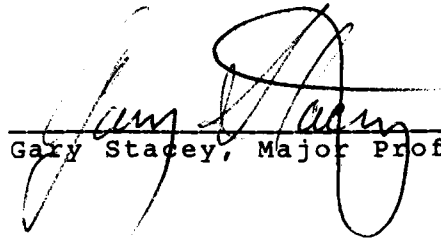


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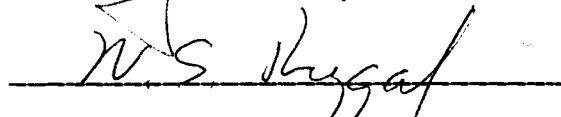
I am submitting herewith a dissertation written by Jae-Seong So entitled "Molecular Cloning and Characterization of Nodulation Genes in Bradyrhizobium japonicum." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

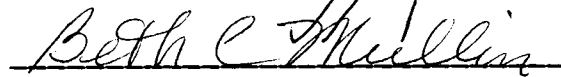


Gary Stacey, Major Professor

We have read this dissertation
and recommend its acceptance:







Accepted for the council:



Vice Provost and Dean of
The Graduate School

MOLECULAR CLONING AND CHARACTERIZATION OF NODULATION GENES
IN BRADYRHIZOBIUM JAPONICUM

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Jae-Seong So

August 1988

ACKNOWLEDGEMENTS

In the course of this study, a number of people have assisted me generously. First of all, I wish to express my gratitude to Dr. Gary Stacey, chairman of my supervisory committee, for his guidance and assistance in completion of this study. Gratitude is also extended to the other members of my committee, Drs. Beth Mullin, Raymond Beck and Stuart Riggsby, for their time and suggestions toward the completion of this study. A special thank goes to Dr. Evans Roth at the Zoology Department for his help in light and electron microscopy. Also, Dr. V. Puvanesarajah of the Carbohydrate Research Center, University of Georgia is appreciated for analysis of polysaccharide samples.

Thanks are due to all my lab co-workers for their helpful discussion and technical assistance, which made my academic years at the University of Tennessee, Knoxville most rewarding.

It is impossible to express the gratitude I owe to my parents for their love and understanding. Finally, but by no means least, I wish to thank my wife, Hwa-Soon Kim and my son, Min-Woo So, without whose love and patience none of this would have been possible.

ABSTRACT

Nodulation of soybean by Bradyrhizobium japonicum is a complex developmental process involving intimate interactions between both plant and bacterial genes. To study this process at the molecular level, 25 symbiotically defective mutants of B. japonicum strains USDA 110 and 61A101C were isolated by random transposon Tn5 mutagenesis. Research was focused on four nodulation-defective mutants of strain USDA 110 and one Nod⁻ mutant of strain 61A101C. The mutated DNA from each of these mutants was cloned and shown to be responsible for the observed phenotype by homogenotization of the cloned fragments into wild-type strains. The four USDA 110 mutations were found to map to two distinct regions; one of which encoded essential functions for histidine biosynthesis. Hybridization of these cloned regions, as well as the cloned 61A101C region, to the previously cloned nod regions of Rhizobium meliloti and nif and nod regions of B. japonicum USDA 110 showed no homology, indicating that these regions define new nodulation loci in B. japonicum. Using the cloned mutated DNA as probes, the wild type clones were successfully obtained from gene libraries by colony hybridization.

Additional attention was placed on the cloned region from 61A101C because of the interesting phenotype of mutation in this locus. The region was found to be well

conserved among various Bradyrhizobium species whereas no homology was detected with Rhizobium species examined. To determine the structure, organization and possible function of the region, it was characterized by physical and genetic means. A detailed restriction map of the region III was generated. The essential nod gene region was narrowed to a 2.9 kb XhoI fragment, which was the smallest fragment responsible for the conserved hybridization pattern. DNA sequences of parts of the 2.9 kb XhoI fragment were determined. Search for homology of the sequences against the DNA sequence data bank failed to find any significant homology to the previously known gene sequences.

In addition, the phenotype of a mutation in the region was characterized. Although the mutant was originally shown to be Nod⁻, in further nodulation experiments with various cultivars of soybean it formed anomalous nodule-like structures at varying frequencies (0 to 54% of plants inoculated) depending on the cultivar used. Ultrastructural analysis of these structures revealed no evidence for bacterial invasion as the plant cells were completely devoid of bacteria. The mutant could induce root hair curling but failed to induce infection thread formation.

The mutant was found to be different from wild type with respect to its cell surface. A large portion of cells in liquid medium formed aggregates resulting in sizable clumps. Moreover, when the cells were collected by

centrifugation, the mutant cells formed a "rocket"-shaped pellet unlike the compact round pellet formed by wild type cells. Chemical analysis of the exopolysaccharide (EPS) samples from the mutant and wild type cells showed that the mutant's EPS contained a considerably higher glucose content, which is presumably due to overproduced beta-2-glucan. Further, the mutant strain JS314 lacked a membrane polypeptide of 14 kd in size, that was present in the wild-type strain.

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INTRODUCTION

The Gram-negative, aerobic, non-spore-forming, rod shaped or pleomorphic soil bacteria, rhizobia can form a morphologically distinct structure, a nodule, on the root of specific host plants. The term rhizobia will be used to denote bacteria of two genera: the fast-growing Rhizobium species and the slow-growing Bradyrhizobium species (Jordan, 1982). General phenotypic differences between these two genera are summarized in Table I. With certain exceptions (Trinick, 1973), the host plants that are nodulated by rhizobia are members of the family Leguminosae. Within this family, there are specificities between a group of plants and a particular Rhizobium or Bradyrhizobium species. Indeed, rhizobia are classified on the basis of their host range (e.g. B. japonicum-soybean, R. meliloti-alfalfa, R. phaseoli-bean, R. leguminosarum-pea, and R. trifolii-clover).

The process of nodule formation is complex, and requires active participation of both host plant and infecting rhizobia. The first step in the establishment of symbiosis is the recognition and invasion of the appropriate legume by the bacteria. Rhizobia attached to the root hairs induce deformations, curling and branching of the normally straight root hairs. The curled root hairs resemble shepherds' crooks that can entrap bacteria in their fold.

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 hr	7-20 hr
Mannitol Medium	Acid production	Alkaline production
Disaccharide Utilization	(+)	(-)
Phosphogluconate Dehydrogenase	(+)	(-)
Chemoautotrophy	(-)	(+)
Antibiotics 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)

At this site, rhizobia penetrate the plant cell wall and a tubular infection thread forms which carries the bacteria into the root cortex. Meanwhile, the cortical cells are induced to divide and are then invaded by the bacteria. Rhizobia are released from the infection thread into the cytoplasm of cortical cells and are surrounded by a host-derived peribacteroid membrane. The rhizobia differentiate into bacteroids within the plant cell. Mature bacteroids can fix atmospheric nitrogen into ammonia, which is transported to the host plant and utilized as a nitrogen source. In return, bacteroids are provided from the host plant with carbon sources which are derived from photosynthesis.

The biochemical mechanisms underlying the nodulation process have been difficult to elucidate, because the events occur transiently in a very small portion of the root cells. Nevertheless, our knowledge of the molecular genetics of the nodulation process has increased rapidly in recent years. Identification of the bacterial genes and gene products involved in nodulation will provide a valuable tool for elucidating the cellular mechanisms by which nodule formation proceeds.

A number of bacterial genes essential for development of the symbiosis (sym genes) have been identified and consist of nif, nod and fix genes. The nif genes are responsible for construction of nitrogenase and fix genes are

essential for nitrogen fixation, but are of unknown function. The nod genes are those involved in the formation of the nodule. In species of Rhizobium, sym genes have usually been located on large indigenous plasmids and have been studied intensively in R. meliloti (Banfalvi et al. 1981; Long et al. 1982; Meade et al. 1982; Ruvkun et al. 1982; Kondorosi et al. 1984; Jacobs et al. 1985), R. leguminosarum (Brewin et al. 1980; Downie et al. 1983; Rossen et al. 1984), and R. trifolii (Scott et al. 1982; Schofield et al. 1984).

In the case of the slow-growing rhizobia, Bradyrhizobium species, earlier studies focused on nif and fix genes (Hennecke 1981; Adams et al. 1984; Fischer and Hennecke 1984; Hahn et al. 1984; Kaluza et al. 1985), but identification and characterization of Bradyrhizobium nod genes are now a topic of intense research. Russell et al. (1985) reported the cloning of the B. japonicum gene region encoding early nodulation functions utilizing the homology between B. japonicum DNA and the cloned nod genes of R. meliloti and R. leguminosarum. Subsequent studies on the cloned region revealed nod genes which are similar in their arrangement and regulation to those of Rhizobium, the so-called "common nod" genes (Lamb and Hennecke 1986; Nieuwkoop et al. 1987). These genes are of obvious importance, but we wanted to identify additional genes which are necessary for soybean nodulation (for example, those involved in host range).

Therefore, a range of symbiotically defective mutants were isolated from two strains of B. japonicum by generalized transposon Tn5 mutagenesis.

Using the mutants thus obtained, attempts were made to isolate and characterize new gene regions in B. japonicum which are essential for nodulation of soybean. As a result, three independent nodulation gene regions were cloned. They were characterized by genetic and molecular means. One of these regions was characterized in detail to determine its structure, organization and possible symbiotic function.

CHAPTER I

REVIEW OF THE LITERATURE

A. Molecular Genetics of Nodulation

Recent developments in broad host-range plasmid vectors, transposon mutagenesis, and recombinant DNA techniques have facilitated the isolation of mutants which are defective in symbiosis, the cloning of the relevant gene regions, and the characterization of those genes. A number of rhizobial genes involved in the formation of effective nodules are collectively called sym genes and further divided into three categories: nif, fix, and nod (Stacey 1988). In Rhizobium species most of the known sym genes are located on plasmids (the Sym plasmids), and the molecular genetics of sym genes in several Rhizobium species (for example, R. meliloti, R. leguminosarum, and R. trifolii) is now well advanced. In contrast, sym genes in Bradyrhizobium species are believed to be on the chromosome, and information on them is now emerging.

1. Identification and Cloning of Nod Genes

In order to understand the nodulation process at the molecular level, it is necessary to isolate the genes involved. Since the nodulation phenotype is only manifested in the presence of the plant, isolation of nodulation genes is problematic. Nevertheless, there have been several

approaches successfully employed for this objective. First, Long et al. (1982) isolated essential nodulation genes from R. meliloti by directly complementing a Nod⁻ mutant. They constructed a gene library in a broad host range vector, introduced the gene bank into a Nod⁻ mutant strain en masse, and then inoculated the resulting transconjugants on alfalfa plants. From the nodules formed, they isolated the bacteria from which the introduced gene fragment was isolated as an extrachromosomal element.

Secondly, many laboratories utilized gene tagging techniques, where a transposon was randomly introduced into the genome of a wild type strain and the resulting mutants were tested individually on an appropriate host plant for their nodulation phenotype (Beringer et al. 1978; Hom et al. 1984; Rostas et al. 1984; Applebaum et al. 1985). Our laboratory also employed this approach to isolate an array of Sym⁻ mutants of B. japonicum (So et al. 1987). A schematic diagram of the random Tn5 mutagenesis in B. japonicum is shown in Figure 1. Once a symbiotic mutation is identified, the mutated nod gene can be cloned directly by selection for a DNA fragment conferring the drug resistance phenotype carried on the transposon. The nonmutated nod gene can then be isolated from a gene library using the mutated gene as a hybridization probe.

