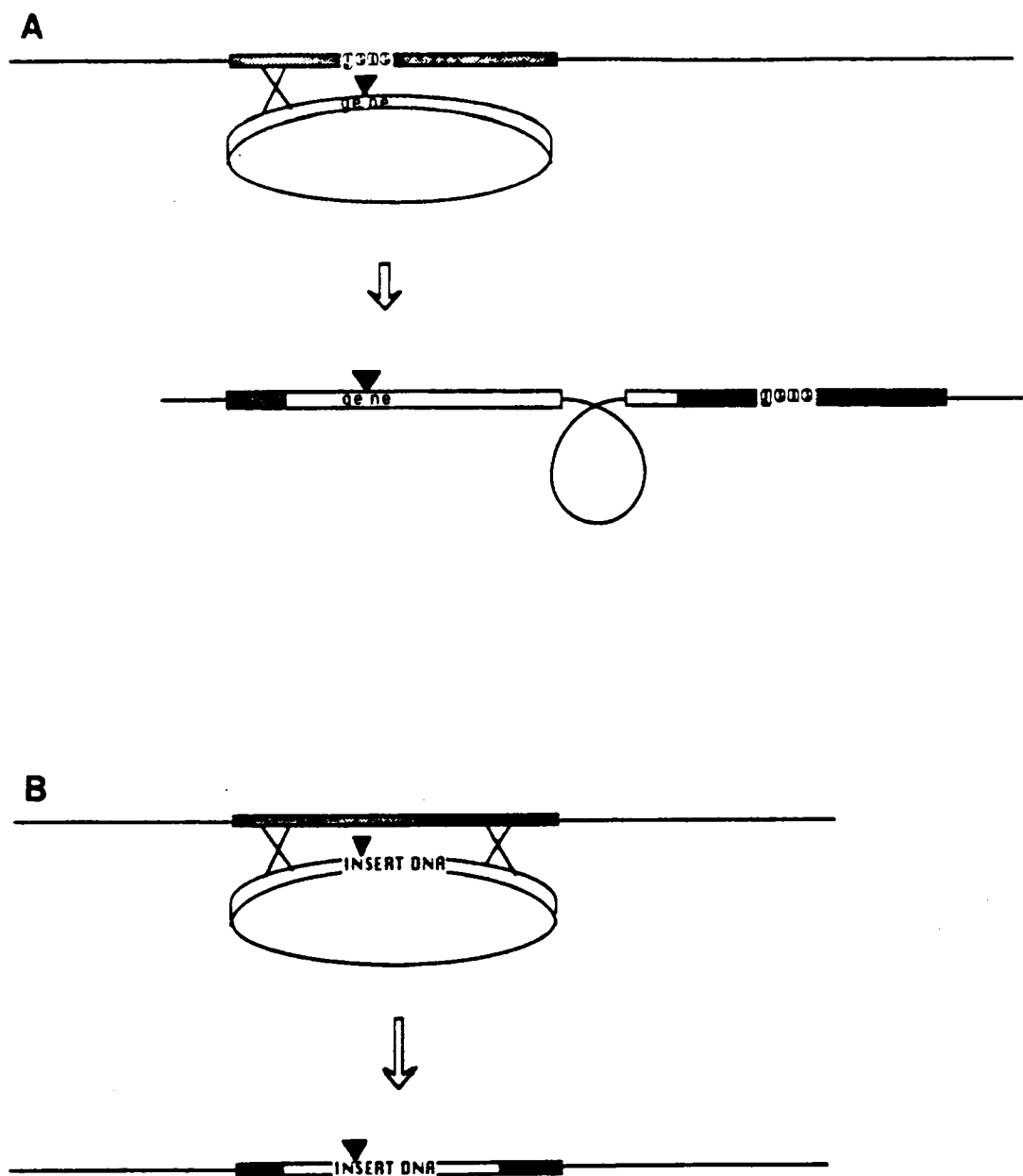


Figure 1. Schematic presentation of homologous recombination. (A) Single cross-over event resulting in the integration of the vector DNA and the mutated gene into the genome. (B) Double cross-over event resulting in the replacement of wild type DNA by the mutated DNA. The triangle indicates a mutation.

Deletion Mutagenesis

Deletions were made in the cosmid clones, pRjUT14 and pRjUT24 as follows. The clones were digested with XhoI and religated so that all internal XhoI fragments were deleted. These deletions were verified by restriction analysis. To facilitate the selection of homogenotized deletion mutants, a kanamycin resistance gene cassette (pUC 4 -kixx, Pharmacia) was ligated into the unique XhoI site of the cosmid derivative in place of the deleted DNA. The kanamycin cassette contains the npt (neomycin phosphotransferase) gene from Tn5 (Beck et al., 1982). The resulting cosmid was transformed into competent E. coli HB101 cells and kanamycin resistant transformants were selected. Deletion mutants of B. japonicum USDA 110 were isolated by marker exchange of the wild type sequence with the deletion derivatives by double reciprocal recombination following triparental conjugation. To distinguish between spontaneous kanamycin resistant mutants (Frequency: 10^{-6}) and true homogenotes, colony hybridizations with ^{32}P labelled pBR322:Tn5 were done. A Tn5 mutant, NAD138, was used as a positive control and a spontaneously generated kanamycin resistant mutant of USDA 110 was used as a negative control.

Plant Infection Tests

The symbiotic phenotype of B. japonicum-Tn5 insertion mutants and deletion mutants was tested on Glycine max cv Essex (L. Merr). The seeds were surface sterilized with 20% chlorox for 10 minutes,

rinsed twice with sterile distilled water and then soaked in 0.01N HCl for 10 minutes. The seeds were again rinsed extensively (5-6 times) with sterile distilled water. G. soja seeds were scarified by soaking in conc. H_2SO_4 for 1 minute, rinsed with water, and soaked in 0.01 N HCl for 10 minutes followed by extensive rinses with water. The small seeded legumes, (i.e., Macroptilium atropurpureum, Trifolium repens) were surface sterilized with 95% ethanol for 1 minute followed by soaking in 0.1% $HgCl_2$ for 5 minutes. The seeds were rinsed with sterile distilled water and soaked overnight in 50 mls of sterile distilled water.

Sterilized seeds were germinated on sterile moist paper or water agar for 2 days in the dark at room temperature. Seedlings were transferred aseptically to sterile plastic growth pouches (Northrup King Seed Co; Minneapolis, Minn.) moistened with 10 mls of sterile plant nutrient solution (Wacek and Brill, 1973) or 35 ml serum vials containing vermiculite saturated with sterile plant nutrient solution supplemented with 1% sucrose. The vermiculite bottles were covered with sterile 18 oz. whirlpak bags (Nasco). Plants were grown for 21 days at 26°C in a growth room at $320\text{ Em}^{-2}\text{ s}^{-1}$ with a 14 - h photoperiod. The plants in pouches were watered as needed with half-strength plant nutrient solution. Plants were scored for the appearance and number of nodules at a 2 day interval, starting 7 days after inoculation. The results of all plant tests were the average of 3 separate experiments with 5 replicates in vermiculite bottles and 15 replicates in pouches.

Siratro plant assays were carried out in test tubes on nitrogen free medium as described previously (Kondorosi et al., 1982). The efficiency of nodulation was determined in terms of the percentage of plants nodulated and/or average number of nodules per plant. Bacteria were isolated from the nodules formed by Tn₅ mutants that caused a delay in nodulation. The nodulation phenotype of these bacteria was verified by plant infection tests.

Nitrogen fixation activity was detected by the acetylene reduction assay (Wacek and Brill, 1976) with a Shimadzu GC - 8A gas chromatograph equipped with a 6 foot (ca. 2m) Poropak R column. The detector was maintained at 100°C and the column at 75°C.

Root Hair Curling Studies

Germinated seedlings were transferred to pouches as above and inoculated with approximately 10^8 cells of the appropriate culture. Plants were watered as needed with half strength plant nutrient solution. At the time of root hair inoculation, the position of the root tip (RT) was marked on the surface of the growth pouch. Four days after inoculation, longitudinal sections of roots around the root tip mark were taken and stained with phosphate buffered saline (per liter of water, 0.43g, KH_2PO_4 ; 1.48g, $\text{Na}_2\text{H}_2\text{PO}_4$; 7.2 NaCl) containing 0.05% toluidine - blue for 15 s, followed by rinsing with phosphate buffered saline (PBS). The root sections were mounted under glass coverslips and were observed with bright field optics for the presence of curled root hairs. At least three

plants were examined for each Bradyrhizobium strain.

Examination of Cortical Cell Division

Plants were infected with USDA 110 or NAD165 and grown as described. Six days after inoculation, roots were randomly selected from each experimental treatment for microscopic analysis using the method of Calvert et al. (1984). Prior to the removal of each root, partial cuts were made through the RT points. A 70 mm segment was excised from each root which represented 30 mm above and 40 mm below the RT mark. Each 70 mm segment was divided into five or six pieces, 8-13 mm long. The root segments were fixed for 2 h with 3% glutaraldehyde in 50 mM potassium phosphate buffer, pH 7.2. Following rinsing with 50 mM potassium phosphate buffer (pH 7.2), the segments were dehydrated in a graded series of ethanol at room temperature, (50%, 70%, 95% ethanol; 30 minutes each followed by 100% ethanol, 2 times, 30 minutes each) followed by 100% xylene, for 30 minutes, 2 times. The dehydrated tissue was infiltrated with paraffin by immersing it in paraffin (Paraplast, American Scientific) 2 times for 20 minutes at 58°C. The pieces were embedded in an embedding mould (Peel away moulds, American Scientific) containing paraffin. The moulded blocks were sectioned by a metal blade to 5 µm thickness using an A-0820 ultra microtome. The sections were mounted and stained with tannic acid-ferric chloride and saffranin-fast green (Jensen, 1962). Each set of serial sections was examined for characteristic features such as

curled root hairs, infection threads and cell division centers in the outer cortex.

Construction of lac Z Fusions

Translational fusions with the lacZ gene from E. coli were made using the mini-Mu-Mud II-lac system (Ratet et al., 1988). The mini-mu-lac vehicles combine the useful properties of Mu, namely high transposition frequency, headful packaging and relatively low insertional specificity (Toussaint and Rebois, 1983) with well established lac fusion methodology (Silhavy and Beckwith 1985). After mini-mu-lac mutagenesis in E. coli, the putative translational gene-lac fusion must be transferred to the appropriate rhizobial strain to allow screening for proper fusions and regulation studies. This requires appropriate selectable markers and an efficient conjugal mobilization system. The mini-mu-lac vehicle used for mutagenesis in this study, was Mud II PR48; a derivative which carried a site for conjugal transfer from the plasmid RP4 (ori T) and an origin of replication of the Agrobacterium rhizogenes Ri plasmid (ori RiHRI) (Ratet et al., 1988). This vehicle allows a one step mini-mu-lac mediated plasmid gene fusion mutagenesis in E. coli, followed by high frequency conjugal transfer to and stable low copy maintenance of the plasmid borne lac-gene fusions in Rhizobiaceae. Mud-II-PR48 also carries selectable markers for chloramphenicol and gentamycin resistance, facilitating the selection of mini-mu- lac insertions.

A 5.3 kb EcoRI fragment from the cosmid pRJUT10, was subcloned into pBR322 (tet^r), to yield pND1013. This plasmid was conjugated into E. coli MC4100 (Muc^{ts}) (MudIIPR48) (Ratet et al., 1988) and Cm^r , tet^r resistant transconjugants were selected. An overnight culture of one such transconjugant, ND527, grown at 30°C was diluted 100-fold in fresh LB containing tet and Cm supplemented with MgCl_2 (to allow absorption of the phage) and was further grown to an O.D of 0.2 - 0.4. The culture was then heat induced at 45°C for 30 minutes to induce the lytic growth of the Mu phage. The heat induced culture was grown at 37°C for 2 h, during which a majority of the cells lysed. The lysate was centrifuged and stored under chloroform at 4°C. Following transduction into strain M8820 (MU^+), the tet^r Cm^r colonies were then screened for plasmids containing mini-Mu-lac insertions. 300 such constructs were individually mobilized into USDA 110 by triparental matings, and tested for the presence of β -galactosidase activity by replica plating on RDY (tet Cm) medium containing X-gal (100ug / ml) with or without genistein, (a flavanoid that was previously shown to induce nod genes in B. japonicum (Banfalvi et al., submitted)). Colonies giving a blue color on plates containing genistein or seed exudate were streak purified and used in induction studies. The position of mini-Mu-lac insertions were mapped by restriction endonuclease digestions of the donor plasmids from E. coli.

Flavanoid Chemicals and Seed Extract

Flavanoid chemicals used in this study were apigenin, coumestrin, daidzein, luteolin, naringenin (Sigma Chemical Co, St. Louis, MO), genistein and biochanin A (ICN Biomedicals.; K&K labs). The flavanoids were made as 1mM stock solutions in 100% ethanol.

The seeds of Glycine max cv. Essex (L.Merr) were surface sterilized as before. One hundred sterile seeds were shaken overnight in 100 ml of sterile distilled water at 30°C. These extracts were concentrated to 10 ml by rotary evaporation, centrifuged to remove particulate matter, filter sterilized and stored at 4°C.

Induction of lac Z Fusions

B. japonicum strains that contained lacZ gene fusions were grown to early logarithmic phase ($A_{600} = 0.1$). The inducer was added (2μM, final concentration) to empty tubes to evaporate the ethanol and 2 mls of culture was added to each tube. The tubes were incubated for 14-16 hours with shaking at 30°C. After incubation 0.5 mls of each culture was assayed in duplicate. These cells were permeabilized by SDS-chloroform and assayed for B - galactosidase activity by the method of Miller (1972). However, chlorophenol - red -B - D galactopyranoside (CPRG, Boehringer - Mannheim) was used as substrate for greater sensitivity in place of 2 - nitro phenyl - B - D - galactopyranoside (ONPG).

Isolation and Analysis of Exopolysaccharides

Six day old B. japonicum cells were harvested by centrifugation at 16,000 g for 20 min. The supernatant fluid obtained after the removal of cells was concentrated by rotary evaporation at 40°C. The concentrated solution was dialyzed extensively for 48 hours with four changes of distilled water and lyophilized to yield the crude exopolysaccharide (EPS) fraction. Proton nuclear magnetic resonance (nmr) spectra were generated with a Bruker AM 500 spectrometer by Dr. Puvanesarajah (Complex Carbohydrate Research Center). The spectra were run at 70°C with 3 - (trimethyl silyl) - 1 - propane sulfonic acid, sodium salt, as the internal reference.

CHAPTER III

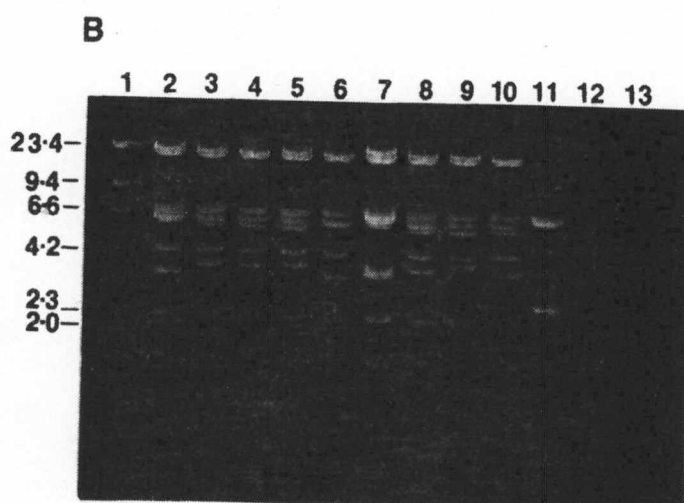
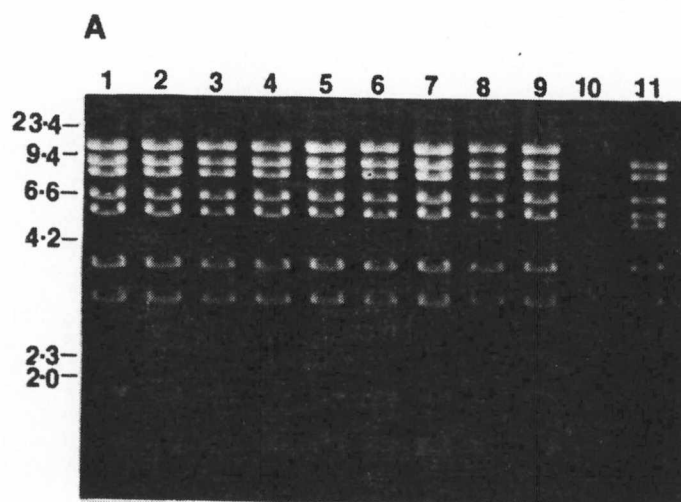
RESULTS

Identification of Symbiotic Loci

Mutagenesis of Cosmid Genomic Clones of *B. japonicum* USDA 110

In a previous study (Russel et al., 1985), a 100 kb DNA region including the common nod genes was isolated as four overlapping cosmid clones, namely pRjUT10, pRjUT2, pRjUT14 and pRjUT24 (Figure 2). To identify additional genes involved in nodulation, three overlapping cosmid clones pRjUT10, pRjUT2 and pRjUT14 were subjected to site-directed Tn5 insertional mutagenesis, using two independent Tn5 donor strains (λ :Tn5 and Pl:Tn5, see Chapter II). Transposition frequency using both donor strains was 10^{-5} - 10^{-6} per recipient cell. A total of 60 unique Tn5 insertions were generated. The precise sites of the Tn5 insertions were determined by restriction mapping analysis, using three different enzymes, EcoRI, HindIII and BamHI. For example, since EcoRI does not cleave Tn5, the presence of Tn5 in the 5.3 kb EcoRI fragment led to the disappearance of the corresponding band and the appearance of a new band corresponding to a DNA size of $5.3 + 5.7$ (size of Tn5) = 11.0 kb (Figure 3A). For precise mapping of the Tn5 insertions in the 5.3 kb EcoRI fragment, BamHI (which cleaves Tn5 once) was used to digest the cosmids containing Tn5 (Figure 3B). HindIII was also

Figure 3. Mapping of Tn5 insertions in the 5.3 kb EcoRI fragment of pRjUT10. (A) EcoRI digests of cosmids containing Tn5 (lane 1 - 9), pRjUT10 (lane 11). (B) BamHI digestions of cosmids containing Tn5 in the 5.3 kb EcoRI fragment lanes (2 - 12), pRjUT10, (lane 13), λ : HindIII (lane 1).



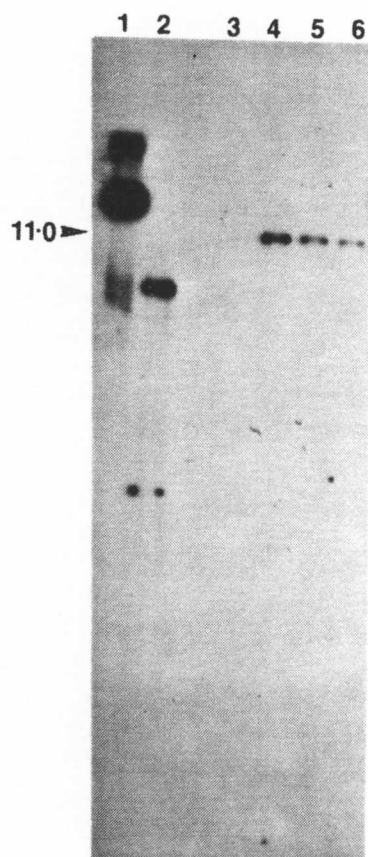
used to confirm the site of Tn5 insertions (data not shown).

All the cosmid clones containing Tn5 insertions were mobilized by triparental matings into Bradyrhizobium japonicum USDA 110, to reconstruct the mutation by homologous recombination. The frequency of transfer of kanamycin and streptomycin resistance into B. japonicum USDA 110 was 10^{-6} per recipient cell, whereas the spontaneous mutation frequency was lower than 10^{-9} . About 200 colonies were isolated from each mating. In order to distinguish between true homogenotes resulting from a double crossover, and co-integrates arising from a single crossover, the transconjugants were scored for the loss of the antibiotic marker on the vector (i.e., tet^r). The frequency of the loss of the marker was $5 \cdot 10^{-6}$.

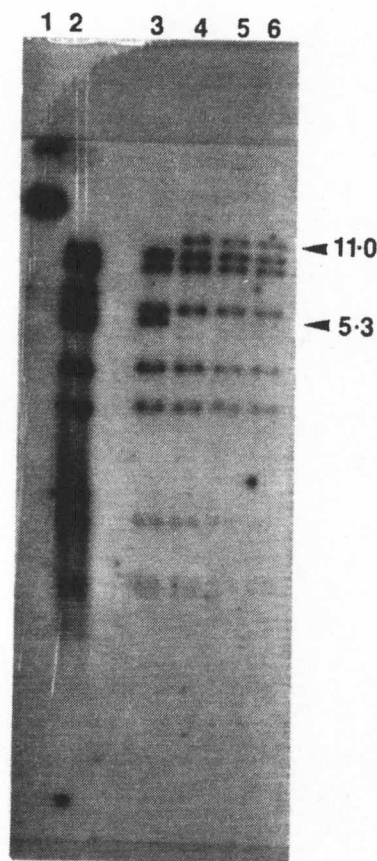
The kanamycin and streptomycin resistant colonies which were sensitive to tetracycline (150 ug/ml) were streak purified. Southern blots of EcoRI digested genomic DNA from the putative mutants were analyzed by hybridization with ³²P labelled pBR322:Tn5, to verify that the Tn5 insertions were in the correct location on the B. japonicum genome (Figure 4A). This was also corroborated by hybridization with radioactively labelled pRJUT10, which showed a decrease in mobility of a specific EcoRI fragment due to the insertion of Tn5. An example of an insertion into a 5.3 kb fragment is shown in Figure 4B.

The Tn5 insertion mutants of B. japonicum were tested as described in Chapter II for their ability to nodulate Glycine max

Figure 4. Hybridization analysis of Tn5 transconjugants of *Bradyrhizobium japonicum* USDA 110. (A) Hybridization of ³²P labelled pBR322:Tn5 to EcoRI digested chromosomal DNAs of mutants (lane 4 - 6); pBR322:Tn5 (lane 1); pRjUT10 (lane 2); wild type DNA (lane 3). (B) The previous probe was boiled off and the same filter was hybridized to radioactively labelled pRjUT10. Note the disappearance of a 5.3 kb fragment, that is present in the wild type, in the cosmids containing Tn5 and the appearance of a new 11.0 kb fragment containing Tn5.



A



B

cv Essex (soybean) and fix nitrogen. Four phenotypic classes were obtained on soybean (G. max cv Essex) plants: (i) delayed Nod: a 2 or more day delay in nodulation, ii) Nod⁻: more than 98% of plants did not have any nodules with the remaining plants assayed showing some nodule like structures after 21- 28 days, iii) Nod⁺ Fix⁻: no significant delay in nodulation compared to the wild type strain, but these mutants could not fix atmospheric nitrogen, iv) Nod⁺ Fix⁺: Tn₅ insertion mutants that were indistinguishable from the wild type. Mutants belonging to the first 2 classes were further characterized in this study.

Mutagenesis of pRjUT14 and pRjUT24

The two overlapping clones pRjUT14 and pRjUT24 together encompassed about a 40 kb DNA region adjacent to pRjUT10 (Figure 2, page 47). To identify genes that encode symbiotic functions, the cosmid clone pRjUT14 was used as a target for site-directed Tn₅ mutagenesis and approximately 35 cosmid derivatives containing Tn₅ insertions were isolated and mapped (Figure 5). The putative mutants were assayed for their nodulation and nitrogen fixation abilities on soybean plants. Figure 6 shows the nodulation kinetics of one mutant, NAD14, that exhibited a delay in nodulation of soybean roots. The Tn₅ insertion mapped to a specific 7.7 kb EcoRI fragment which was verified by restriction endonuclease mapping and Southern hybridization analysis. To substantiate the importance of this DNA region in symbiotic functions, deletions

Figure 5. Nodulation characteristics of *B.japonicum* mutants with Tn5 insertions mapping within pRjUT14. The sites of insertions are indicated by triangles and the symbols underneath correspond to different Nod and Fix phenotypes, wild type (●), negative (○) and delayed nodulation (d). Abbreviations : R, EcoRI.

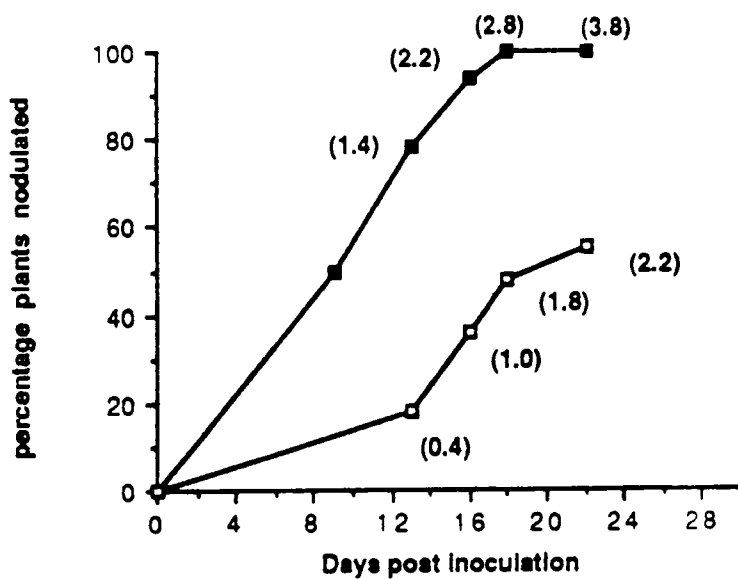


Figure 6. Nodulation kinetics of *B. japonicum* wild type (■) and Tn5 mutant NAD14 (□) on *Glycine max* var. Essex. Mutant NAD14 showed a 4 day delay in nodulation. The numbers in brackets indicate the average number of nodules per plant

were made in pRjUT14. All internal Xho I fragments adding up to 30 kb DNA were deleted and replaced with a 1.7 kb XhoI cassette fragment containing the neomycin phosphotransferase (npt) gene which confers kan^r (Beck et al., 1982). The resulting clone, AN262 (Figure 7) which was kanamycin resistant was mobilized into B. japonicum by the triparental mating system. A similar deletion of DNA contained in pRjUT24 (about 40kb) was made, followed by the insertion of the 1.7 kb XhoI cassette fragment as above yielding clone AN264 (Figure 7). Both these clones contained approximately 4 kb of B. japonicum DNA that flanked the kanamycin gene, and this facilitated marker exchange of genomic wild type DNA with the deleted, cloned DNA by homologous recombination.