Thirdly, once a nod gene region is obtained, additional nod genes can be identified after mutagenizing neighboring

Figure 1. Random Tn₅ mutagenesis scheme. Tn₅ insertions are symbolized by arrowheads. Large circles represent the chromosome of bacteria. Small circles are plasmids. Tn₅ carried on the suicide plasmid pSUP1011 was mobilized into the recipient B. japonicum with help of tra functions provided from a helper plasmid pRK2073. In the B. japonicum background, the suicide plasmid pSUP1011 is degraded because it cannot replicate. At the same frequency, Tn₅ on the introduced plasmid hops onto the chromosome of B. japonicum resulting in an insertion mutation selectable by resistance to kanamycin and streptomycin. The resulting mutants were tested individually on plant to determine their symbiotic phenotype.

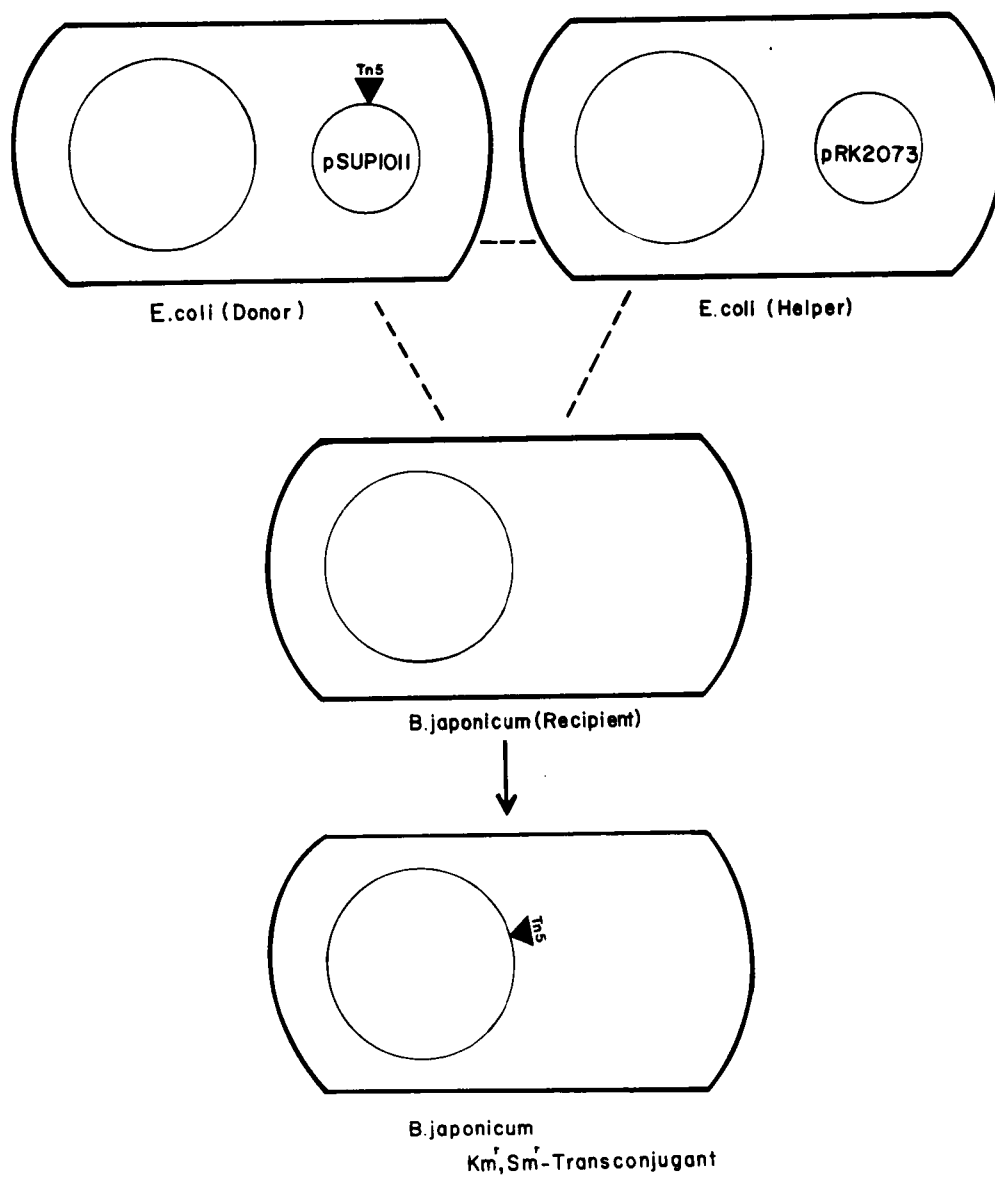


Figure 1

regions by site-specific mutagenesis and subsequent nodulation tests of the mutants. This strategy is based on the observation that many sym genes of Rhizobium species are clustered, on large indigenous plasmids or on the chromosome (Banfalvi et al. 1981; Downie et al. 1983). This clustering of nod genes was found to be the case for B. japonicum species as well (Nieuwkoop et al. 1987; Deshmane et al. in preparation). Transposon Tn5 saturation mutagenesis of the DNA region adjacent to the cloned common nod region (ca. 100 kb) and subsequent nodulation assay of the mutants allowed identification of several nod loci which appeared to be essential for nodulation (Deshmane et al. in preparation).

In addition, several laboratories have isolated nod genes by DNA-DNA hybridization using previously cloned nod genes from different species of rhizobia as probes. For example, Russell et al. (1985) used the homology between the cloned R. meliloti nod genes and B. japonicum to isolate the nod genes from this slow-growing rhizobium by hybridization. Similar approaches were used in other slow-growing rhizobia (Marvel et al. 1986; Noti et al. 1985).

An approach different from the above methods was to look for the acquisition of nodulation functions by bacterial species that previously lacked them. For example, in several cases when the Rhizobium Sym plasmid was transferred to an Agrobacterium tumefaciens strain which had been cured of its Ti (tumor inducing) plasmid, the recipient gained the

capacity to induce ineffective nodules on an appropriate legume host (Hooykaas et al. 1981; Wong et al. 1983). More recently, Hirsch et al. (1984) reported that a 20 to 40 kb fragment of the R. meliloti Sym plasmid, when introduced into A. tumefaciens, can be expressed and results in the formation of ineffective nodules on alfalfa roots. The nodules exhibited normal meristem and vascular development, but often were devoid of bacteria and lacked infection threads. This method, however, has only been utilized to study previously identified nod genes, not to discover new loci.

2. Cloned Nod Genes

The first nodulation genes identified were those found to encode root hair curling ability (Rossen et al. 1984; Jacobs et al. 1985; Russell et al. 1985). The appearance of the curled root hair is the earliest observable sign of successful infection by rhizobia. These root hair curling genes are clustered and functionally and structurally conserved among the various Rhizobium and Bradyrhizobium species examined (Djordjevic et al. 1985; Kondorosi et al. 1984; Marvel et al. 1985; Torok et al. 1984; Rossen et al. 1984; Egelhoff et al. 1985; Scott 1986).

Genetic complementation and DNA sequence studies have identified four nod genes involved in root hair curling, designated nodABCD. The nodD gene is transcribed divergently from the nodABC genes. The nodABC genes are in

one transcriptional unit, which at least in R. leguminosarum also contains two other genes nodIJ. The nodIJ genes also have been shown by hybridization to be well conserved among other rhizobia (Nieuwkoop et al. 1987; Banfalvi and Stacey unpublished data). Mutations in the nodABC genes were invariably Nod⁻, whereas mutants defective in the nodIJ genes showed a few days delay in nodule formation. Mutations in nodD of R. meliloti gave a leaky phenotype (Jacobs et al. 1985), whereas nodD mutations in R. leguminosarum were Nod⁻ (Downie et al. 1985). In B. parasponia and B. japonicum, an additional gene was found between the nodD and nodA genes (Scott 1986; Nieuwkoop et al. 1987). Nucleotide sequence analysis of the region upstream of nodABC genes of B. parasponia revealed the existence of an open reading frame (ORF) of 408 bp which was designated nodK and appeared to be organized in a single transcriptional unit with nodABC (Scott 1986). In B. japonicum, an ORF similar in position to the nodK gene was identified (Nieuwkoop et al. 1987), and shown to be in the same transcriptional unit as nodABC (Banfalvi et al. submitted). The latter ORF was given a different name, nodL, as the ORF was overall only 30% homologous, at the amino acid level, to B. parasponia nodK. Hybridization data indicated that the nodL gene is broadly conserved among Rhizobium and Bradyrhizobium species (Banfalvi et al. submitted).

The nodD gene was found to be present in multiple copies in almost all Rhizobium species with the exception of R. leguminosarum (Rodriguez-Quinonez et al. 1987). This reiteration of nodD genes is also likely in B. japonicum as multiple bands were found when genomic DNA was hybridized with a nodD probe (Schell and Stacey, unpublished data), explaining the finding that a mutation in nodD did not completely abolish nodulation ability of B. japonicum (Nieuwkoop et al. 1987). This reiteration of nodD gene also explains the above mentioned leaky phenotype of nodD mutation in R. meliloti. In R. meliloti three copies of nodD (nodD1, nodD2 and nodD3) were detected (Gottfert et al. 1986; Rodriguez-Quinones et al. 1987; Honma and Ausubel 1987). Single nodD2 mutants had the same nodulation phenotype (i.e. 4 to 7 days delay in nodulation) as the nodD1 mutants, while a double nodD1-nodD2 mutant exhibited a more severe delay in nodulation, indicating that at least two copies of functional nodD genes are necessary for the efficient nodulation of alfalfa (Medicago sativa) (Gottfert et al. 1986). Recently, it was shown that all three nodD copies are involved in nodulation to an extent depending on the actual host (see below).

At present, very little is known about the biochemical role of the common nod genes. The nodABC genes appear to encode gene products which are somehow involved in the formation (or modification) of a signal(s) which induces

root hair curling and cortical cell division. It is likely that the signal(s) could be a plant growth regulator(s) or other molecules which are interacting with plant cell walls or membranes. For example, in the R. leguminosarum-pea symbiosis, research indicated that the bacteria synthesize a factor(s) which inhibits overall root growth in response to the host plant's exudate. The synthesis of this factor(s) required the nodABC and D genes (van Brussel et al. 1986). Mutations in these genes resulted in both inability to induce root hair curling and a change in the mode of plant root growth. From these data, however, it could not be excluded that the factor(s) may be a molecule of plant origin that is modified by the nodABC gene products. As the factor(s) was found to be soluble, the isolation and characterization of it will be possible and of great significance in elucidating the function of the nodABCD genes.