Isolation of kanamycin resistant transconjugants. The mobilization of deletion plasmids AN262 and AN264 into B. japonicum and selection on RDY medium containing kanamycin (200ug/ml) and chloramphenicol (30ug/ml) led to the isolation of transconjugants. To distinguish between the spontaneous kanamycin resistant (frequency 10^{-6}) mutants and truly homogenotized deletion mutants, colony hybridizations were done using a ³²P - labelled pBR322:Tn5 as a radioactive probe. Several transconjugants (frequency 10^{-8}) were found to contain Tn5 sequences and were the result of homologous recombination.

Hybridization analysis of genomic DNAs from the putative deletion mutants, wild type as well as the cosmid clones (AN262 and

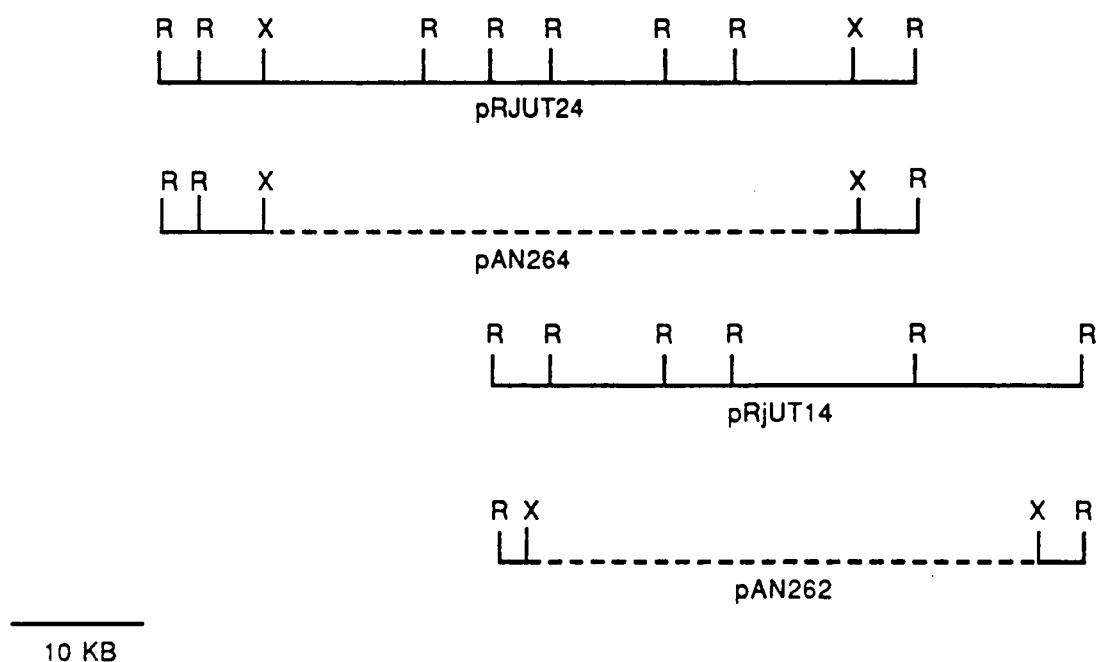


Figure 7. Schematic diagram of the deletion derivatives of pRJUT14 and pRJUT24, AN 262 and AN 264 respectively. The dotted lines indicate the region of deleted DNA in pRJUT14 and pRJUT24 respectively. Abbreviations: R, EcoRI. X, XhoI.

AN264), led to the identification of the deletion mutants, NAD163 and NAD164 respectively (Figures 8 and 9). When probed with pRJUT14 and pRJUT24, respectively, the mutants showed only the junction fragments hybridizing to the probe, indicating that the rest of the DNA was deleted.

Nodulation kinetics of deletion mutants NAD163 and NAD164.

The deletion mutants NAD163 and NAD164 were tested for their ability to nodulate Glycine max cv. Essex. Figures 10 and 11 show the nodulation kinetics of NAD163 and NAD164. NAD163 was delayed in its ability to nodulate G. max cv. Essex by about 2-3 days as compared to USDA 110. NAD164 nodulated soybean at a reduced efficiency as compared to the wild type. The nodulation kinetics exhibited by mutants NAD14 (Tn5 insertion mutant described earlier) and NAD163 and NAD164 taken together, suggest that perhaps the 7.7 kb EcoRI fragment encoded functions that affected the efficiency of nodulation. The efficiency of nodulation is defined in terms of percentage of plants nodulated and /or average number of nodules per plant.

Molecular Characterization of Symbiotic Genes in pRJUT10

Previous work in this laboratory identified a 40 kb DNA region of B. japonicum USDA 110 (cosmid pRJUT10) which encoded early nodulation functions (e.g. root hair curling, Hac) (Russel et al., 1985). To locate the nod genes identified in other Rhizobium strains, radioactive probes of specific nod genes from R. meliloti

Figure 8. Verification of the deletion in the mutant NAD163. Hybridization of Southern blots of Eco RI digested DNAs of deletion mutant NAD163 and B. japonicum USDA 110 (lanes 1 and 3 respectively) and Xho I digested genomic DNAs from the same strains (lanes 2 and 4 respectively) with radioactively labelled (^{32} P) pRjUT14 as a hybridization probe.

1 2 3 4

23.4—

9.4—

6.6—

4.2—

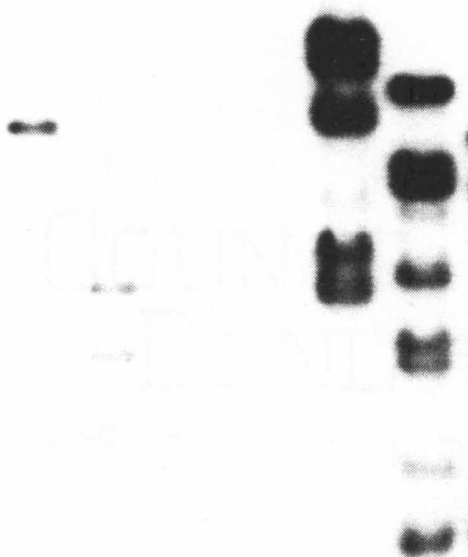
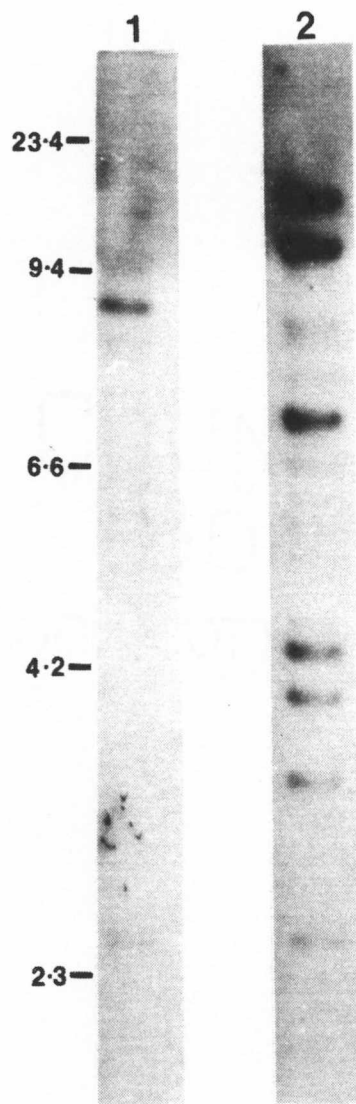


Figure 9. Verification of the deletion in the mutant NAD 164. Hybridization of Southern blots of EcoRI digested genomic DNAs of deletion mutant NAD 164 (lane 1) and B. japonicum USDA110 (lane 2) with radioactively labelled pRjUT24.



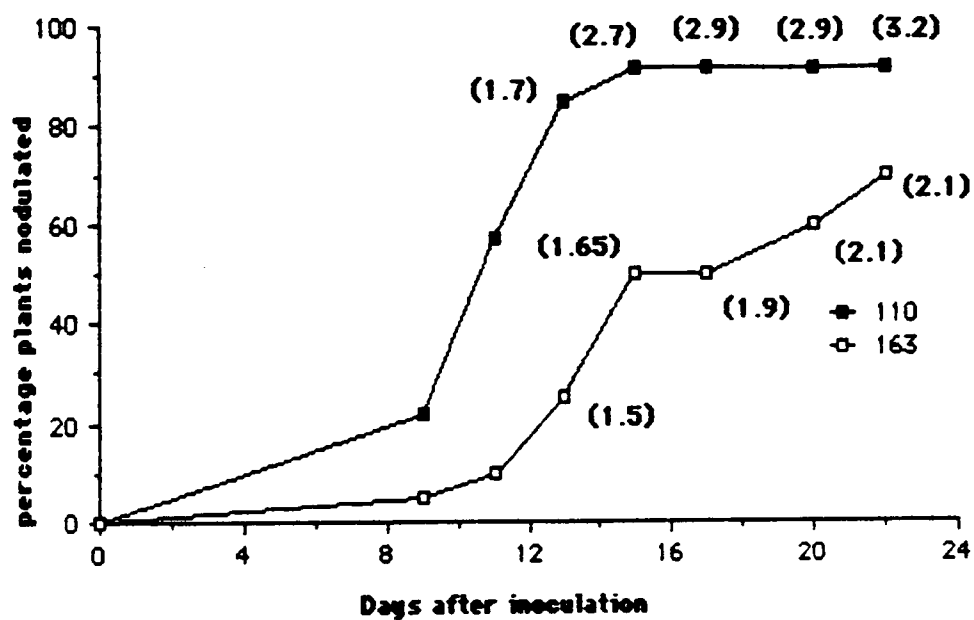


Figure 10. Nodulation kinetics of *B. japonicum* USDA 110 (■) and deletion mutant NAD 163 (□) on *Glycine max* var. Essex. The numbers in the bracket indicate the average number of nodules per plant.

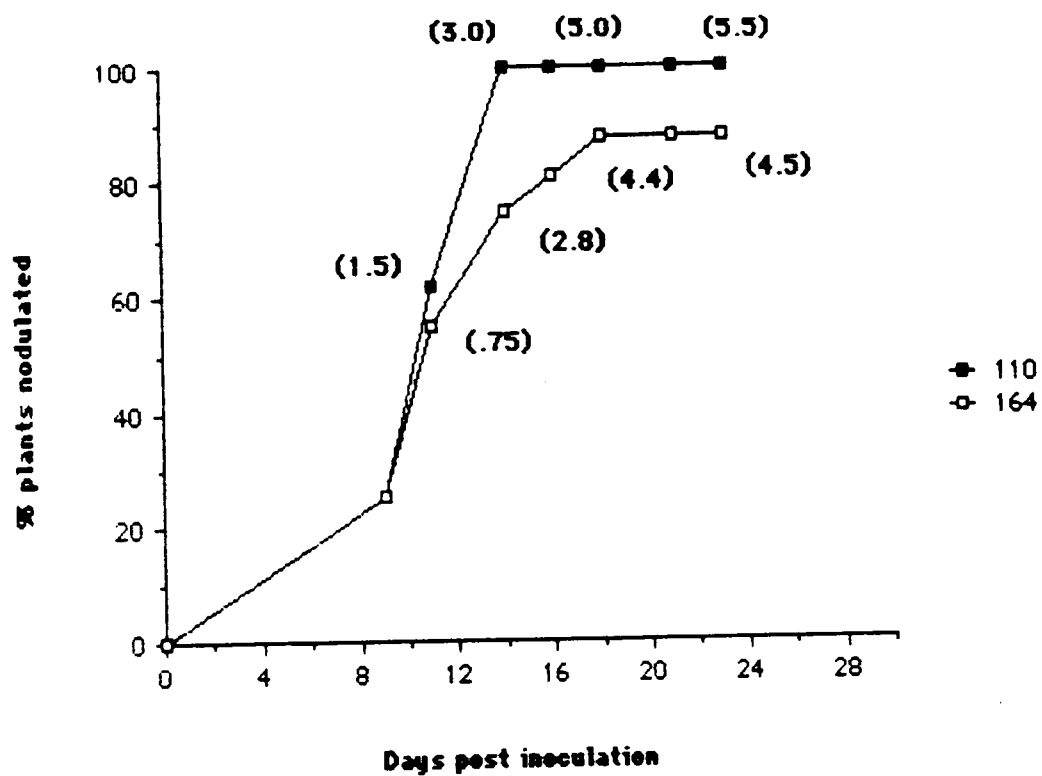


Figure 11. Nodulation kinetics of *B. japonicum* USDA 110 (■) and deletion mutant NAD 164 (□) on *Glycine max* var. Essex. The numbers in brackets indicate the average number of nodules per plant.

(nodABCD and R. leguminosarum (nodIJ) were hybridized to Southern blots of several restriction digests of pRjUT10. The nodABCIJ and nodD loci were localized to two HindIII fragments of 5.6 and 3.9 kb in pRjUT10 (Nieuwkoop et al., 1987). To delimit the common nod genes and identify other symbiotic genes within this region, pRjUT10 was subjected to site-directed mutagenesis. Several regions of symbiotic functions were identified as shown in Figure 12. The previously characterized common nod region was functionally verified by Tn5 insertions in nodB, nodC and nodD. The Tn5 insertion- mutants in nodB and nodC were unable to curl root hair of soybean, (Figure 13) indicating that the products of these genes were directly or indirectly involved in initiating the root hair curling response. Tn5 insertion in the nodD gene resulted in a delayed nodulation phenotype on soybean (Nieuwkoop et al., 1987). A fix region (represented by mutant NAD90) was identified downstream of nodABCIJ and was subsequently found to be fixA and nifA (Nieuwkoop et al., 1987) (Figure 12). Hybridization analysis using a DNA fragment from Rhizobium spp MPIK3030 known to encode host specificity functions (Bachem et al., 1985), led to the identification of a homologous region downstream of nodABCIJ in the 9.5 kb EcoRI fragment of pRjUT10. This region was further localized to a 2.9 kb HindIII-EcoRI fragment. To test the possibility that this region encoded functions required for nodulation of Macroptilium atropurpureum (siratro) which is a normal host of Rhizobium spp. MPIK3030, the symbiotic phenotype of

Figure 12. Nodulation characteristics of Bradyrhizobium japonicum mutants with the Tn5 insertion mapping within pRJUT10. The sites of insertion are indicated by triangles and the symbols underneath correspond to different Nod and Fix phenotypes : wildtype, (●); negative (○); and delayed nodulation (d). Abbreviations: R, EcoRI. The locations of fix A and nif A are taken from Lamb and Hennecke ().



Figure 13. Root hair curling of Glycine max 4 days after inoculation with A) USDA 110 B) Hac^- mutant. The roots were inspected by the method of Puppke (1980).

A



B



an insertion mutant NAD138, (Tn5 insertion in the 2.9 kb HindIII-EcoRI fragment) was tested on soybean and siratro. While NAD138 showed little or no difference in its ability to nodulate soybean when compared with the wild type USDA 110, it could not elicit nodules until after 24 days on siratro (Figure 14). Thirty six days after inoculation, the average number of nodules per nodulated siratro plant was 7, 8 and 3 for strains MPIK3030, USDA 110 and NAD138, respectively. No differences in nodule color or size could be detected by the three strains.

DNA sequence analysis of the siratro hsn region has defined an open reading frame of 1.1 kb in size (Maria Schell, personal communication). The siratro hsn gene is transcribed in the same direction as nodABC and encodes a 33 kd protein as determined by expression in E. coli maxicells (Sancar et al., 1985) (Tony Nieuwkoop, personal communication).

A mutant (NAD27), with an insertion in a 5.8 kb EcoRI fragment of pRJUT10, downstream of nodD, was found to cause a delay of 7-8 days in nodulation of soybean (Figure 15). Insertion of lacZ to test transcriptional fusions in this locus showed induction by the addition of soybean root exudate or genistein indicating that this locus was under the control of a host - inducible promoter (David Gerhold, personal communication). The location of Tn5 in both NAD138 and NAD27 was verified by hybridization analysis with pRJUT10 DNA (Figure 16).

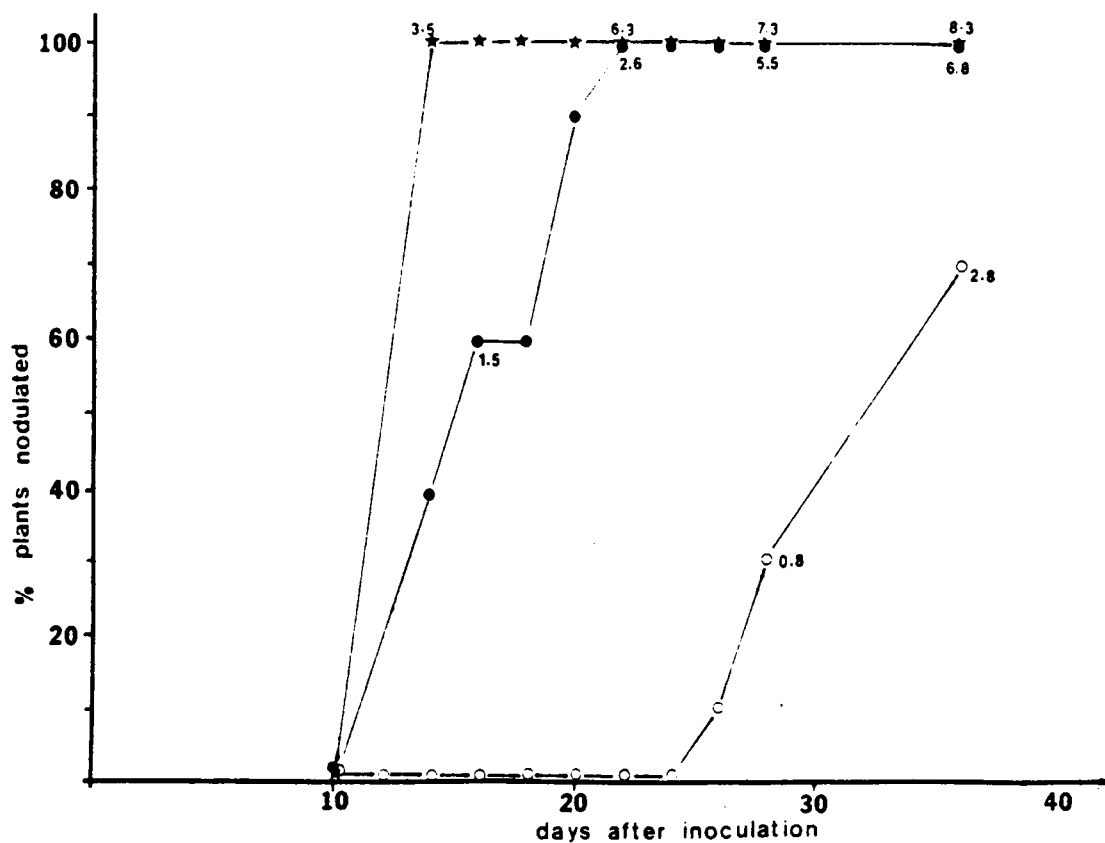


Figure 14. Nodulation kinetics of MPIK3030 (★), USDA 110 (●) and mutant NAD138 (○) on siratro. The kinetics are shown as a percentage of plants nodulated (15 plants). The average number of nodules per nodulated plant is indicated by the numbers adjacent to the data points.

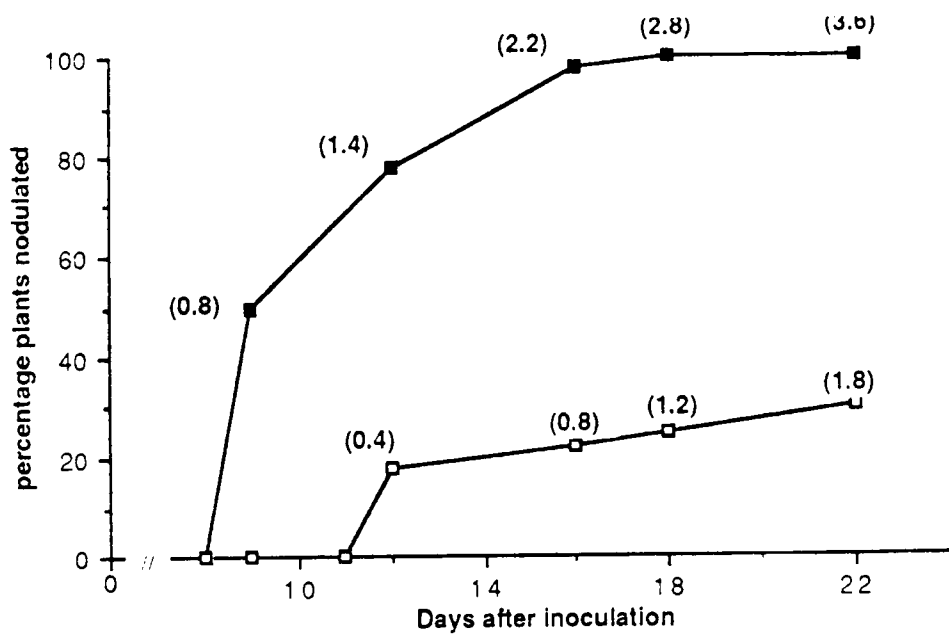
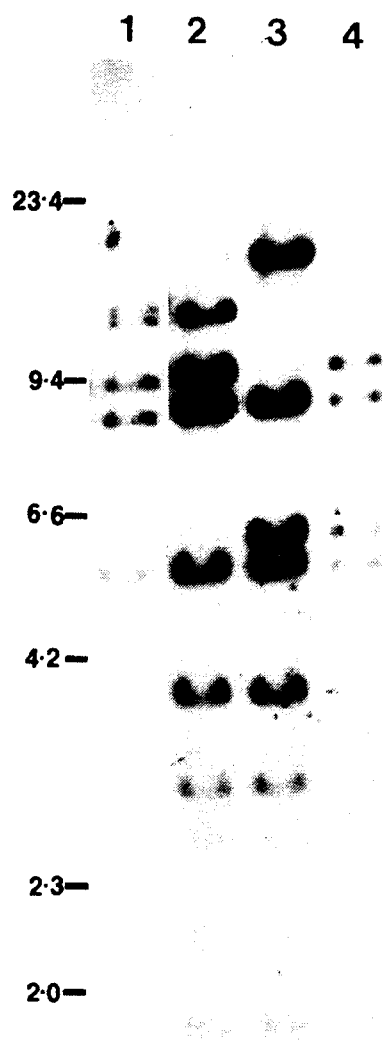


Figure 15. Nodulation kinetics of *B. japonicum* USDA 110 (■) and a mutant, NAD 27 (□) on *Glycine max* cultivar Essex. The kinetics are shown as percentage of plants nodulated (15 plants). The numbers in brackets indicate the average number of nodules per plant.

Figure 16. Hybridization analysis of Tn5 mutants. Hybridization of a Southern blot of EcoRI digested genomic DNAs from Tn5 insertion mutants of B. japonicum USDA110, nod D⁻ mutant AN314 (lane 1), NAD27 (lane 2), NAD138 (lane 3) and wild type (lane 4) with ³²P - radioactively labelled pRjUT10.



Identification and Molecular Characterization of Symbiotic

Genes on the 5.3 KB EcoRI Fragment of PRJUT10

Site Directed Mutagenesis of the 5.3 kb EcoRI Fragment of pRjUT10

A Tn5 insertion in the 5.3 kb EcoRI fragment of *B. japonicum* contained within pRjUT10 (mutant NAD165) (Figure 17) led to a Nod⁻ phenotype on soybean (cultivar Essex), but was able to form nodules on two other hosts, namely *G. soja* and siratro. Moreover, it was linked to the common nod genes and hybridization analysis indicated that none of the previously characterized nod genes from other rhizobia, namely nodeFGH from *R. meliloti* and the hsn region from *Rhizobium* spp. MPIK3030, showed homology with this region. These results indicated that the 5.3 kb EcoRI fragment contained a new nodulation region. To define the limits of the symbiotic functions encoded in this region, mutants were constructed by homogenotization of the wild type DNA with cloned mutated DNA containing Tn5 insertions in this region. The presence of Tn5 was verified by restriction enzyme mapping and hybridization analysis (Figures 3 and 4, pages 48 and 51). The location of Tn5 insertions in the 5.3 kb EcoRI fragment of pRjUT10 is shown in Figure 18. All insertion mutants were tested for their ability to nodulate soybean cultivar Essex. Three symbiotic regions were identified based on the phenotype of the mutants. Mutants with Tn5 insertions between the three regions had the same nodulation phenotype as the wild type. An insertion mutant NAD165, in region I was unable to form

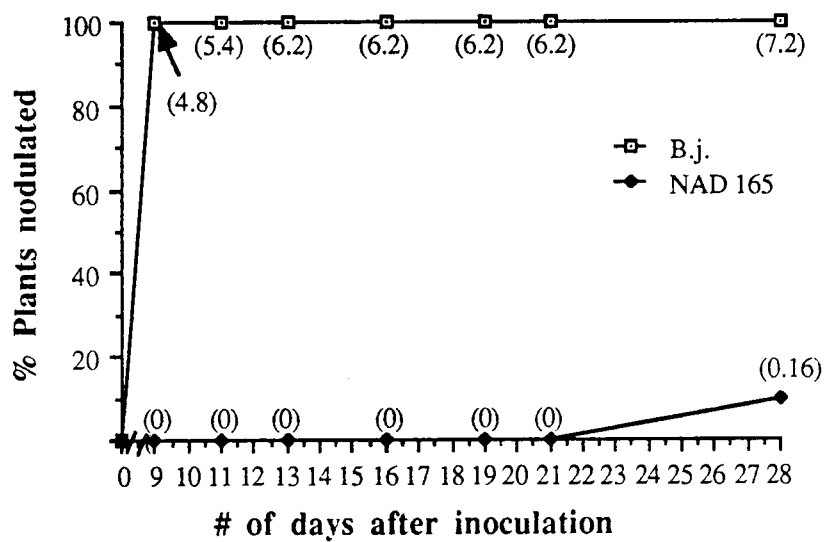


Figure 17. Nodulation kinetics of *B. japonicum* USDA 110 (□) and mutant NAD 165 (◆) on *Glycine max* cultivar Essex. (The numbers in brackets indicate average # of nodules.)

Figure 18. Restriction map of pRjUT10 and an expanded map of the 5.3 kb Eco RI fragment. The location of Tn5 insertions are shown by arrows. Abbreviations : nod, nodulation; fix, fixation; d, delayed nodulation; +, wild type; -, mutant; B, BamHI; H, HindIII; R, EcoRI.