Another important step toward understanding the function of the nod genes was to determine their DNA sequences. The DNA sequences of the nodABC genes indicated large hydrophobic domains in the encoded proteins (Jacobs et al. 1985; Torok et al. 1984; Rossen et al. 1984), suggesting that some of these gene products might be found in the cell membrane. Indeed, the antibody against the nodC protein of R. meliloti was used to localize the protein in the membrane, and nodulation was reduced significantly by adding

the antibody along with R. meliloti inocula (John et al. 1985). Recent work by the same group has suggested that nodC is a transmembrane protein which may act as a surface receptor (John et al. 1988). The transmembrane orientation of the NodC protein was confirmed by surface-specific radiolabelling and proteolysis experiments. Significantly, in nodules of various legumes, a truncated form of the NodC protein was detected immunologically (John et al. 1988), indicating that the nodC gene is expressed not only in the early step of nodulation but in later stages as well and possibly modified post-translationally.

The the nodD gene product has been extensively studied and appears to function as a positive regulator of nod gene transcription as will be discussed below. As for the other common nod genes, nodIJ, little information has been published. When the predicted protein sequence of the nodI gene of R. leguminosarum was compared with a protein sequence data bank, the nodI amino acid sequence was found to be similar to that of a series of bacterial gene products, hisP, malK, oppD and pstB which are involved in the active transport of histidine, maltose, oligopeptide and phosphate respectively (Higgins et al. 1986). These proteins are membrane-associated ATP-binding proteins. Based on these observations, it was postulated that the nodI gene product might be involved in some way in the ATP-dependent transport of some molecules (Downie et al. 1987).

This is an interesting hypothesis because it is now known that there are small molecular weight signal compounds excreted from the host plant that regulate nod gene expression (see below).

Nodulation phenotypic analyses of mutations in the regions adjacent to the common nod regions on different host plants identified another set of genes responsible for conferring host range ability. In R. meliloti, Horvath et al. (1986) have utilized site-directed Tn5 mutagenesis and subsequent nodulation tests of the mutants on different host plants to identify four additional nodulation genes [nodFEGH = hsnABCD (host specificity of nodulation)] which determined host-range specificity and were located several kilobases away from the common nod genes. Tn5 insertion mutations in nodH abolished root hair curling and nodulation ability completely, while nodFEG mutants were still able to nodulate their natural plant host Medicago sativa and Melilotus albus, but to different extents. Mutations in the hsn genes are not complemented by nodulation genes of other Rhizobium species, unlike mutations in the common nod genes. An analogous set of genes located close to the common nod genes were found in R. leguminosarum, R. trifolii and Rhizobium sp. NGR234 (Downie et al. 1983; Djordjevic et al. 1985; Bassam et al. 1986). In R. trifolii, mutations in nodF and E were shown to affect host-range, as the mutants gained the ability to nodulate Pisum sativum (peas), but showed only

poor nodulation ability on the normal host plant, Trifolium repens (white clover) (Djordjevic et al. 1985). Also, in B. japonicum a locus situated close to the common nod genes has been identified and cloned, which encodes a host specific nodulation function (Nieuwkoop et al. 1987). A mutation in this locus resulted in a Nod^- phenotype on siratro, but retained a Nod^+ phenotype on soybean.

The function of the hsn genes is unknown, although there are some interesting clues. A protein sequence of R. leguminosarum nodF gene product deduced from the DNA sequence was shown to be similar to the acyl-carrier protein from Escherichia coli and barley, especially around the pantothenate-binding region (Shearman et al. 1986). Most of the amino acids conserved between nodF of R. leguminosarum and the acyl carrier proteins of E. coli and barley were also found to be conserved with the nodF products of R. meliloti (Debelle and Sharma 1986) and R. trifolii (Schofield and Watson 1986). The predicted isoelectric points of the nodF products and acyl-carrier proteins were found to range from 4.1 to 4.3 (Debelle and Sharma 1986), supporting the hypothesis that the nodF gene product could act as an acyl carrier protein. No significant homology between the predicted sequence of nodE product and other known proteins was reported. Interestingly, however, the nodE products of R. leguminosarum and R. meliloti were found to share 86% sequence homology which is the highest sequence

conservation of the nod genes analyzed in different Rhizobium/Bradyrhizobium species (Debelle and Sharma 1986), suggesting that only minor differences in protein sequences could determine the host-range of Rhizobium species. In contrast to the nodFE genes, the nodG gene was identified only in R. meliloti. When the amino acid sequence of the nodG gene product of R. meliloti was compared to the protein sequence data bank, one protein, the ribitol dehydrogenase of Klebsiella aerogenes, was found to share a significant homology (Debelle and Sharma 1986). Based on this homology, it was hypothesized that during the infection process the legume host provided ribitol as a carbon source for the invading bacteria and that the ability to catabolize this compound facilitated bacterial growth and infectivity (Debelle and Sharma 1986).

3. Regulation of Nod Genes

The nodulation process appears to require interaction and regulation of bacterial and plant genes. Studies on the regulation of bacterial genes involved in nodulation have been aided by placing a reporter gene (for example, beta-galactosidase) under the control of the promoter from cloned nod genes. Mulligan and Long (1985) showed that a nodC::lacZ fusion in R. meliloti was induced to form beta-galactosidase only in the presence of alfalfa seed and root extracts. A functional nodD gene was essential for induction, suggesting that nodD is a positive regulatory

gene. The nodD gene transcript is expressed constitutively (Mulligan and Long 1985). Similar findings have been reported for the nodABC genes of R. trifolii (Innes et al. 1985), R. leguminosarum (Rossen et al. 1985), and B. japonicum (Kosslak et al. 1987; Banfalvi et al. submitted). In R. leguminosarum, expression of the nodD gene was found to be subject to autoregulation as the nodD::lacZ expression was repressed in strains containing an multicopy cloned nodD gene (Rossen et al. 1985). Shearman et al (1986) showed that a region of the nodD protein shared some homology with the region of the araC protein. The araC protein interacts with DNA regulatory sequences that control the autoregulation of araC and in the presence of arabinose, activates other genes involved in the catabolism of this sugar (Lee et al. 1981). Recently, comparisons of the deduced amino acid sequence of the nodD protein of R. fredii with those of known DNA-binding regulatory proteins showed that it was similar to the product of lysR of Escherichia coli, another DNA-binding regulatory protein (Applebaum et al 1988).

The nod gene inducers present in seed and root extracts were identified as various flavonoids. Each Rhizobium was found to be optimally induced by a unique, substituted flavonoid such as luteolin for alfalfa-R. meliloti (Peters et al. 1986), 7,4 dihydroxy flavone for clover-R. trifolii (Redmond et al. 1986), and naringenin for peas and

Vicia sativa-R. leguminosarum (Firmin et al. 1986; Zaat et al. 1987). These molecules were shown to be remarkably potent as nod gene induction was seen at concentrations as low as 200 nM (Firmin et al. 1986). Synthesis of the nod gene inducers by plants takes place via the general phenyl propanoid metabolic pathway. This pathway appears to be intimately involved in plant-microbe interactions. For example, in addition to the inducers of nod genes in rhizobia, acetosyringone, produced by a branch metabolic pathway leading to lignin biosynthesis, was shown to be an inducer of vir (virulence) genes in Agrobacterium tumefaciens (Stachel et al. 1986). The expression of vir genes is essential for the establishment of crown gall disease by A. tumefaciens. The phenyl propanoid pathway also leads to the synthesis of flavonoid-derived phytoalexins in many plants (reviewed in Smith and Banks 1986). Phytoalexins are antimicrobial chemicals produced by plants in response to pathogen attack.

In Bradyrhizobium species, an analogous but significantly different regulatory mechanism of the common nod genes was observed. As in Rhizobium species, the expression of B. japonicum nodABC genes was induced in response to root extract of its host soybean (Kosslak et al. 1987; Banfalvi et al. submitted). Also, the activation of nodABC::lacZ fusions by inducing molecules showed a concentration dependence similar to that reported for the

induction of Rhizobium nod genes with flavones. However, a significant difference between Rhizobium and Bradyrhizobium is that the nodD gene is inducible in B. japonicum, but not in Rhizobium. This result led us to postulate that an additional, divergent nod box-like sequence in front of the nodD gene functions to allow induction of nodD (Banfalvi et al. submitted). An additional difference is that the soybean-derived inducer compounds for B. japonicum are isoflavones (primarily genistein and daidzein), rather than flavones.

In all Rhizobium and B. japonicum species examined, a conserved DNA sequence of about 40 nucleotides has been found 200 to 250 bp upstream of the translational start site of most of the known nod genes, and has been named the "nod box" (Rostas et al. 1986). For example, in R. meliloti, a copy of the nod box is present 5' of all the nod genes, with the exception of the nodD gene. The nod boxes have been proposed to be involved in the transcriptional regulation of adjacent nod genes. Deletion analysis experiments confirmed that the nod box was essential for nod gene induction (Rostas et al. 1986; Spaink et al. 1987a). The presence of nod box sequences 5' of nod and hsn genes strongly suggests coordinated regulation of these genes. The activation of nod and hsn genes was postulated to be mediated by binding of the nodD gene product to the nod box thereby activating transcription of adjacent nod genes (perhaps by binding of

RNA polymerase to the nod or hsn promoters) (Rostas et al. 1986; Spaink et al. 1987a). Indeed, in R. leguminosarum, using the gel retardation technique, it has been shown that a complex between protein and the DNA fragment containing the nod box is formed. This result only occurred when the nod box sequence was exposed to cell extracts of strains with a functional nodD gene (Hong et al. 1987). Interestingly, the nodD gene product appeared to bind to the regulatory DNA even in the absence of inducer compounds because the patterns of DNA-protein complex formation were the same as those obtained with cell extracts that had not been exposed to inducer (Hong et al. 1987). At a recent meeting, similar results with R. meliloti were presented indicating 1) antibody directed against the nodD protein removed the specific DNA binding activity from nodD protein-rich extracts, and 2) a synthetic oligonucleotide identical to the 3' half of the nod box blocked the formation of the electrophoretically retarded complexes (Fischer et al. 1988. Proceedings of 11th Annual Symposium on Plant Physiology, University of California, in press).