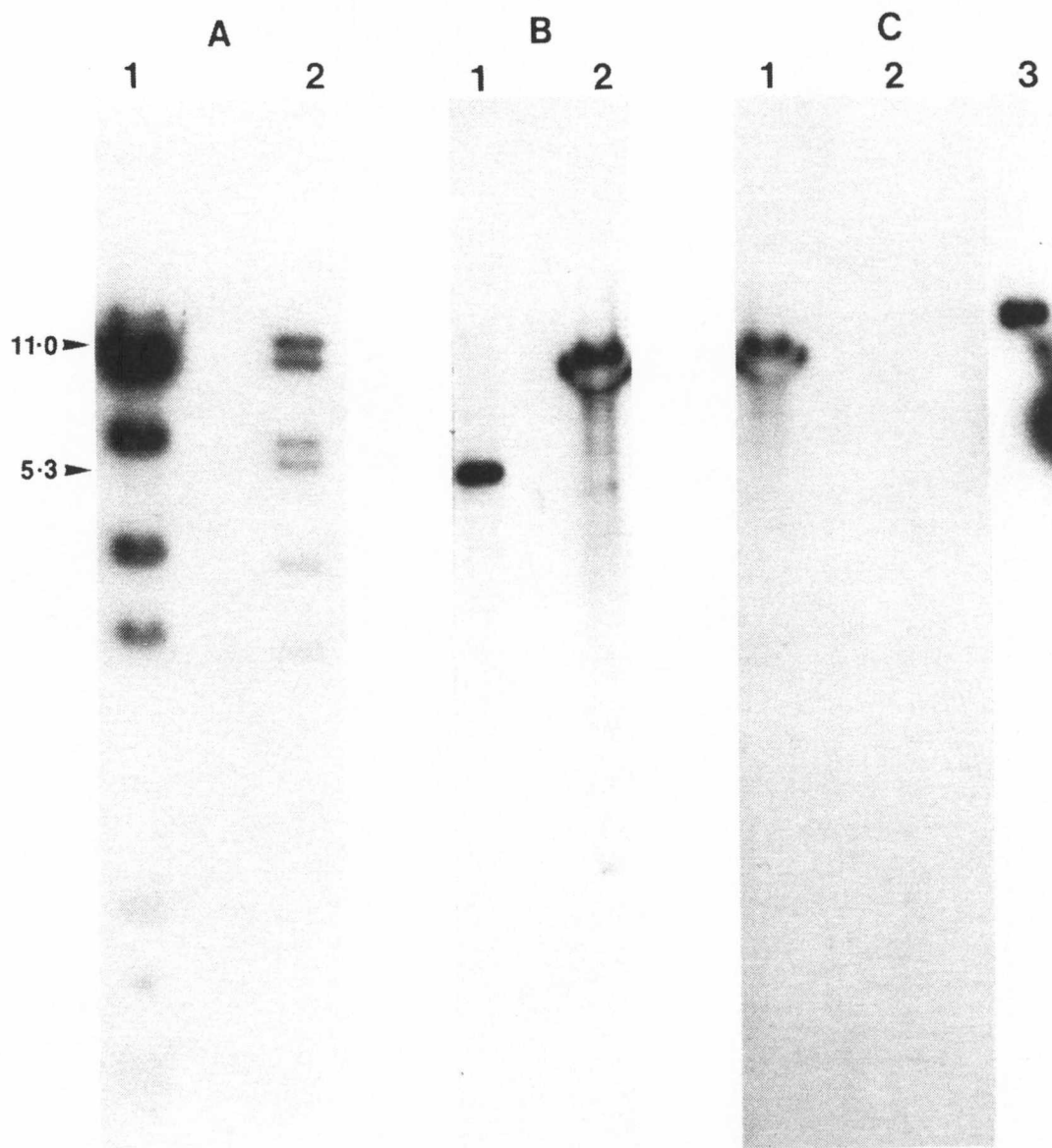
nodules until 18 days post inoculation (Figure 17) at which point nodules were formed due to kan^S revertants. Approximately 20% of the plants were nodulated by the mutant after 24 days whereas 100% of the plants were nodulated by the wild type strain after 14 days. The location of Tn₅ insertion in NAD165 was verified by Southern hybridization analysis (Figure 19). The average number of nodules per plant, 24 days after inoculation was 10 for USDA 110 and 0.8 for NAD165. Other mutants with Tn₅ insertions located proximally to that in NAD165 such as NAD167 and NAD168 could form nodules on 80% of the plants after 21 days. The nodulation locus represented by mutants NAD165, NAD167 and NAD168 was designated as nodT. Due to the difference in phenotype within these mutants, the locus may contain more than one gene. Since a subclone of the 5.3 kb EcoRI fragment was mutagenized in this experiment, Nod⁺ mutations flanking nodT were not obtained. Therefore, nodT may extend beyond the EcoRI site shown in Figure 18.

Mutants with insertions in region II, represented by NAD172, NAD173 and NAD174 showed a delayed nodulation phenotype on soybean. The nodulation kinetics of NAD174 indicated that there was a 8 day delay in nodulation as compared to the wild type USDA 110 (Figure 20). After 21 days, the mutant formed half as many nodules per plant as the wild type. This nodulation locus was designated as nodU.

Mutants with insertions in region III, represented by NAD180 and NAD181 showed a shorter delay of about 4 days in nodulation as

Figure 19. Verification of the presence and location of Tn5 in mutant NAD 165. Hybridization of Southern blots of Eco RI digested genomic DNA from B. japonicum Nod⁻ mutant, NAD165 (lane 1) and wild type, USDA 110 (lane 2) with radioactively labelled A)pRjUT10 and C)pBR322:Tn5. lane 3: pBR322: Tn5. (B) gel purified radioactively labelled 5.3 kb EcoRI fragment was probed against genomic DNAs from USDA 110 (lane 1) and NAD165 (lane 2).

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100% COTTON FIBER



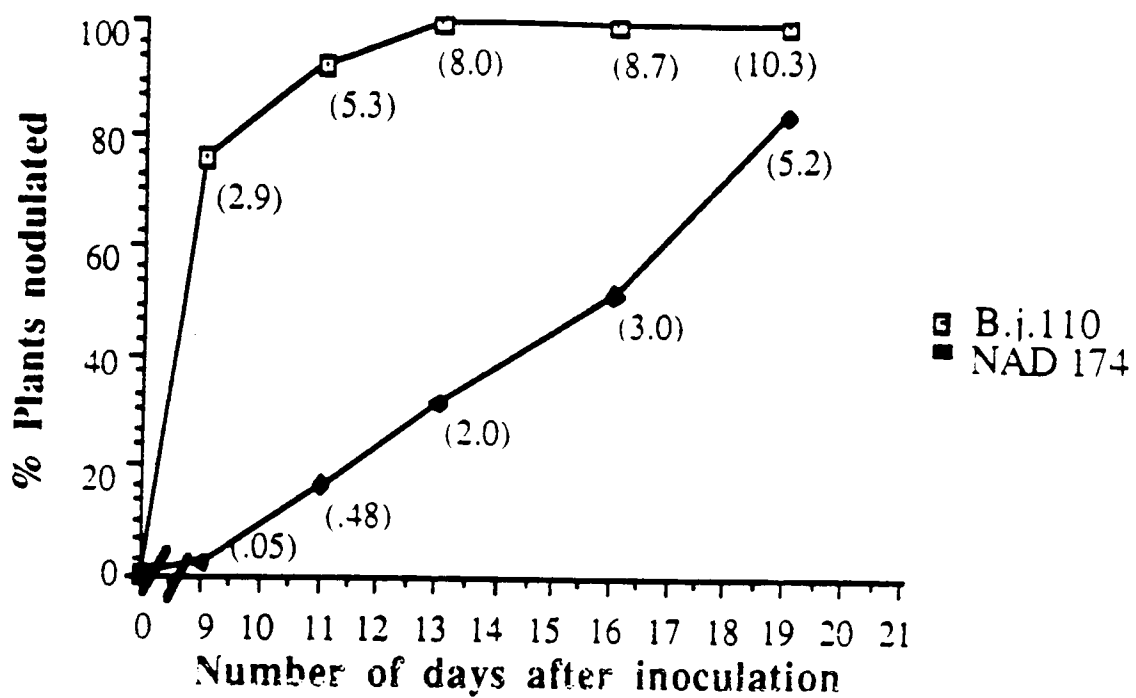


Figure 20. Nodulation kinetics of *B. japonicum* USDA 110 (○) and a mutant, NAD 174 (◆) on *Glycine max* cultivar Essex. (The numbers in brackets indicate average # of nodules.)

compared to the wild type (Figure 21). The average number of nodules per plant infected by NAD180 were 8.3 as compared to 10 by the wild type. This nodulation locus was designated as nodV. Since other mutations in the EcoRI fragment adjacent to nodV have a similar phenotype (see Figure 12, page 67), the nodV locus may extend beyond the 5.3 kb EcoRI fragment and may contain more than one gene.

Characterization of the Nodulation Defective Mutant NAD165

Nodulation kinetics on other hosts. Since the mutant NAD165 was unable to form nodules on cultivar Essex, it was of interest to see if it could nodulate other cultivars or other hosts of USDA 110. The mutant was unable to nodulate all cultivars of soybean tested except cultivar Peking (Table III). These results implied that the nodT locus was essential for the nodulation of most cultivars of soybean. The mutant NAD165 was also tested for its ability to nodulate G. soja, siratro, cowpea and pigeonpea. The results showed that NAD165 did nodulate G. soja and nodule-like structures were elicited on siratro (Table III). Curiously, NAD165 which could not nodulate cultivar Essex even after 18 days could nodulate G. soja after 10 days (Figure 22) and nodule like structures were seen on siratro after 14 days. Bacteria from crushed nodules of G. soja were plated on non-selective medium and subsequently tested for the presence of Tn5 by plating on medium containing streptomycin and kanamycin (150 ug/ml each). The

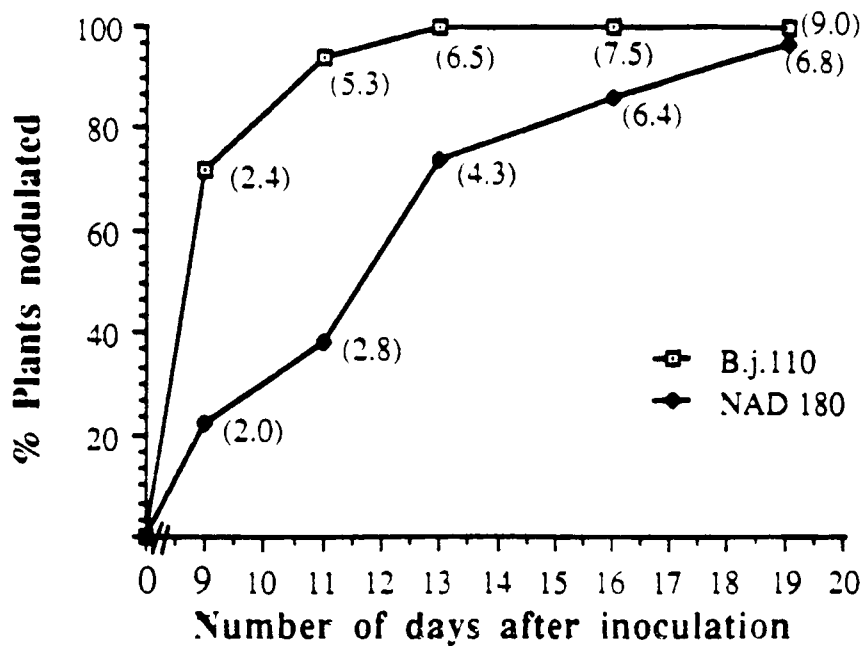


Figure 21. Nodulation kinetics of *B. japonicum* USDA 110 (□) and mutant NAD 180 (◆) on *Glycine max* cultivar Essex. (The numbers in brackets indicate average number of nodules.)

TABLE III. Nodulation phenotype^{*} of NAD165 on different cultivars of soybean and alternate hosts.

Legume	Inoculum	
	USDA 110	NAD165
<u>Glycine max</u> (soybean)		
cv Essex	+	-
cv Williams	+	-
cv Hardee	+	-
cv Forrest	+	-
cv Harasoy	+	-
cv Peking	+	+/-
<u>Glycine soja</u>	+	+
<u>Macroptilium atropurpureum</u> (siratro)	+	+/-
<u>Vigna unguiculata</u> (cowpea)	+	-
<u>Cajanus cajan</u> (pigeonpea)	+/-	-
<u>Pisum sativum</u>		
cv Wandopea	-	-
cv Snappea	-	-
<u>Trifolium repens</u> (clover)	-	-

* - The plants were harvested 21 days after inoculation. The results were obtained from at least 15 replicates.