Initial observations indicated that the nodD genes of different rhizobia were functionally equivalent; for example, when nodD of R. meliloti was introduced into a nodD mutant of R. trifolii, the failure of this mutant to nodulate clover was reversed (Fischer et al. 1985). However, the concept of nodD being a "common" nod gene has

been revised. It is now known that the specificity of response to different flavonoids is dependent on the nodD gene and in part, determines host range specificity (Horvath et al. 1987; Spaink et al. 1987b; Honma and Ausubel 1987; Gyogypal et al. 1988). For example, by using nodA-lacZ gene fusions of R. meliloti, it was shown that the nodD gene of the broad host range Rhizobium sp. MPIK3030 induces expression of these fusions by interacting with plant factors from siratro (Macroptilium atropurpureum, a host of MPIK3030) and from the non-host alfalfa (Medicago sativa), whereas the R. meliloti nodD genes only interact with alfalfa exudate (Horvath et al. 1987). In line with these results, the nodD1 gene of R. meliloti was unable to suppress the nodD1 mutation of the broad host range Rhizobium sp. MPIK3030 (analogous to NGR234), while introduction of the nodD1 gene of MPIK3030 into R. meliloti conferred the ability to nodulate Macroptilium atropurpureum (a host of MPIK3030) which is normally not infected by R. meliloti (Horvath et al. 1987). Presumably, Macroptilium root exudate contains an inducer that is recognized by the product of the nodD gene of strain MPIK3030 but not by the nodD gene product of R. meliloti. Furthermore, by constructing a chimeric nodD gene containing the 5' region of the MPIK3030 nodD and the 3' section of the R. meliloti nodD, the carboxy-terminal part of NodD was shown to determine flavonoid-specificity, which is in line with the

previous finding that the 3' ends of nodD1 and nodD2 of R. meliloti are more diverged than their 5' ends (Gottfert et al. 1986). Recently, by testing nodulation phenotype on different host plants of different nodD-specific mutants (nodD1, nodD2, nodD3, or nodD1,2 double mutant), it was shown that there are functional differences between the nodD copies, suggesting that the three nodD genes within R. meliloti are allelic forms with divergent flavononid specificity, rather than equivalent copies of a gene widespread among rhizobia (Gyorgypal et al. 1988). Further, the contribution of the three nodD copies of R. meliloti to nodulation was found to depend on the nodABC promoter-inducing substances present in the exudates of the actual host plants (Gyorgypal et al. 1988). That is, the transcription of nodABC is induced to differing degrees by a spectrum of compounds if different nodD genes are present. In summary, it is now clear that the nodD gene product interacts with certain plant inducer compounds by its carboxy-terminus part to transmit the plants' signal to bacteria. However, it remains to be shown formally if inducer molecules bind directly to the nodD gene product, or whether the plant factor has a more indirect effect on transcription. For example, it cannot be ruled out that the phenolic compounds act at a common membrane site and that a secondary signal is responsible for the induction of the nod genes either in combination with the nodD product, or

perhaps by activating the nodD product itself. The membrane-located virA gene product of A. tumefaciens has been reported to act in this way, responding to the presence of acetosyringone and similar phenolic compounds, by activating the virG product (Winans et al. 1986). Knowing that the nodD gene product binds to the regulatory DNA even in the absence of inducer, Hong et al. (1987) proposed that the nodD protein-inducer interaction causes a change in the conformation of the bound nodD protein such that it opens up the nod gene promoters and so allows their transcription when inducers are present.

As mentioned above, different rhizobia have specificities for certain phenolic compounds for optimal induction of nod genes. Moreover, the nod genes are not only activated by plant-specific compounds; other plant compounds antagonize their induction; for example, isoflavones genistein and daidzein which were found to be the best inducers of gene expression in B. japonicum were potent antagonists of nod gene induction in R. leguminosarum (Firmin et al. 1986). Therefore, it seems likely that, during the normal nodulation process, the regulation of nod genes is subject both to activation and to antagonism of activation by different plant-specified molecules. Indeed, in clover, it was shown that the coumarin umbelliferone and the isoflavonoid formononetin, both of which are present in the root exudate of this legume, were effective in

antagonizing the induction of nod genes of R. trifolii (Djordjevic et al. 1987). By growing clover seedlings on agar containing an indicator for beta-galactosidase and seeded with a strain of R. trifolii containing a nodC-lacZ fusion, it was noted that the beta-galactosidase activity of the Rhizobium cells was not uniform throughout the root area. Inhibitory compounds were released from the root tip of clover while stimulatory compounds were detected at or near the zone of emerging root hairs (Djordjevic et al. 1987). This finding is consistent with the biology of infectivity shown for several legumes (Bhuvaneswari et al. 1980; Halverson and Stacey 1986). The root hairs of many legume plants are transiently infectible (Bhuvaneswari et al. 1980) and so the rapid response by the bacterium to the presence of the stimulatory compounds is probably a necessary adaption, maximizing the possibility of infection of the highly-infectible, preemergent root hair cells.

B. Polysaccharides in Nodulation

Cell surface polysaccharides of rhizobia, particularly lipopolysaccharides (LPS), exopolysaccharides (EPS), and low molecular weight beta-2-glucans, have been postulated to be involved in the infection and nodulation of legumes for a number of years (for example, Sanders et al. 1978; Abe et al. 1982; Bhagwat and Thomas 1983). The importance of these cell surface polysaccharides in nodulation has been

supported mainly through investigation of mutant strains of rhizobia defective in nodulation and cell surface polysaccharides. (see below)

1. Lipopolysaccharides (LPS)

The involvement of LPS in nodulation was first suggested by early work on bacteriophage mutants of Rhizobium (Kleczkowska 1950). Maier and Brill (1978) reported that two Nod⁻ mutants of B. japonicum lacked a surface antigen that was present on the wild-type parental strain. This surface antigen was associated with the O-antigen portion of the LPS. More recently, when the cell surface polysaccharides of wild-type and a nonnodulating mutant of B. japonicum were compared, the CPS and EPS did not differ in their sugar composition but the LPS isolated from two strains differed considerably (Puvanesarajah et al. 1987). In the latter study, as the nonnodulating mutant strain lacked a complete LPS moiety, it was suggested that a complete LPS structure might be necessary for nodulation. However, since the mutants in the above studies did not have defined mutations, it is difficult to say whether or not these data imply a role for LPS in nodulation.

Noel et al. (1986) reported that two Tn5-induced R. phaseoi mutants having altered LPS induced nodule structures with abnormal infection thread that ceased development within the root hair. The nodulation defects in the two mutants were found to be solely due to the single Tn5 insert

because revertants restored both normal LPS structure and wild-type nodulation. Recently, chemical and immunological studies on one of the above two Tn5-induced mutants of R. phaseoli showed that the defect in nodule development was correlated with the missing antigenic O-chain of the mutant's LPS (Carlson et al. 1987), which was consistent with the previous results of Maier and Brill (1978). Thus, at least in B. japonicum and R. phaseoli, O-antigen portion of LPS appears to be a necessary factor in nodule development.

2. Exopolysaccharides (EPS)

In Rhizobium species, EPS has been implicated as an important factor for successful nodulation. By screening with the fluorescent stain Calcofluor which binds to beta-linked polysaccharides, Leigh et al. (1985) isolated a number of Tn5-induced R. meliloti mutants that were deficient in the production of EPS (Exo⁻). These mutants had non-fluorescent colony phenotype due to their lack of EPS, and thereby formed ineffective (non-nitrogen fixing) nodules on alfalfa. Microscopic studies on these pseudonodules revealed that the nodulation step associated with the presence of EPS appeared to be invasion rather than the formation of the nodules (Finan et al. 1985). These mutants did not curl root hairs or form infection threads or bacteroids, but they appeared to penetrate the epidermis directly. Bacteria were found only in the intercellular

spaces. Leigh et al (1985) classified R. meliloti Exo⁻ mutants into six distinct groups (A through F) on the basis of complementation analysis with recombinant plasmids and sensitivities to different bacteriophages. The exoABE and F loci were found to map to a second megaplasmid distinct from the Sym plasmid (Finan et al. 1986). The exoC and D loci were mapped to the chromosome (Hynes et al. 1986). Recently, however, ExoE⁻ mutants were demonstrated to carry deletions in the exoA-exoB loci of the second megaplasmid (Finan 1988). Thus, in R. meliloti, genes required for effective nodulation are located on more than one megaplasmid.

A similar nodulation phenotype has been reported for specific mutations in other species of Rhizobium. A number of transposon Tn5-induced Exo⁻ mutants of R. trifolii (Chakravorty et al. 1982) and strain NGR234 (Chen et al. 1985) were still able to infect host legumes but were unable to initiate nitrogen-fixing nodules. Examination of nodules induced by the Exo⁻ mutants of R. trifolii and strain NGR234 by electron microscopy showed that nodules were poorly developed, contained fewer dividing cells and intracellular bacteria, and gave little or no indication of the presence of bacteroids (Chen et al. 1985). Recently, it was demonstrated for R. trifolii and Rhizobium sp. NGR234 that a Fix⁺ phenotype could be restored by the addition of EPS or oligosaccharide from the appropriate parent strain to a

Exo⁻Fix⁻ mutant just prior to inoculation of the plant (Djordjevic et al. 1987). The defective nodulation properties were corrected only by the addition of homologous EPS or oligosacchride, indicating that these compounds have more than a passive role.

In R. phaseoli, the symbiotic plasmid was found to contain two genes that regulate EPS synthesis and thereby affect nodulation phenotype (Borthakur et al. 1985). One of the genes, termed as psi (polysaccharides inhibition), was located close to the common nod genes. When cloned in multicopy vectors, it caused inhibition of EPS synthesis in R. phaseoli and abolished nodulation ability. psi::Tn5 mutant strains induced non-fixing nodules on Phasolus beans. Based on these results, Borthakur et al. (1985) has postulated that psi is normally expressed only in the nodule and serves to repress genes (e.g. those for EPS synthesis) which are normally expressed in free-living cultures but not in the bacteroids. The other gene, termed as psr (polysaccharide restriction), was identified and located 13 kb from psi, which when introduced, conferred an EPS⁺ phenotype to R. phaseoli strains containing multicopy psi (Borthakur et al. 1987). The molecular nature of the interaction between these two genes is unknown, but by constructing psi::lacZ fusions it was shown that psr repressed transcription of psi (Borthakur et al. 1987).

3. Beta-2-glucan

One of the characteristics that relates Agrobacterium and Rhizobium/Bradyrhizobium is their ability to synthesize low-molecular-weight beta-2-glucans (Dedonder et al. 1964; Puvanesarajah et al. 1985). In Agrobacterium, the chv (chromosomal virulence) loci were found to be essential for oncogenic transformation of dicotyledonous plants and for attachment to plant cells (Douglas et al. 1985). It has been shown that mutations in the chv region of A. tumefaciens result in the loss of synthesis of a beta-2-glucan (Puvanesarajah et al. 1986). Mutants having a nodulation phenotype similar to the one described above for Exo^- mutants were isolated in R. meliloti (Dylan et al. 1986) by mutagenizing a DNA fragment which hybridized to the chv loci of A. tumefaciens; this new locus was termed ndv (nodule development) locus. Phenotypic studies on a mutant of R. meliloti analogous to the one isolated by Dylan et al. (1986) demonstrated that the mutant did not form beta-2-glucan, it was not motile because it lacked flagella, and it formed ineffective pseudonodules (Geremia et al. 1987), indicating a highly pleotropic effect of the mutation. In both Agrobacterium and Rhizobium, beta-2-glucan was found to be synthesized through the formation of a protein-bound intermediate (Zorrengruieta et al. 1985). More recently, by radiolabeling the protein with UDP[^{14}C]-glucose, the intermediate protein was found to be a 235 kd protein

(Zorrengrueta and Ugalde 1986) which is likely the chvB gene product (Nester, personal communication). Both A. tumefaciens Chv⁻ and R. meliloti Ndv⁻ mutants described above lacked a 235 kd inner membrane protein which acted as an intermediate in beta-2-glucan synthesis. Although no specific roles for these glucans in the infection process have been reported, it appears that the glucan is essential for the formation of effective nitrogen-fixing nodules, at least in R. meliloti. Recently, Miller et al. (1986) postulated that in A. tumefaciens the beta-2-glucan is crucial to maintaining the appropriate osmotic pressure within the periplasmic space. This is an attractive hypothesis because the lack of beta-2-glucan, as described above, has several pleiotropic effects which could be explained by a collapse of the periplasmic space.

CHAPTER II

MATERIALS AND METHODS

A. Bacterial Culture Media and Growth Conditions

B. japonicum strains were grown at 30 C on AMA medium (Wacek and Brill 1976), RDM medium (Bishop et al. 1976), YS medium (RDM medium ommiting gluconate andlutamate) or defined medium (Mort and Bauer 1982). To test for an auxotrophic phenotype, B. japonicum strains were inoculated in the defined medium as described by Davis et al. (1980). Escherichia coli strains were grown at 37 C in M9 minimal medium, LB medium, or YT medium (Miller 1972).

Medium formulations were as follows (unless indicated otherwise, all reagents are from Sigma Chemical Co., St. Louis, MO): AMA (per liter of deionized water); 10 g mannitol, 1 g yeast extract (Difco Laboratories, Detroit, MI), 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g NaCl , 0.5 g K_2HPO_4 , 0.005 g FeCl_2 or 0.0011 g FeSO_4 , pH 7.5. RDM (per liter of deionized water); 5 g Na-gluconate, 1 g Na-glutamate, 1 g yeast extract, 0.12 g KH_2PO_4 , 0.12 g MgSO_4 , 1 ml trace element stock solution (per liter of deionized water); 5 g CaCl_2 , 0.145 g Boric acid, 0.125 g FeSO_4 , 0.075 g CuSO_4 , 0.0043 g MnSO_4 , 0.108 g ZnSO_4 , 0.125 g Na-molybdate, 7 g nitriloacetate, pH 7.5. M9 minimal medium (per liter); 6 g Na_2HPO_4 , 3 g KH_2PO_4 , 0.5 g NaCl , 1 g NH_4Cl , pH 7.0. After autoclaving, 10 ml of 0.01M CaCl_2 solution, 1 ml of a $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ stock solution

(20 g/100 ml), 10 ml of 20% glucose solution and 0.5 ml of a 1% thiamine-HCl solution were added. LB (Luria-Bertani) medium (per liter of deionized water); 10 g Tryptone (Difco), 5 g yeast extract, 10 g NaCl, pH 7.0. YT medium (per liter of deionized water); 8 g Tryptone, 5 g yeast extract, 5 g NaCl, pH 7.0. To make solid medium, 17.5 g of purified agar (Difco) was added per liter of broth.

Antibiotics were added to the medium to the final concentrations for B. japonicum, 100 ug/ml kanamycin, 200 ug/ml of streptomycin and 150 ug/ml tetracycline, and for E. coli, 50 ug/ml ampicillin, 15 ug/ml tetracycline and 50 ug/ml kanamycin. The bacterial strains, plasmids and bacteriophages used in this study are listed in Table II.

B. Total Genomic and Plasmid DNA Isolation

1. Large-scale Genomic DNA Isolation from B. japonicum

Total genomic DNA was isolated from B. japonicum as described (Russell et al. 1985). Cells from a culture in 500 ml of RDM medium were harvested after 4 to 5 days of growth, pelleted, washed in TE buffer (10 mM Tris hydrochloride, 0.5 mM EDTA; pH 7.4), and suspended in 10 ml of 0.1 M Tris hydrochloride (pH 7.4)-0.1 M EDTA-0.2 M NaCl. Chicken egg white lysozyme (100 mg; Sigma Chemical Co., St. Louis, MO) was added to the cell suspension, which was then incubated for 60 min at 37 C. After this period, 1 ml of 25% sodium dodecyl sulfate (SDS) was added, followed by the

TABLE II. Bacterial Strains, Plasmids and Bacteriophages Used in This Study

Strains	Plasmids	Phages	Relevant Characteristics	Reference or Source
<u>B. japonicum</u>				
USDA 110			Nod ⁺ Fix ⁺ wild-type	Kuykendall and Elkan (1976)
USDA 110 E1-8d1			Deletion mutant of USDA 110	H. Hennecke
USDA 46			Nod ⁺ Fix ⁺ wild-type	D. Kuykendall
USDA 76			Nod ⁺ Fix ⁺ wild-type	D. Kuykendall
USDA 122			Nod ⁺ Fix ⁺ wild-type	D. Kuykendall
USDA 123			Nod ⁺ Fix ⁺ wild-type	D. Kuykendall
USDA 6			Nod ⁺ Fix ⁺ wild-type	D. Kuykendall
AN6			Nod ⁺ Fix ⁺ wild-type	D. Kuykendall
61A101C			Nod ⁺ Fix ⁺ wild-type	Nitragin Co.
ML100			His ⁻ Nod ⁻ mutant of USDA 110	So et al. (1987)
ML112			His ⁻ Nod ⁻ mutant of USDA 110	So et al. (1987)
ML115			Nod ⁻ mutant of USDA 110	So et al. (1987)
ML116			His ⁻ Nod ⁻ mutant of USDA 110	So et al. (1987)
ML117			His ⁻ Nod ⁻ mutant of USDA 110	So et al. (1987)
ML118			Nod ⁻ mutant of USDA 110	So et al. (1987)
ML119			Nod ⁻ mutant of USDA 110	So et al. (1987)
ML120			Fix ⁻ mutant of 61A101C	So et al. (1987)
ML121			Fix ⁻ mutant of 61A101C	So et al. (1987)
ML124			Fix ⁻ mutant of 61A101C	So et al. (1987)
ML125			Fix ⁻ mutant of 61A101C	So et al. (1987)
ML126			Fix ⁻ mutant of 61A101C	So et al. (1987)
ML127			Nod ⁻ mutant of 61A101C	So et al. (1987)
ML128			Fix ⁻ mutant of 61A101C	So et al. (1987)
ML129			Nod ⁻ mutant of 61A101C	So et al. (1987)
ML142			Nod ⁻ mutant of 61A101C	So et al. (1987)
ML143			Fix ⁻ mutant of 61A101C	So et al. (1987)

TABLE II (continued)

Strains Plasmids Phages	Relevant Characteristics	Reference or Source
ML144	Nod ⁻ mutant of 61A101C	So et al. (1987)
ML145	Fix ⁻ mutant of 61A101C	So et al. (1987)
ML146	Fix ⁻ mutant of 61A101C	So et al. (1987)
ML147	Fix ⁻ mutant of 61A101C	So et al. (1987)
ML148	Fix ⁻ mutant of 61A101C	So et al. (1987)
ML149	Fix ⁻ mutant of 61A101C	So et al. (1987)
ML150	Fix ⁻ mutant of 61A101C	So et al. (1987)
ML151	Fix ⁻ mutant of 61A101C	So et al. (1987)
JS831	13.5 kb::Tn5 homogenote	This study
JS721	6.0 kb::Tn5 homogenote	This study
JS314	5.5 kb::Tn5 homogenote	This study
<u>Bradyrhizobium</u> sp. cowpea strain 32H1	Nod ⁺ Fix ⁺ wild-type	K. Scott
<u>B. parasponia</u>	Nod ⁺ Fix ⁺ wild-type	
<u>Bradyrhizobium</u> sp. peanut strain	Nod ⁺ Fix ⁺ wild-type	A. Kondorosi N. Brewin B. Rolfe B. Rolfe
<u>R. meliloti</u>	Nod ⁺ Fix ⁺ wild-type	
<u>R. leguminosarum</u>	Nod ⁺ Fix ⁺ wild-type	
<u>R. trifolii</u>	Nod ⁺ Fix ⁺ wild-type	
<u>Rhizobium</u> sp. NGR234	Nod ⁺ Fix ⁺ wild-type	
<u>R. fredii</u>	Nod ⁺ Fix ⁺ wild-type	H. Keyser H. Keyser
USDA 193	Nod ⁺ Fix ⁺ wild-type	
USDA 194	Nod ⁺ Fix ⁺ wild-type	

TABLE II (continued)

Strains Plasmids Phages	Relevant Characteristics	Reference or Source
<u>Agrobacterium tumefaciens</u>		
A6	Virulent, wild-type	E. Nester
A136	Avirulent, Ti-plasmid cured	E. Nester
<u>Escherichia coli</u>		
HB101	F ⁻ <u>hsdR</u> <u>hsdM</u> <u>recA</u> <u>thi-1</u> <u>leuB6</u> <u>proA2</u> <u>lacZ4</u> <u>supE44</u>	Davis et al. (1980)
DH1	F ⁻ <u>recA</u> <u>endA1</u> <u>gyrA96</u> <u>thi-1</u> <u>hsdR17</u> <u>supE44</u> <u>lambda</u>	Manniatis et al. (1982)
JM107	<u>endA1</u> <u>gyrA96</u> <u>thi</u> <u>hsdR17</u> <u>lac</u> <u>proAB/F'</u> <u>proAB</u> <u>lacI^q</u>	Yanisch-Perron et al. (1985)
<u>Plasmids</u>		
PRK2073	<u>tra</u> ⁺ (RK2:ColE1)	Leong et al. (1982)
PSUP1011	<u>Km^rNm^r</u> (by Tn5) <u>Cm^r</u> <u>oriT</u> (RP4)	Simon et al. (1983)
PSUP101	<u>Cm^r</u> <u>oriT</u> (RP4)	Simon et al. (1983)
PHC79	<u>Tc^rAp^r</u> cosmid	Hohn (1979)
ptac11	Ap ^r expression vector	Amann et al. (1983)
PRj676	pBR322 clone of USDA 110 <u>nifK</u> genes and adjacent region	Hennecke (1981)
PRjUT25	PHC79 clone of USDA 110 <u>nifH</u> gene and adjacent region	Schell and Stacey (unpublished)
PZB15	pBR329::3.2 kb <u>EcoRI</u> fragment containing the <u>nodIJ</u> genes of <u>R. leguminosarum</u>	Nieuwkoop et al. (1987)

TABLE II (continued)

Strains Plasmids Phages	Relevant Characteristics	Reference or Source
PPP346	Cosmid clone of 24 kb fragment of <u>R. meliloti</u> Sym plasmid containing common <u>nod</u> and <u>hsn</u> genes	Putnok and Kondorosi (1987)
PRJUT10, 14, 24	pHC79 clones of USDA 110 common <u>nod</u> genes region	Russell et al. (1985)
PJS51, 56, 72, 76, 712, 713, 718, 724, 727	pHC79 clones containing 11.7 kb <u>EcoRI</u> <u>nod</u> :: <u>Tn5</u> fragment	This study
PJS81, 82, 83, 84, 85, 86, 88, 91, 92, 93, 94	pHC79 clones containing 19.2 kb <u>EcoRI</u> <u>nod</u> :: <u>Tn5</u> fragment	This study
PJS34, 35, 36, 313, 314, 315, 316	pHC79 clones containing 11.2 kb <u>EcoRI</u> <u>nod</u> :: <u>Tn5</u> fragment	This study
PBJ502, 507, 701, 702, 703, 704, 705	pHC79 wild-type clones corresponding to the 11.7 kb <u>EcoRI</u> <u>nod</u> :: <u>Tn5</u> fragment	This study
PBJ801, 803, 804, 805	pHC79 wild-type clones corresponding to the 19.2 kb <u>EcoRI</u> <u>nod</u> :: <u>Tn5</u> fragment	This study
PBJ101	pHC79 wild-type clone corresponding to the 11.2 kb <u>EcoRI</u> <u>nod</u> :: <u>Tn5</u> fragment	This study
PHK8315, 8382, 818, 817, 822, 87, 81	pHC79 subclones of pJS83	This study
PHK759, 727, 710, 72	pHC79 subclones of pJS718	This study
PHK51	pHC79 clone of 11.2 kb <u>EcoRI</u> fragment of pJS51	This study
PHK32, 34	ptac11 clones of 5.5 kb <u>EcoRI</u> fragment of pBJ101 in both orientations	This study