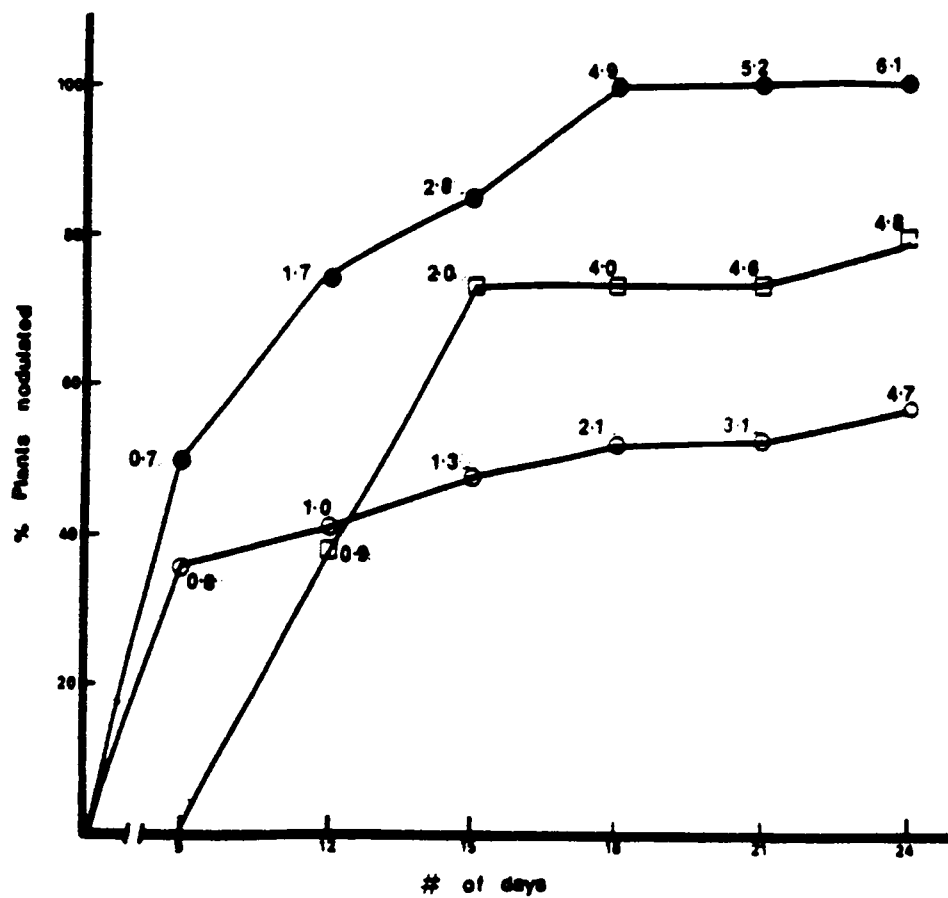
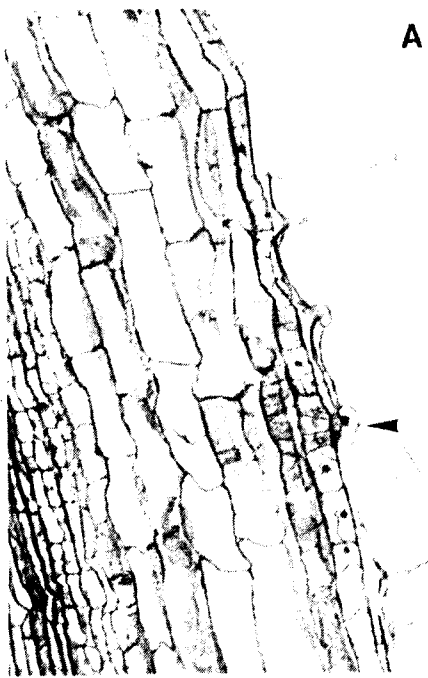


Figure 22. Nodulation kinetics of *B. japonicum* USDA 110 (●) and mutants, NAD 174 (□) and NAD 165 (○) on *Glycine soja*. The numbers at the data points on the graph indicate the average number of nodules per plant.

results indicated that Tn₅ was present as they were kan^r and str^r. Bacteria could not be isolated from nodules on siratro. To test the possibility that the Tn₅ insertion in NAD165 led to an increased or altered host - range, the mutant was inoculated on the Pisum sativum cultivars, wando pea and snap pea and Trifolium repens (clover). However, NAD165 could not form nodules on any of the plants tested (Table III).

Symbiotic phenotype of NAD165. Microscopic studies were done to determine the precise step at which the block in symbiosis occurred in mutant NAD165. The ability to curl root hair is the first visible step in the legume-rhizobium symbiosis. Mutant NAD165 was found to elicit the curled root hair response, suggesting that the block in symbiosis was beyond this step. Thick sections of paraffin embedded root sections were stained with tannic acid-saffranin-ferric chloride (Jensen, 1962) and observed under the microscope. The roots infected with USDA 110, showed very clear cortical cell division associated with curled root hair (Figure 23). Activity of the nodule meristem had caused a local swelling of the root (Figure 24). At least ten such centers of meristematic activity were observed per inoculated root. This stage of nodule development has been termed emergence (Calvert et al., 1984). In contrast, however, roots inoculated with NAD165 did not show any cortical cell division associated with the curled root hair (Figure 23). The outer cortex was completely devoid of any

Figure 23. Light microscopy of soybean roots inoculated with A) wild type B) mutant, NAD 165. Both micrographs are of longitudinal sections of roots, 6 days after inoculation. The wild type induced cell division in the outer cortex under a curled root hair. The newly divided cells have stained darkly, indicating that they were enriched in cytoplasm. In contrast, note the absence of meristematic activity in the roots infected by the mutant. Magnification : 100X.

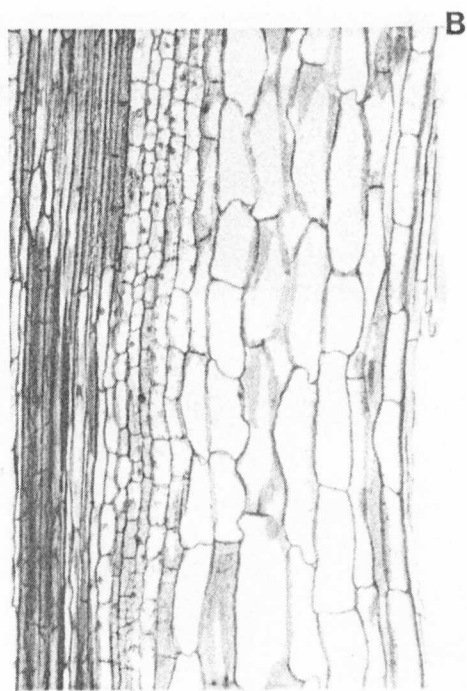
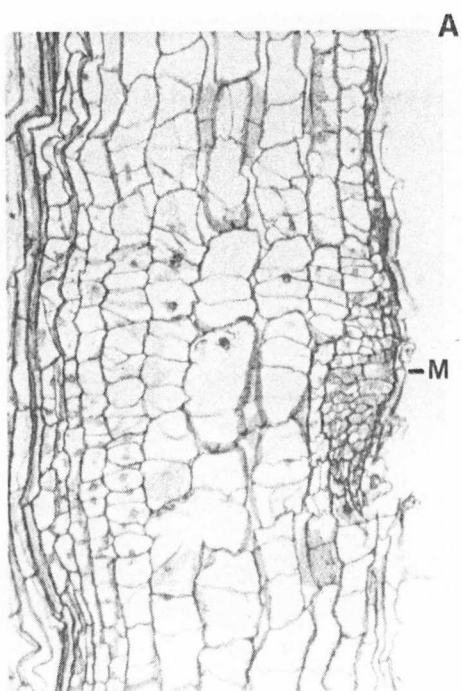


A



B

Figure 24. Light micrographs of Glycine max (soybean) roots inoculated with A) wild type B) mutant, NAD 165. Both micrographs are of longitudinal sections of roots, 6 days after inoculation. The light micrographs shown here represent later stage of infection by the wild type. The activity of the nodule meristem is more pronounced, causing a local swelling of the root. At least, 10 such centers of meristamatic activity were observed in the root sections, infected by the wild type. On the other hand, the roots infected by the mutant showed an absence of such centers in the outer cortex.



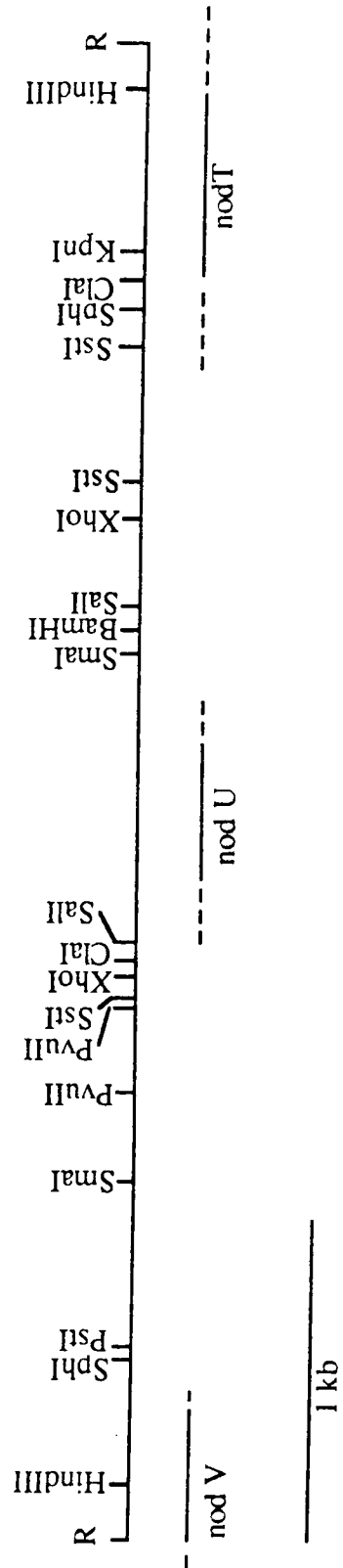
meristematic activity (Figure 24). This indicated that the mutation in NAD165 resulted in a block in symbiosis at a stage after root hair curling, but not later than the initiation of cortical cell division. Infection threads were not seen in the roots inoculated with either the wild type or the mutant.

Chemical analysis of the outer surface of NAD165. To test the possibility that the mutation in NAD165 led to an alteration in the components of the cell surface, crude preparations of exopolysaccharide (EPS) from NAD165 were analyzed by ^1H NMR spectroscopy and compared to the EPS of USDA 110. The results indicated that the nmr spectra of the EPS of NAD165 showed very few differences to that of USDA 110 (Dr. V. Puvnesarajah, personal communication).

Physical Characterization of the 5.3 kb EcoRI Fragment

Restriction endonuclease mapping. The phenotype of Tn5 insertions in the 5.3 kb EcoRI fragment suggested that the locus had at least three DNA regions encoding symbiotic functions. In order to study this nodulation region at the molecular level, the 5.3 kb EcoRI fragment of pRJUT10 was subcloned into the plasmid vector, pBR329, to give clone pGSE3 (Table II, page 24). A detailed restriction endonuclease map of the region was constructed using this subclone, as shown in Figure 25.

Figure 25. Restriction map of the 5.3 kb EcoRI fragment. The exact relative distances and order of the restriction sites very close to each other, such as PvuII, SstI, XhoI, ClaI and SalI have not been determined.



Hybridization analysis. The previously characterized nodulation genes from several rhizobial species did not hybridize to the 5.3 kb Eco RI fragment. It was of interest to see if the genes located on this fragment were present in other Rhizobium / Bradyrhizobium species. Therefore the 5.3 kb EcoRI fragment was hybridized to chromosomal DNAs from several strains of B. japonicum, as well as strains from Rhizobium. The results showed that this region was conserved in all the B. japonicum strains tested (Figure 26). The hybridization pattern could distinguish the B. japonicum symbiotic type I strains (represented by USDA 110, USDA122) from symbiotic type II strains (represented by 61A101C, USDA 76) (Stanley et al., 1985). Homologous sequences were also detected in B. parasponia and Bradyrhizobium spp. 32H1. The Rhizobium strains showed no homology to these probes (Figure 27) even at low stringency. Identical results were obtained when two internal SstI fragments (Figure 25) of 0.4 and 1.8 kb were used as probes (data not shown). These results indicated that the region encoded on the 5.3 kb EcoRI fragment was unique to the Bradyrhizobium species.

Regulation of Genes Located on 5.3 kb EcoRI Fragment

Isolation of lacZ fusions. The regulation of the genes located on the 5.3kb EcoRI fragment was examined using lacZ transcriptional/translational gene fusions. The 5.3 kb EcoRI fragment was cloned into pBR322 yielding pND1013 which was

Figure 26. Hybridization of Southern blots of EcoRI digests of genomic DNAs of B.japonicum strains to radioactively labelled, gel purified 5.3 kb EcoRI fragment of pRjUT10. Abbreviations : 46, USDA 46; 76, USDA 76; 112, USDA 112; 122, USDA 122; 123, USDA 123; 110, USDA 110; UT10, pRjUT10.