TABLE II (continued)

Strains Plasmids Phages	Relevant Characteristics	Reference or Source
<u>Bacteriophages</u>		
M13mp18, 19	M13 phage vectors	Bethesda Research Laboratories
fFR <u>nodC</u>	M13 mp8::0.7 kb <u>nodC</u> fragment of R. <u>meliloti</u>	F. Rodriguez-Quinones
fMG <u>nodD</u>	M13 mp18::275 bp <u>nodD</u> fragment of R. <u>meliloti</u>	M. Gottfert
fBH <u>hsnA</u>	M13 mp18::0.5 kb <u>hsnA</u> fragment of R. <u>meliloti</u>	B. Horvath
fBH <u>hsnB</u>	M13 mp18::0.5 kb <u>hsnB</u> fragment of R. <u>meliloti</u>	B. Horvath
fBH <u>hsnC</u>	M13 mp18::1.6 kb <u>hsnC</u> fragment of R. <u>meliloti</u>	B. Horvath
fFR <u>hsnD</u>	M13 mp18::320 bp <u>hsnD</u> fragment of R. <u>meliloti</u>	F. Rodriguez-Quinones

Abbreviations: Ap, ampicillin; Tc, tetracycline; Cm, chloramphenicol; Km, kanamycin;
Nm, neomycin

addition of 0.5 ml of predigested proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, IN). Proteinase K was predigested for 15 min at 37 C in 0.01 M Tris hydrochloride (pH 8.0)-5 mM CaCl_2 . The cell suspension containing SDS and proteinase K was incubated at 65 C for 1 hr. The cell lysate obtained by this method was then mixed directly with CsCl to give a refractive index of 1.4. This mixture was centrifuged in a Ti60 fixed-angle rotor (Beckman Instruments, Inc., Palo Alto, Calif.) at 40,000 rpm for 48 hr at 20 C. The high-molecular weight DNA was obtained by puncturing the side of the tube with a 16-gauge needle and collecting those fractions showing high viscosity.

2. Small-scale Genomic DNA Isolation from *B. japonicum*

Genomic DNA was isolated in small-scale essentially as described by So et al. (1987). Cells were grown to stationary phase in 10 ml RDM broth, pelleted and washed with 10 ml TE buffer containing 100 mM NaCl and 0.1% Sarcosyl. After harvesting, cells were resuspended in 5 ml TE buffer containing 1% SDS and 0.5 mg of proteinase K. The mixture was then incubated for 2 hr at 37 C. The lysate was extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), then extracted 4-times with an equal volume of ether. DNA was precipitated with 2.5 volumes of ethanol at -20 C overnight and pelleted by centrifugation in an SW 41 rotor at 15,000 rpm for 30 min. The resulting pellet was suspended in 50 μl TE buffer.

3. Large-scale Plasmid DNA Isolation

CsCl/ethidium bromide bouyant density gradient ultracentrifugation was employed as described (Manniatitis et al. 1982). E. coli cells harboring plasmid were grown in 500 ml of LB medium at 37 C until O.D.₆₀₀ = 0.4, at which time chloramphenicol was added to the culture to the final concentration of 170 ug/ml. The culture was further incubated with vigorous shaking for 12 to 16 hr. The cells were harvested by centrifugation at 8,000 rpm for 10 min in a Beckman JA-17 rotor, resuspended in 10 ml of TE buffer (pH = 7.5). The cells were pelleted as above in a 30 ml Corex tube which had been baked overnight in a dry oven at 400 C. The cell pellet was resuspended in 13.5 ml of TE buffer containing 15% sucrose (wt/vol) plus 13.5 mg lysozyme, and incubated at 37 C for 1 hr. After this period, 14 ml of TE buffer containing 0.1% (vol/vol) Triton X-100 (Sigma) was added to the solution and gently mixed prior to incubation at room temperature for 30 min. The mixture was centrifuged at 15,000 rpm for 1 hr with a Beckman JA-17 rotor. The supernatant was poured into a glass 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. Cesium chloride (26.13 g) was added to the solution and completely dissolved. Ethidium bromide (13.9 mg) was added to the mixture, which was subsequently poured into a Quick-Seal tube (Beckman Inc., Palo Alto, CA, 25 x 89 mm).

The mixture in the tube was then centrifuged at 35,000 rpm for 48 hr in a Ti60 rotor at 20 C. Plasmid DNA was viewed with a long-range wavelength ultraviolet lamp (UVP Inc., San Gabriel, CA) and collected by puncturing the side of tube with a 16 gauge needle. Ethidium bromide was removed by extraction with an equal volume of sec-butanol five times. The solution was diluted 2-fold with TE buffer and the plasmid DNA was precipitated with 2.5 volumes of ethanol overnight. The DNA was collected by centrifugation at 15,000 rpm for 20 min in an SW41 rotor.

4. Small-scale Plasmid DNA Isolation

Plasmid DNA was isolated in small-scale essentially as described by Manniatis et al. (1982). Three milliliters of overnight culture in LB broth was centrifuged in an 1.5 ml Eppendorf tube for 2 min. The cell pellet was resuspended in 100 ul of Solution I (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA pH 7.5, 5 mg/ml lysozyme) and incubated after vortexing for 5 min at room temperature. Two hundred microliters of Solution II (0.2 N NaOH with 1% SDS in deionized water) was added to the tube, which was then rocked sharply and placed on ice for 5 min. After this period, 150 ul of precooled Solution III (3 M K-acetate, pH 5.6) was added to the tube, mixed briefly and incubated on ice for another 5 min. The mixture was centrifuged for 5 min in a microfuge. The supernatant was transferred to an 1.5 ml Eppendorf tube, extracted twice with a mixture of

phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform, and precipitated with a 2.5 volumes of ethanol at room temperature for 5 min. Plasmid DNA was pelleted by centrifugation in a microfuge for 5 min and resuspended in 50 ul of 0.1X TE buffer containing 100 ng of ribonuclease A.

C. Standard DNA Manipulation

1. Restriction Endonuclease Digestion and Agarose Gel Electrophoresis of DNA

Restriction digestions of DNA samples were carried out with commercially obtained restriction endonucleases [Bethesda Research Laboratories (BRL), Gaithersburg, MD] as recommended by the manufacturer, and DNA was electrophoresed in horizontal agarose (SeaKem, FMC Corp., Rockland, ME) slab gels. Sizes of DNA fragments were estimated using HindIII-cut bacteriophage lambda DNA as size markers. DNAs in gels were stained with ethidium bromide for 20 min and visualized under ultraviolet light (Fotodyne Foto System, New Berlin, WI). Photographs of these gels were taken using Polaroid type 55 positive/negative film or type 667 high speed film (Polaroid Corp., Cambridge, MA) and processed as recommended by the manufacturer.

2. Southern Blot Hybridization

For hybridization, gels treated sequentially in 0.2 M HCl for 10 min, denaturation solution (1.5 M NaCl and 0.5 M

NaOH) for 1 hr and neutralizing solution (1 M Tris-HCl, pH 8.0 and 1.5 M NaCl) for 1 hr were blotted onto nitrocellulose filters (Schleicher & Schuell Inc., Keene, NH) in 10X SSC (1X SSC is 0.15 M NaCl plus 0.015 M sodium citrate) as described by Southern (1975). The nitrocellulose blots were then washed briefly in 2X SSC and vacuum dried for 2 hr at 80 C. The blots were prehybridized for at least 4 hr at 37 C in 3X SSC containing 50% formamide, 0.2% Ficoll, 0.2% bovine serum albumin, 0.2% polyvinylpyrrolidone and 50 ug of sheared and denatured salmon sperm DNA per ml. They were then hybridized for 16 hr at 37 C in the same solution with a labeled probe (see section 3). For high stringency hybridization, the filters were washed in 0.1X SSC plus 0.1% SDS(wt/vol) at 65 C. For low stringency, 2X SSC solution containing 0.1% SDS was used for washing at 37 C.

3. Preparation of Labeled Probes and Autoradiography

Radioactively labeled probes were prepared by nick-translation with ^{32}P -dCTP (3,000 Ci/mmol; New England Nuclear, Boston, MA) to a specific activity of approximately 5×10^7 cpm/ug of DNA (Rigby et al. 1976) using a nick translation kit provided from BRL. The single strand specific probes were labeled as described by Sanger et al. (1977) using the Klenow fragment of DNA polymerase I, ^{32}P -dCTP, and a 17-mer primer DNA (BRL). Autoradiography was carried out with X-ray film (Kodak X-Omat AR, Eastman Kodak

Co., Rochester, NY) in the presence of 1 or 2 intensifying screens at -70 C for 1-5 days.

D. Construction of Genomic Library and Colony Hybridization

1. Library Construction

A clone bank was constructed by partially digesting approximately 50 ug of DNA with EcoRI. The digested DNA was size-fractionated by centrifugation (SW 41 rotor at 150,000 x g for 7.5 hr at 4 C) in a 10-30% continuous sucrose gradient in 1X TE buffer containing 0.7 M NaCl. From the gradients, 0.5 ml fractions were collected and analyzed by agarose gel electrophoresis after diluting with an equal volume of TE buffer. The fractions corresponding to 25-50 kb fragments were pooled, diluted with an equal volume of TE buffer and then precipitated with 2.5 volumes of ethanol at -20 C overnight. The DNA was pelleted by centrifugation (100,000 x g for 1 hr in an SW 41 rotor), resuspended in 0.2 ml TE buffer, ethanol precipitated and redissolved in 10 ul 0.1X TE buffer.

Vector DNA was prepared by digesting pHC79 DNA with EcoRI and then treating the DNA with calf intestinal alkaline phosphatase (Amersham, Arlington Heights, IL) as recommended by the manufacturer. B. japonicum DNA (1 ug) was mixed with vector DNA (2 ug) and allowed to ligate overnight at 4 C in a total volume of 12 ul with T4 ligase (New

England Bio-Labs, Beverly, MA). The DNA is then packaged into bacteriophage lambda as described by Hohn (1979) using a lambda in vitro packaging kit (Amersham). The packaged mixture was used to transfect E. coli strain DH1 that had been grown in the presence of 0.4% maltose. Transfectants were plated onto LB agar containing tetracycline and ampicillin, and incubated at 37 C overnight. Clones thus obtained were stored individually in microtiter dishes (Dynatech Laboratories Inc., Alexandria, VA) in the presence of 10% dimethyl sulfoxide at -70 C.

To isolate the Tn5 mutated DNA, colonies were replica plated from frozen microtiter dishes onto LB agar containing ampicillin, tetracycline (encoded by pHC79 vector DNA) and kanamycin (encoded by Tn5), and incubated at 37 C overnight. The appearing colonies were purified by streaking on LB agar plates containing ampicillin, tetracycline and kanamycin to obtain a single isolated colony. Plasmid DNA was isolated from the purified colonies as described above, and analyzed by restriction enzyme EcoRI digestion and agarose gel electrophoresis. To confirm that the cloned DNA carried Tn5, hybridization to Southern blotted genomic DNA was performed using Tn5 probe (i.e. pSUP1011 DNA) as described above. An EcoRI restriction map around the Tn5 insertion site was generated by examining the EcoRI restriction profiles of overlapping clones.

2. Colony Hybridization

For screening of the library, essentially the procedures described by Manniatis et al. (1982) were used. Colonies from frozen microtiter dishes were replica plated onto LB agar containing ampicillin and tetracycline (encoded by pHC79 vector DNA) and grown for 6 hr. Colonies were transferred to nitrocellulose filters (7.1 x 11 cm) and prepared for hybridization by treating sequentially in 0.5 M NaOH for 7 min, 1.0 M Tris hydrochloride (pH 7.5) for 2 min, 0.5 M Tris hydrochloride (pH 7.5) containing 1.5 M NaCl for 4 min, and 1X SSC containing proteinase K (2 mg/ml) for 15 min. Filters were then baked at 80 C for 2 hr prior to hybridization.

E. Bacterial Genetics Procedures

1. Homogenotization

Site-directed mutagenesis of the B. japonicum chromosome using Tn5 insertions on cloned fragments was performed essentially as described previously (Ruvkun and Ausubel 1981). The cloned mutated DNA was introduced into the wild-type strain of B. japonicum by tri-parental mating to induce double-crossover, homologous recombination as depicted in Figure 2. B. japonicum cells were grown in YS broth to early stationary phase and concentrated 5-fold by centrifugation and resuspension in YS broth. The E. coli helper strain (pRK2073) and E. coli donor strain were grown

Figure 2. Recombination scheme following conjugation of a mutated clone into the wild-type strain. Route A: double crossover resulting in the formation of marker exchange. Route B: single crossover resulting in the formation of a co-integrate. Routes C and D: co-integrate resolution resulting in the formation either wild-type (C and F) or mutant genomic structure (D and E). Homologous DNA is drawn as thick line; the thin line represent vector DNA. The wavy lines designate flanking chromosomal DNA. Tn5 insertions are symbolized by arrowheads. Adapted from Hahn and Hennecke (1984).

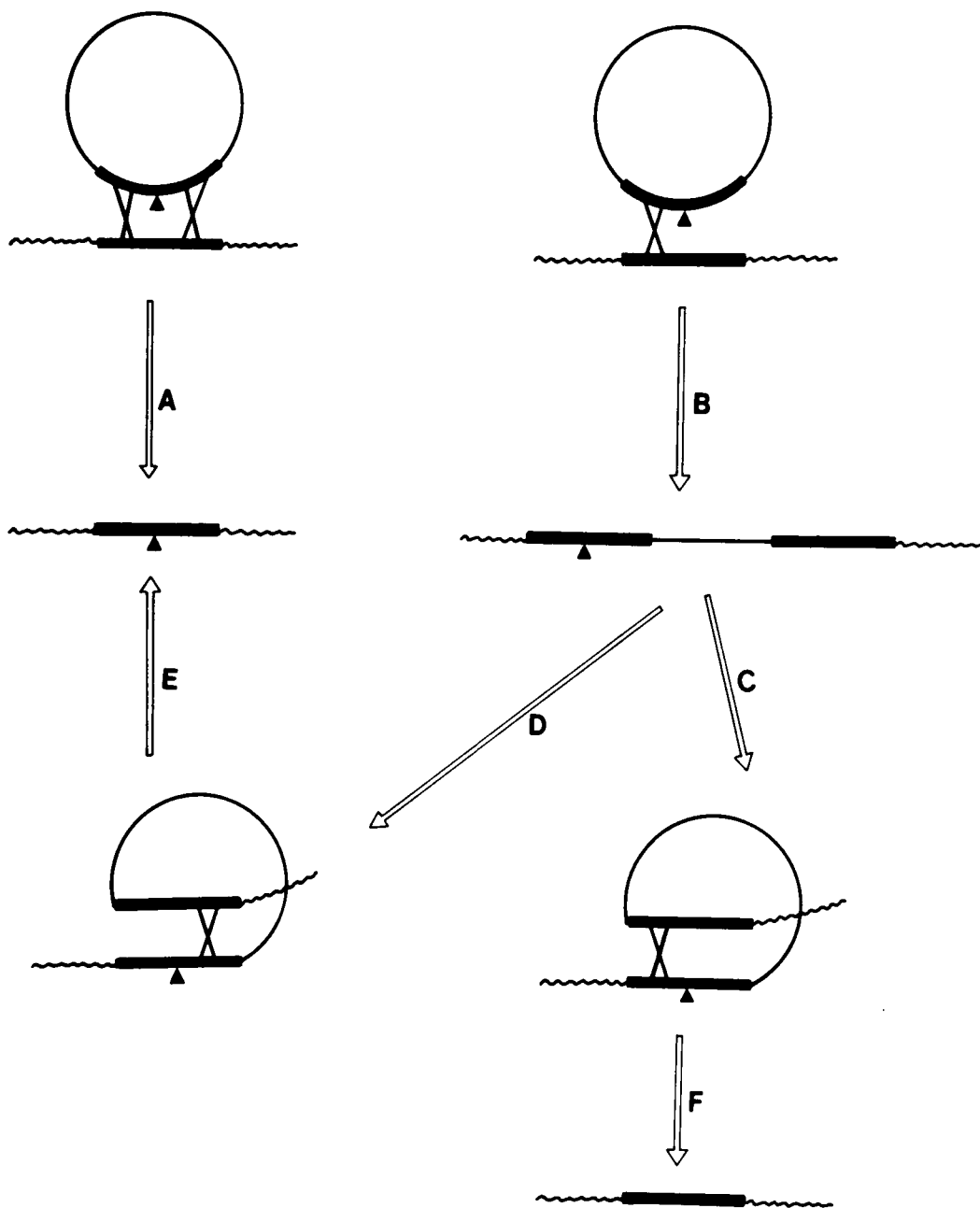


Figure 2

in 5 ml LB overnight. For mating, 0.2 ml B. japonicum (ca. 10^8 cells), 0.1 ml helper and 0.1 ml donor strain (ca. 10^8 cells) were mixed, plated on YS agar, and incubated at 30 C for 2-4 days. The resulting colonies were harvested with 3 ml YS-salts (YS broth without yeast extract) containing 0.01% (wt/vol) Tween 80. A 0.3 ml aliquot of the suspension was plated onto RDM containing kanamycin, streptomycin (both encoded by Tn₅) and chloramphenicol (to counter select E. coli) and incubated at 30 C for 8-10 days. To distinguish double crossover events (true replacements) from single-crossover events (co-integration), resulting kanamycin and streptomycin (Km/Sm)-resistant transconjugants were tested on RDM containing kanamycin, streptomycin and tetracycline (encoded by pH_{C79} vector DNA). After incubation at 30 C for 8-10 days, double-crossover transconjugants were identified by tetracycline sensitivity. True replacement events were further confirmed by hybridization of the putative homogenote's genomic DNA with Tn₅ or vector sequences.

2. Transformation of E. coli

Plasmid DNA was introduced into E. coli essentially as described by Manniatis et al. (1982) with minor modifications. Fifty milliliters of LB broth was inoculated with 0.5 ml of an overnight grown culture of an appropriate E. coli strain and incubated at 37 C with vigorous shaking to an O.D.₆₀₀ = 0.3. Cells were harvested by centrifugation at 8,000 rpm in a Beckman JA-17 rotor for

10 min. From this point all procedures were carried out at 4 C. Cell pellets were washed with 10 ml of precooled 20 mM NaCl solution, spun down as above, resuspended in 2 ml of 30 mM CaCl₂, and kept on ice for at least 20 min. DNA to be introduced (ca. 100 ng) was added to 200 ul of the competent cell suspension, and then kept on ice for at least 30 min. After heat-shock (42 C for 2 min), 1 ml of LB broth was added to the suspension, which was then incubated for 1 hr before plating on appropriate selection plates (0.1 to 0.2 ml per plate).

F. DNA Sequencing

The detailed procedures for the sequencing reaction, polyacrylamide gel electrophoreses and autoradiography described in several laboratory manuals were followed interchangeably (Bethesda Research Laboratories, Gaithersburg, MD; New England Biolabs Inc., Beverly, MA; United States Biochemical Corp., Cleveland, OH).

1. Preparation of M13 Phage Vector DNA

The modified M13 phage vector DNA (M13 mp18 and M13 mp19) was prepared as follows. One liter of YT medium was inoculated with a single colony of E. coli strain JM107 grown overnight on a M9 minimal medium. The culture was incubated at 37 C with vigorous shaking until O.D.₆₀₀ = 0.1. One milliliter of phage stock (ca. 10¹⁴ plaque-forming units) was added to the culture, which was further incubated

another 6 to 8 hr. The cells were harvested by centrifugation at 10,000 rpm in a Beckman JA-17 rotor for 10 min at 4 C. Replicative form DNA was isolated as regular plasmid DNA by isopycnic centrifugation in CsCl/ethidium bromide as described earlier.

2. Subcloning

The M13 vector DNA and the insert DNA to be sequenced were digested with appropriate restriction enzymes, and ligated with T4 DNA ligase (New England Biolabs, Beverly, MA) as recommended by the manufacturer. The ligation mixture was used to transform JM107 cells as described earlier. The transformants were mixed in 3 ml of YT soft agar (0.7% agar) with 0.2 ml of plating culture (JM107 culture at O.D.₆₀₀ = 1.0), 0.1 ml of 2% (wt/vol) X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactoside) in dimethylformamide and 0.02 ml of 100 mM IPTG (isopropyl-beta-D-thiogalactopyranoside). The mixture was then plated on a YT agar plate. After incubation at 37 C overnight, resulting colorless plaques were picked to isolate single-stranded template DNA for sequencing reactions.

3. Single-Stranded Template DNA Isolation

For single-stranded template DNA isolation, 10 ml of YT medium seeded with 0.1 ml of JM107 plating culture was inoculated with the recombinant M13 phages from a single, isolated, colorless plaque and incubated with vigorous

shaking at 37 C for 5 to 8 hr. The cells were pelleted by centrifugation at 10,000 rpm in a Beckman JA-17 rotor for 10 min at 4 C. The supernatant was poured off into a clean tube, to which 2 ml of 2.5 M NaCl solution containing 20% polyethylene glycol was added, and the mixture was placed on ice for 60 min. The phage particles were collected by centrifugation at 12,000 rpm in a Beckman JA-17 rotor for 10 min at 4 C. Single stranded DNA was extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1), and then precipitated with 2.5 volumes of ethanol at -20 C overnight. The DNA was pelleted by centrifugation in a microfuge for 5 min and suspended in 10 ul of 0.1X TE. The concentration of DNA was determined by running an agarose gel (0.7%) with 1 ul of sample along with the standard single-stranded DNA of known concentration (Bethesda Research Laboratories, Gaithersburg, MD).