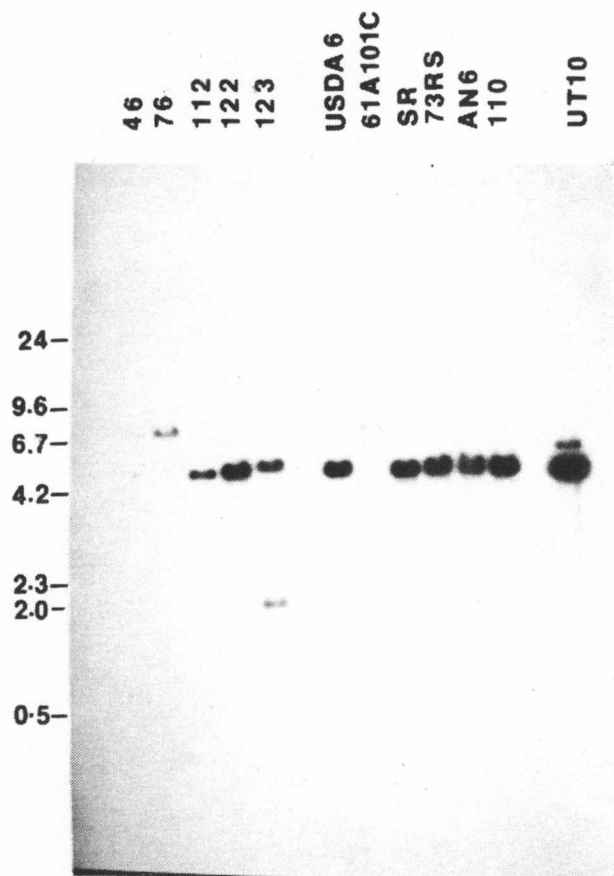
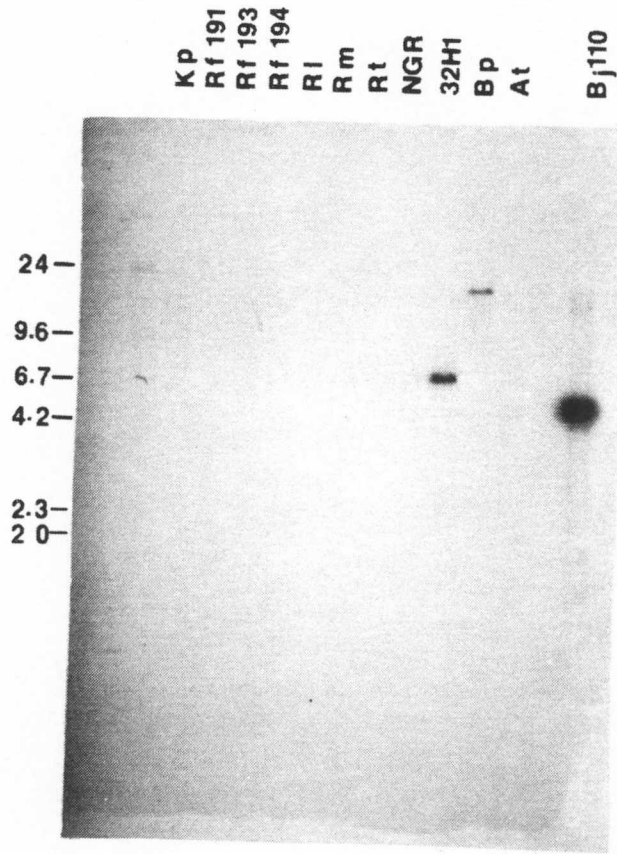
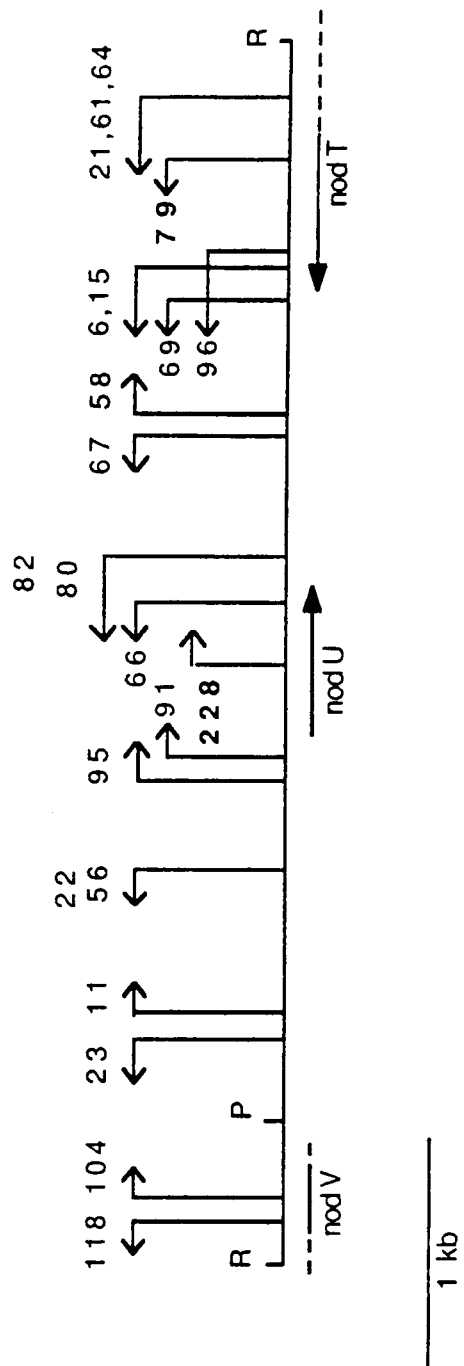


Figure 27. Hybridization of the Southern blot of EcoRI digests of genomic DNAs of various Rhizobium / Bradyrhizobium strains to radioactively labelled gel purified 5.3 kb EcoRI fragment of pRjUT10. Abbreviations : Kp, Klebsiella pneumoniae; Rf, Rhizobium fredii; Rl, Rhizobium leguminosarum; Rm, Rhizobium meliloti; Rt, Rhizobium trifolii; NGR, Rhizobium species strain NGR234; 32H1, Bradyrhizobium species cowpea strain 32H1; Bp, Bradyrhizobium parasponiae; At, Agrobacterium tumefaciens; B.j 110, Bradyrhizobium japonicum USDA 110.



transformed into E. coli MC4100 (MudIIPR48). This strain contained the mini-Mu-lac vehicle which was deficient in transposition and packaging as well as a Mu phage (with a temperature sensitive repressor) that was integrated into the chromosome. This helper Mu phage provided in trans the transposase and packaging functions. Mini-mu-lac insertions were isolated as described in Chapter II. Several insertion fusions of lacZ were generated in the 5.3 kb EcoRI fragment (Figure 28). Three hundred clones containing lac fusion insertions were mobilized into B. japonicum by the triparental mating technique and the transconjugants were selected for tet^R (150 ug/ml) and Cm^R (200 ug/ml). All transconjugants containing translatable fusions were assayed for the induction of lacZ. The precise locations and orientation of the Mu-lac insertions in nodT and nodU were mapped with the aid of restriction enzymes, EcoRI and PstI. The clone pND79 contained a nodT-lacZ fusion that was inducible in B. japonicum (NAD201). Similarly, a clone pND228 containing a nodU-lacZ fusion was also inducible in B. japonicum (NAD202) (Figure 28). The strains NAD201 and NAD202 were used in further studies.

Induction of nodT expression by plant factors. Fifteen different flavonoid compounds were used to induce 2 ml cultures of a nodT:lacZZ fusion strain, NAD201, for 14-16 hrs. A three-fold induction of nodT:lacZ fusion was detected in presence of soybean seed exudate. Apigenin, biochanin A, coumestrin, daidzein and



genistein showed a 2 fold induction of the lacZ fusion (Table IV). All other compounds tested could not induce expression. Previous studies on the regulation of common nod genes have shown that the nod gene product was necessary for the induction by plant flavones. In order to determine if the nodD gene product was required for the induction of nodT, the plasmid pND79 was mobilized into a nodD⁻ mutant, AN314. In the absence of a functional nodD, the nodT locus was not induced by any of the compounds tested suggesting that the nodD gene product may be involved in the regulation of nodT. The nodU:lacZ fusion was also induced by daidzein, genistein and soybean seed exudate. Several fusions in the nodV locus were tested, but were not induced by either soybean seed exudate or genistein. Since the nodV locus is at the extreme end of the 5.3 kb EcoRI fragment, it is possible that the promoter region and perhaps the 5' end of the gene spans the adjacent fragment. The lacZ fusions in the adjacent region have been shown to be inducible by soybean seed exudate (Dave Gerhold, personal communication). These results indicate that the recognition of these unique nodulation loci was very similar to the regulation of the nodLABC genes in Bradyrhizobium japonicum USDA 110 (Banfalvi et al., submitted).

TABLE IV. Expression of B β galactosidase by nodT:lacZ and nodU:lacZ fusions in response to Flavanoid Compounds

Inducer compound [@]	<u>nodT:lacZ</u> [*]		<u>nodU:lacZ</u> [*]		<u>nodL:lacZ</u> ^{\$}
	USDA 110	AN314	USDA 110	USDA 110	
None	56.35 +/- 8.0	250.0	6.5 +/- 1.5		33
Apigenin	130.4 +/- 10.9	214.2	ND		272
Biochanin A	114.0 +/- 5.5	250.0	13.4 +/- 0.0		735
Coumestrin	119.5 +/- 0.0	232.1	ND		99
Daidzein	130.4 +/- 0.0	232.1	17.2 +/- 4.7		593
Genistein	142.7 +/- 10.0	232.1	49.8 +/- 0.0		1,281
Luteolin	61.0 +/- 4.0	250.0	6.25 +/- 0.0		67
Naringenin	48.27 +/- 8.0	232.1	6.85 +/- 0.0		33
Soybean seed exudate	183.0 +/- 2.0	267.8	81.35 +/- 12.8		1,637

* = The values represent the mean +/- standard deviation of two replicates. ND, not determined.
 @ = All compounds were used at 2 uM concentrations, with the exception of crude soybean seed exudate.

\$ = The data shown in this column was obtained from Banfalvi et al (submitted) and is shown for comparison.

CHAPTER IV

DISCUSSION

Identification of Symbiotic Loci

A characteristic feature of the symbiotic genes in Bradyrhizobium species until now appeared to be the lack of a tight clustering of genes, unlike that found in Rhizobium species. This view was largely based on the fact that the nifKDH and common nod genes appeared to be unlinked. However, it is now apparent from this and other studies that the sym genes are indeed clustered in B. japonicum. Site-directed Tn5 mutagenesis of cloned DNA led to the identification of several symbiotic loci in this study (Figures 5 and 12, pages 54 and 67). Such an approach has been successful in the identification of genes involved in nodulation and nitrogen fixation in other Rhizobium/Bradyrhizobium species (Ruvkun et al., 1982; Fuhrmann and Hennecke, 1982; Jagadish and Szalay, 1984; Downie et al., 1985.; Djordjevic et al., 1985, Forrai et al., 1983). Tn5 mutagenesis has been successfully employed in the genetic analysis of a number of bacterial species, for example, Agrobacterium loci involved in tumour formation and opine catabolism (Garfinkel and Nester, 1980 and chromosomal genes and operons of Escherichia coli and Salmonella typhimurium, such as his (Bieck and Roth, 1980) and lac (Berg et al., 1980, Miller et al., 1980). The transposition frequency obtained in this study was

comparable to that observed in other systems (Hahn and Hennecke, 1984). Restriction enzyme digestion analysis confirmed the lack of site specificity of Tn5 transposition on the cosmid DNA (Figures 5 and 12, pages 54 and 67) which was consistent with other reports (Meade et al., 1982; Ruvkun et al., 1982; Hahn and Hennecke, 1984; Kondorosi et al., 1983). Although the high intrinsic antibiotic resistance level of Bradyrhizobium often makes selection difficult, putative Tn5 mutants could be easily selected because Tn5 allowed the expression of kanamycin and streptomycin resistance markers in Rhizobium / Bradyrhizobium species (Putnoky et al., 1983). This also enabled the counter selection of spontaneous kan^r colonies. Addition of chloramphenicol (30 ug/ml) aided in the exclusion of E. coli donors and helpers. The frequency of transfer of the cosmids containing Tn5 to Bradyrhizobium japonicum was comparable to that observed by Hahn and Hennecke (1984). Most of the Tn5 mutants obtained by double cross-over and marker replacement were found to be stable (Figures 4, 16, 19, pages 51, 74, 81). However, one Nod⁻ mutant NAD112, containing a Tn5 insertion in the 5.3 kb EcoRI fragment of pRjUT10 appeared to be unstable. Initial hybridization analysis of the mutant had demonstrated the presence of the Tn5. However, subsequent analysis of this mutant indicated that the Tn5 was no longer located on the chromosome. It is possible that the Tn5 transposed out of the genome inducing a small, but crucial, deletion in the 5.3 kb EcoRI fragment which led to a symbiotically

defective phenotype. Genetic instability of Tn5 insertion mutants as well as Tn5 induced genome rearrangements have been reported previously (So et al., 1987; Hahn and Hennecke, 1984). However the peculiarity of this mutant was that it was kan^r and str^r. One possible explanation was that the Tn5 had induced DNA rearrangements which led to pleiomorphic mutations. Perhaps, the outer surface of this mutant was altered so as to become resistant to the antibiotics. This result suggests that caution should be exercised in the analysis of Tn5 insertion mutants.

The Tn5 insertion mutants were analyzed for their ability to nodulate Glycine max. The results indicated that the common nod genes encoded functions important in early stages of nodule formation (Figure 13, page 69). The arrangement of these genes in B. japonicum was similar to that found in other Rhizobium species (Figure 12, page 67) (Torok et al.; Djordjevic et al., 1985; Downie et al., 1985) and complemented mutations in their equivalent genes (Russel et al., 1985; Nieuwkoop et al., 1987). Hybridization and sequence analysis have confirmed that the common nod genes from B. japonicum shared a high degree of homology with those from other Rhizobium species (Nieuwkoop et al., 1987).

The host range of Bradyrhizobium species is, in general, much wider than that of Rhizobium species. Therefore, it is possible that several loci could be involved in determining the host range. One such hsn locus was identified and localized approximately 8 kb from the common nod genes by hybridization to the siratro hsn genes

of Rhizobium sp. strain MPIK3030. Tn5 insertion mutations in this region prevented B. japonicum from nodulating siratro (Figure 14, page 72) but only caused a small delay in nodulation of soybean. This region appeared to encode the siratro hsn functions of B. japonicum. This type of differential nodulation response has a precedent in several Rhizobium species (Djordjevic et al., 1985; Horvath et al., 1986; Surin and Downie, 1988). Mutants in region III of R. trifolii which were defective in nodulation of one of its hosts, white clover, were able to nodulate subterranean clover (Djordjevic et al., 1986). Recent reports have indicated that mutations in nodLMN from R. leguminosarum also show a differential nodulation response on its alternate hosts (Surin and Downie, 1988). In R. meliloti, some of the hsn mutants were incapable of nodulating Medicago sativa, although it could still nodulate Melilotus albus (Horvath et al., 1986).

A second DNA region of B. japonicum that is involved in a differential nodulation response has been identified in this study. Mutations in the nodT locus affected nodulation of Glycine max (Figure 17, page 77) and Vigna unguiculata (Table III, page 86), but the nodT:Tn5 mutant, NAD165 elicited nodules on Glycine soja (Figure 22, page 87) and induced nodule like structures on siratro (Table III, page 86). In this respect, mutant NAD165 has the same phenotype as R. fredii strains that do not nodulate North American cultivars of soybean (for example, Essex, Williams), but can

nodulate G. soja and G. max cultivar Peking. Interestingly, there have been recent reports (Buenedia Claveria et al., 1988) that mutants of R. fredii strains that can effectively nodulate North American cultivars can also nodulate effectively different plants belonging to the cowpea group. These host specificity loci are probably necessary for the recognition of the host(s) or in nodule initiation (Djordjevic et al., 1986). Another explanation could be that the hsn gene products may serve as signal(s) to herald a compatible bacterial-plant interaction. These signals could possibly result in a modified outer surface of the bacterium. Several genes have been identified in the fast-growing rhizobia that seem to have sequence homology with genes encoding proteins such as amidotransferases (Surin and Downie, 1988), acyl carrier proteins (Evans and Downie, 1986), ribitol dehydrogenase (Debelle et al., 1987) etc. These proteins may play a role in modifying sugars, lipids and protein molecules of the outer surface (Downie and Johnston, 1986). An exhaustive analysis of the the surface chemistry of hsn mutants from Rhizobium/Bradyrhizobium species may elucidate the role of hsn gene products.

Additional nodulation regions that show a delay in the nodulation of soybean have been identified by Tn5 mutagenesis, (Figures 5 and 12, pages 54 and 67). A mutant, NAD27 which had a Tn5 insertion in the 5.8 kb EcoRI fragment of pRjUT10 nodulated soybean with a delay of 4 days as compared to the wild type (Figure 15, page 73). Insertion of lacZ fusions have indicated that this

region is regulated by plant factors. The mobilization of this region into B. japonicum USDA 123 extended its host range to include certain cultivars of soybean that could not be nodulated by the wild type (Sadowsky et al., submitted). Therefore, this region appears to be involved in cultivar specific nodulation. The 7.7 kb EcoRI fragment located on pRjUT14 was also identified as a nodulation region. A Tn5 insertion mutant NAD14 (Figure 6, page 56) and deletion mutants NAD163 and NAD164 (Figures 10 and 11, pages 64 and 65) showed a delay suggesting that this region was perhaps not essential in nodulation, but contributed to the overall efficiency of nodulation. Putnoky et al. (1987) have described a region in R. meliloti, linked to the common nod region that is essential for efficient nodulation (efn) of alfalfa. The efn region also contained the conserved putative nod promoter sequence, or nod box. Interestingly, hybridization analysis using a clone containing a nod box linked to nod genes from R. meliloti revealed homologous sequences adjacent to the nodulation region located in the 7.7 kb EcoRI fragment (David Gerhold, personal communication). These results taken together suggest that while these DNA regions are not essential for nodulation of soybean cultivar Essex, they somehow aid in the fine-tuning of the process.

Characterization of the 5.3 kb EcoRI fragment

The analysis of the nodulation ability of Tn5 mutants in the 5.3 kb EcoRI fragment of pRjUT10 led to the definition of three

nodulation loci that were linked to the common nod genes (Figure 18, page 78). Hybridization studies suggested that these loci were not homologous to any of the nod genes described earlier. Mutations in nodU and nodV resulted in a delay in nodulation of soybean (Figures 20 and 21, pages 83 and 85) suggesting that these loci were not essential in the nodulation of soybean. They may be involved in enhancing the nodulation and/or competitiveness of soybean or may be necessary for the nodulation of other hosts. In contrast, mutation of nodT strongly inhibited the nodulation of soybean, cowpea and pigeon pea, indicating that the nodT locus may be involved in some as yet unknown aspect of recognition which is critical in the nodulation of soybean, cowpea and pigeon pea, but not of G. soja or siratiro (Table III, page 86). Therefore, the nodT locus, and perhaps nodU and nodV, may be involved in efficient nodulation of soybean. These three genes appear to be functionally equivalent to the nod LMN genes of R. leguminosarum (Surin and Downie, 1988) or nodEFGH of R. meliloti (Horvath et al., 1986) or the region III of R. trifolii (Djordjevic et al., 1986). Mutants in the nodL gene of R. leguminosarum inhibited the nodulation of pea, but those in nodM and nodN showed only a delay in the nodulation of pea. Similarly, the nodH mutants of R. meliloti showed a Nod⁻ phenotype on Medicago sativa; but nodEFG mutants show a delayed phenotype. Hybridization analysis amongst several members of the Rhizobium species, with nodTUV as probes showed the

absence of analogous sequences in the fast growing rhizobia (Figure 27, page 99). A similar analysis amongst members of the Bradyrhizobium group (Figure 26, page 97) revealed that not only were these sequences conserved in all strains tested, but the pattern could also distinguish the STI and STII types as described by Stanley et al. (1985). Curiously, the fast growing strains of R. fredii (which can nodulate soybean) do not contain any DNA sequences homologous to these nodulation regions. It is intriguing that two strains that nodulate soybean are so divergent in their DNA sequences. Perhaps, the absence of nodTUV loci prevent, at least in part, R. fredii strains to nodulate North American cultivars of soybean. Other DNA regions required for the nodulation of soybean by B. japonicum (such as nodS) (Jae So, personal communication) also do not seem to share any homology with the DNA from R. fredii. One application of this feature is that these DNA regions could be used as probes to identify ecological variations of the genus Bradyrhizobium, and thus can be used in their classification (manuscript in preparation).

The mutants in the nodT locus were also characterized at the phenotypic level. Light microscopic studies on the roots inoculated with the mutant NAD165, indicated that the mutation in nodT blocked the nodulation process at the stage of cortical cell division (Figures 23 and 24, pages 89 and 91). It is not clear if the mutant could initiate the formation of infection threads, as the technique used did not allow easy visualization of these

structures, even in the wild type. However, two possibilities are implicit with this observation: (1) The mutant did not penetrate the root hair cell, and therefore could not initiate infection. (2) The mutant lacked the signal factor which normally stimulated cortical cell division. Calvert et al, (1984) have reported that the cortical cell division in soybean roots is induced even before infection thread formation. They have observed the formation of pseudoinfections, which are small local centers of cortical cell division not associated with infection threads. The existence of such pseudoinfections suggests that the mechanism(s) which initiate cortical cell division may be distinct from those which initiate penetration and infection thread formation. Truchet et al. (1984) and Hirsch et al. (1984) have made similar conclusions based on anatomical analyses of pseudo-nodules on alfalfa. Bauer et al. (1985) have subsequently shown that bacteria could induce cell divisions in the host root cortex even though they remain separated from the root surface. More recently, Djordjevic et al. (1988) reported that in clover, cortical cell division was stimulated even before the entry of the bacterium into the root hair.

The nodABC genes from Rhizobium meliloti were necessary and sufficient for the nodule induction of one of its hosts, Neptunia oleracea (Debelle et al., 1986). Previously, Hirsch et al. (1982) had isolated Nod⁻ mutants of R. meliloti that induced root hair-curling of alfalfa but localized mitotic activity within the

interior of the root was not observed. More recently, Bender et al. (1987) have reported that nodulation of Parasponia by Rhizobium sp. NGR234 did not need nodABC gene products, but the initiation of cell division needed the expression of Parasponia specific hsn genes which was regulated by nodD. Lewin et al. (1987) have identified three host specificity loci in Rhizobium sp. NGR234. Transfer of any of the three hsn loci to appropriate recipients were able to induce root hair curling, while only Hsn1 conferred the ability to nodulate the host, suggesting that host range genes may act in controlling host specificity at stages later than curling of root hairs. Initiation of cell division seems to be host specific in some Rhizobium-host interactions. As the nodT mutant (NAD165) did not initiate cortical cell division, it is tempting to speculate that the nodT gene product may be an inducer of cortical cell division or is involved indirectly in its induction. Cytokinins which are effective inducers of plant cell division have been reported to be synthesized by Rhizobium spp. (Giannattasio and Coppola, 1969.; Phillips and Torrey, 1970.). Bauer et al. (1985) have shown that B. japonicum secreted inducers of cortical cell division in a host-specific manner. However, it remains to be seen whether such functions are encoded by nodT.

Induction of nodT and nodU by Host Factors

Expression of nod genes has been shown to be regulated by a number of plant factors in many Rhizobium species (Mulligan and

Long, 1985; Innes et al., 1985; Rossen et al., 1985). In B. japonicum, the expression of nodLABC and nodD is regulated by soybean seed exudate (Banfalvi et al., submitted). Apigenin and isoflavones, such as genistein and daidzein, have been shown to be inducers of nodL: lacZ and nodD: lacZ fusions. This induction exhibits species specificity such that luteolin, an inducer of nod genes in R. meloloti, did not induce nod genes in B. japonicum (Banfalvi et al., submitted). This specificity has been found to be mediated by the nodD gene product (Wijffellmann et al., 1986; Horvath et al., 1987).

The nodT and nodU genes appear to be regulated in similar manner to the other nod genes from B. japonicum. The induction levels for nodT: lacZ and nodU: lacZ fusions were low when compared to the nodL: lacZ fusion (Table IV, page 105). This could be due to several reasons. The fusions in nodL were constructed differently from those in nodT or nodU, and it may be that the difference in the constructs was reflected in activity of β -galactosidase. It is also possible that the level of β -galactosidase activity reflects the level of induction of the genes, and therefore only a transient induction and a small concentration of the gene products was required. The distance of the promoter from the lacZ insertion in nodT: lacZ and nodU: lacZ fusions is not known. However, the promoter is about 5-75bp away from the lacZ gene in the nodL: lacZ fusion construct and that could possibly reflect in the β -galactosidase activity. It is also

possible that the location of lacZ fusion insertion in an open reading frame may result in a change in the amino-terminal of the B galactosidase protein. This change would reflect in the B galactosidase activity. It is also possible that the induction of the nodT locus is negatively regulated by another gene product. The hsn gene fusions in other Rhizobium spp. also seem to be induced at a lower level as compared to nodABC (Djordjevic et al., 1987; Surin and Downie, 1988; Downie et al., 1986). None of the fusions in the nodV locus were induced by soybean seed exudate or any of the isoflavones. Since the nodV locus lies at the extreme end of the 5.3 kb EcoRI fragment; it is possible that the promoter and perhaps even a part of the gene lies in the adjacent fragment. The lacZ fusions in the adjacent EcoRI fragment to nodV are inducible and show B-galactosidase levels similar to nodT and nodU (D. Gerhold, personal communication). Therefore, it is likely that the transcript for nodV initiates outside of 5.3 kb EcoRI fragment. Interestingly, the induction of nodT locus appeared to require the nodD gene product since in a mutated nodD background, nodT could not be induced (Table IV, page 105). However, unlike nod genes that are regulated by nodD, this locus did not contain the conserved nod box sequence as seen by hybridization analysis. DNA sequence analysis will determine if an altered form of this sequence, or some other regulatory motif is present in the upstream regulatory region. Although host inducible loci without a nod box

like sequence have been reported previously in R. fredii (Sadowsky et al., 1988) and hybridization analysis and partial sequencing does not indicate the presence of a nod box like sequence in other host-inducible regions of B. japonicum (Dave Gerhold and Sohail Luka, personal communication), the role of nodD gene product in their regulation has not been studied.

The nodD gene product has been shown to bind to the nod box, by the gel retardation assay (John et al., 1988; Fisher et al., 1988). It is possible that the same nodD gene product or possibly another nodD-like gene product can recognize a different sequence as a promoter. Applebaum et al. (1988) have identified the presence of a second copy of nodD (nodD₂) in R. fredii 191 which may be involved in the regulation of exopolysaccharide synthetic genes. Sadowsky et al. (1988) have reported another sequence which is possibly involved in plant specific control of R. fredii gene expression. Other nodulation loci sequenced in our laboratory, for example, the siratro hsn region and the nodS locus, also do not show any nod box like sequence. It is therefore likely that additional DNA sequences may serve as regulatory elements of Bradyrhizobium specific symbiotic genes. The high levels of expression of nodT :lacZ fusions in AN314 (nodD:Tn5) seem to suggest that nodD may also negatively regulate the expression of nodT. Complementation of the nodD:Tn5 mutation in the presence of nodT:lacZ fusion will be able to clarify the role of nodD in the regulation of nodT.

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VITA

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In the pursuit of higher education, she left her country and came to USA. She attended East Tennessee State University for a few months before entering the graduate program at the Dept. of Microbiology, University of Tennessee, Knoxville in March 1984. Nirupama is married to another microbiologist, Dr. Prakash Sista and will join him at North Carolina. She has accepted a post-doctoral position with Dr. Joseph Pagano, at Lineberger Cancer Research Center, University of North Carolina, Chapel hill.