4. Sequencing Reaction, Polyacrylamide Gel Electrophoresis and Autoradiography

Sequencing reactions were carried out using a commercially available sequencing kit (United Biochemical Corp., Cleveland, Ohio) and alpha ^{32}P -dATP as recommended by the manufacturer. Samples from the sequencing reaction were loaded on a standard 8% polyacrylamide/urea sequencing gel prepared as described (BRL Sequencing Manual). Following electrophoresis, the gel was lifted onto a Whatmann 3MM paper (Whatmann Inc., Clifton, NJ), wrapped with Saran wrap

(Network Service Co., Chicago, IL), and then exposed to X-ray film (Kodak X-Omat AR) overnight. Sequence data were analyzed on an IBM PC with Beckman Microgenie software.

G. Protein Isolation and Gel Electrophoreses

This was performed in conjunction with Mark Barbour, Department of Microbiology, University of Tennessee. Total proteins were isolated from B. japonicum cells grown in RDM to an O.D.₆₀₀ = 0.7, and cytoplasmic and membrane-associated fractions of the protein samples were separated by published procedures (Maagd and Lugtenburg 1986). Cells were harvested at 4 C by centrifugation with a Beckman JA-17 rotor at 10,000 rpm and were suspended in 10 ml of 50 mM Tris-HCl (pH = 8.5)-20% (wt/vol) sucrose-0.2 mM dithiothreitol supplemented with 0.2 mg of DNase and RNase per ml. Cells were disrupted by three passages through a French pressure cell at 25,000 lb/in². The broken cell suspension was treated with 0.2 mg of lysozyme per ml for 30 min, subsequently diluted with 2 volumes of 50 mM Tris-HCl (pH = 8.5), and centrifuged for 20 min at 900 x g to remove unbroken cells. The supernatant fluid was supplemented with KCl to a final concentration of 0.2M and centrifuged for 2 hr at 15,000 rpm in a Beckman JA-17 rotor. The supernatant fluid thus obtained was kept apart as the cytoplasmic fraction. The resulting pellet, containing the unseparated cell membranes was carefully suspended in 0.5 to 1.0 ml of

20% (wt/vol) sucrose-5 mM EDTA-0.2 mM dithiothreitol (pH = 7.5) and referred to as the membrane fraction. Proteins in the two fractions were analyzed by sodium dodesyl sulfate polyacrylamide gel electrophoreses (SDS-PAGE). SDS-PAGE was carried out through 1 mm thick slab gels of 10 or 15% acrylamide overlayed with a 4% stacking gel prepared by the procedures described by Laemmli (1970). Proteins were electrophoresed at room temperature and at a constant 8 mA until the tracking dye ran off. The gels were fixed in 40% ethanol containing 7% acidic acid, subsequently stained with Coomassie blue for 2 to 4 hrs, and destained with 40% ethanol containing 7% acidic acid.

H. Plant Infection Test

1. Bottle Assay

Seeds of Glycine max (purchased locally from Mayo Co. or obtained as a generous gift from Dr. R. Bernard, University of Illinois, Urbana, IL) were surface sterilized in 20% (vol/vol) chlorox for 10 min, rinsed twice with sterile distilled water, soaked in 0.01 N HCl for 10 min and rinsed with sterile distilled water five-times. Sterilized seeds were germinated on sterile, moist filter paper for 2-3 days in the dark at room temperature. Seedlings were transferred aseptically to sterile 35 ml serum vials containing vermiculite saturated with plant nutrient solution (Wacek and Brill 1976), and inoculated with 1 ml of an early stationary phase

culture of B. japonicum. A sterile, 18-oz Whirlpac bag (Nasco Inc., Oakville, Conn.) was placed over each vial. The vials were then maintained in a growth room under the following conditions: 26 C; light intensity of 510 microeinsteins $\times M^{-2} \times s^{-1}$; a 15-hr photoperiod. After 21 to 28 days, the vials were opened to visually determine the nodulation phenotype.

2. Acetylene Reduction Assay

To determine the nitrogen fixing ability of nodules formed by the inoculated B. japonicum, nodules were placed in a 35 ml vial, which was sealed with a serum stopper. Acetylene was injected to provide a final partial pressure of 0.1 atm. After incubation at room temperature for 1 to 5 hr, the acetylene reduction activity was determined by injecting the gas sample (1 ml) from the vial into a gas chromatograph and measuring the amount of ethylene formed. A Shimadzu GC-8A gas chromatograph (Shimadzu Corp., Kyoto, Japan), fitted with a stainless steel column packed with Porapak-R and a flame ionization detector, was used to separate and measure the hydrocarbons. The operating conditions were as follows: nitrogen carrier gas pressure, 6 kg/cm²; air pressure, 0.1 kg/cm²; hydrogen gas pressure, 0.7 kg/cm²; injector, detector, and column temperatures were maintained at 100 C, 100 C and 75 C, respectively.

3. Pouch Assay

To determine the time course of nodulation, seedlings germinated as above were transferred aseptically to sterile growth pouches (Northrup-King Seed Co., Minneapolis, Minn.) moistened with 10 ml of sterile plant nutrient solution. Seedlings were inoculated as above, and were watered as needed with half-strength plant nutrient solution. At regular time intervals, nodules were counted.

4. Root Hair Curling Assay

To examine root hair curling ability, seedlings inoculated, and subsequently grown for 1 to 4 days in a plastic growth pouch as described above, were immersed in phosphate-buffered saline (per liter of water: 0.43 g of KH_2PO_4 , 1.48 g of Na_2HPO_4 , and 7.2 g of NaCl) containing 0.05% (wt/vol) toluidine blue for 15 s and then rinsed with phosphate-buffered saline. After staining, roots were excised, sectioned longitudinally, and mounted in phosphate-buffered saline under glass cover slips. Roots were examined visually with bright-field optics for the presence of curled root hairs. At least 10 inoculated plants were examined for each B. japonicum strain.

5. Isolation of Bacteria from Nodules

Nodules were aseptically removed from the root, rinsed in 70% ethanol, washed twice in sterile distilled water, and immersed in 0.1% HgCl_2 in 0.06 N HCl for 5 min. Following

this pretreatment, nodules were washed 5 times in sterile distilled water and crushed in 0.5 ml of AMA broth. Nodule isolates were then streaked onto AMA agar and incubated at 30 C.

I. Exopolysaccharide Isolation and Analysis

1. Isolation

The wild-type strain 61A101C and the mutant strain JS314 of B. japonicum were grown on defined medium (Mort and Bauer 1980). The culture ($\text{O.D.}_{600} = 0.7$) was centrifuged at 10,000 rpm in a Beckman JA-17 rotor for 30 min at 4 C. The supernatant fluid thus obtained was filtered through a 0.45 μm Milipore filter (Millipore Corp., Bedford, MA), and concentrated to 1/10 original volume by evaporation under reduced pressure at 40 C using a Rotavapor R110 (Brinkmann Instruments Inc., Westbury, NY). The concentrated solution was exhaustively dialyzed against deionized water (5 changes, about 4 l of water per change) and lyophilized using a Labconco freeze drier 5 (Labconco Corp., Kansas City, MO). This polysaccharide fraction is referred to as EPS.

2. Chemical Analysis (performed by V. Puvanesarajah, Carbohydrate Research Center, University of Georgia)

The composition of the EPS isolated from wild type and mutant strain JS314 was analyzed by gas liquid chromatography (GLC) following appropriate derivatization of sugars as

described (Puvanesarajah et al. 1986). GLC was performed with a Perkin-Elmer Sigma 3B gas chromatograph with (i) a packed column of 3% SP2330 on 100/200 mesh Supelcoport (Supelco Inc., Bellefonte, PA) or (ii) a packed column of 3% SP2340 on 100/200-mesh Supelcoport. For the identification of neutral sugars, samples of EPS were treated with 2 N tri-fluoroacetic acid at 120 C for 2 hr, reduced with sodium borohydride, and analyzed by GLC as alditol acetates.

J. Light and Electron Microscopy

1. Sample Preparation (performed in conjunction with Dr. Evans Roth, Department of Zoology, University of Tennessee)

Bacterial samples. The wild-type strain 61A101C and mutant JS314 of B. japonicum were grown in a defined medium as described eariler. Drops of varying dilutions were placed on parlodion-coated copper grids to obtain a proper number of bacteria per grid, allowed to air dry, and observed in an electron microscope.

Nodule samples. At days 20 to 23 after inoculation, nodules (or in the case of plants inoculated with JS314, outgrowth structures on the primary roots) were dissected so that one central slice was obtained from each and a portion of the root remained attached for orientation later. They were fixed at room temperature for 1 to 2 hr in 3% glutaraldehyde buffered to pH 6.8 with 0.05 M phosphate buffer, followed by 1% osmium tetroxide for the same time

(similarly buffered). After fixation, the tissues were dehydrated through an acetone series (starting with 25%, about 20 min per step) and infiltrated and embedded in Spurr low-viscosity medium. Sections a few micrometers thick were often cut and stained with Azure B for light microscopy so that the general nature of the nodules could be assessed. For electron microscopy, sections were mounted on copper grids without substrates and stained with both uranyl acetate and lead citrate.

2. Microscopy (performed by Dr. Evans Roth)

For light microscopy, a Zeiss photomicroscope was used with Nomarski interference optics. For electron microscopy, a Hitachi H600 microscope was used with either 75- or 100-kV beams.

CHAPTER III

RESULTS

PART I

A. Isolation and Characterization of Tn5-induced Mutants of B. japonicum

1. Verification of Tn5 Transposition

A total of 3,626 Km/Sm-resistant mutants of USDA 110 and 3,300 of strain 61A101C were generated by random Tn5 mutagenesis. Verification that the Km/Sm-resistant B. japonicum isolates contained Tn5 within their genome was obtained by hybridization of Southern blotted genomic DNA with nick translated Tn5 probe (i.e. pSUP1011 DNA). Results obtained with 17 61A101C and 18 USDA 110 Km/Sm-resistant isolates chosen randomly confirmed the presence of Tn5 (Figure 3). Restriction endonuclease BglIII cleaves Tn5 at two positions (one in each of its inverted repeat sequences) which are separated by a distance of approximately 2.7 kb (Jorgensen et al. 1979). Thus, any B. japonicum isolate containing Tn5 should have a 2.7 kb fragment that would hybridize to the Tn5 probe. In addition, insertion of Tn5 into the genome of B. japonicum should result in the presence of two additional junction fragments showing homology to both the right and left ends of Tn5. If transposition occurred randomly, these junction fragments

Figure 3. Southern hybridization analysis of BglII digested genomic DNA from 35 randomly chosen Km/Sm-resistant transconjugants. DNAs were fractionated through a 0.7% agarose gel, Southern blotted onto a nitrocellulose filter and hybridized with the nick translated pSUP1011 DNA (A) and pSUP101 DNA (B). Size markers on left are in kb. Lanes 1 through 18, Km/Sm-resistant USDA 110 isolates; lane 19, USDA 110 wild-type strain; lanes 20 through 36, 61A101C Km/Sm-resistant isolates; lane 37, 61A101C parental strain; lane 38, pSUP1011 DNA. Fragment sizes in kb indicated at left correspond to the pSUP1011 DNA cut with BglII. The uppermost band in lane 4 and the band larger than 10 kb in lane 32 are due to incomplete digestion of the DNA samples. Size markers on left are in kb.

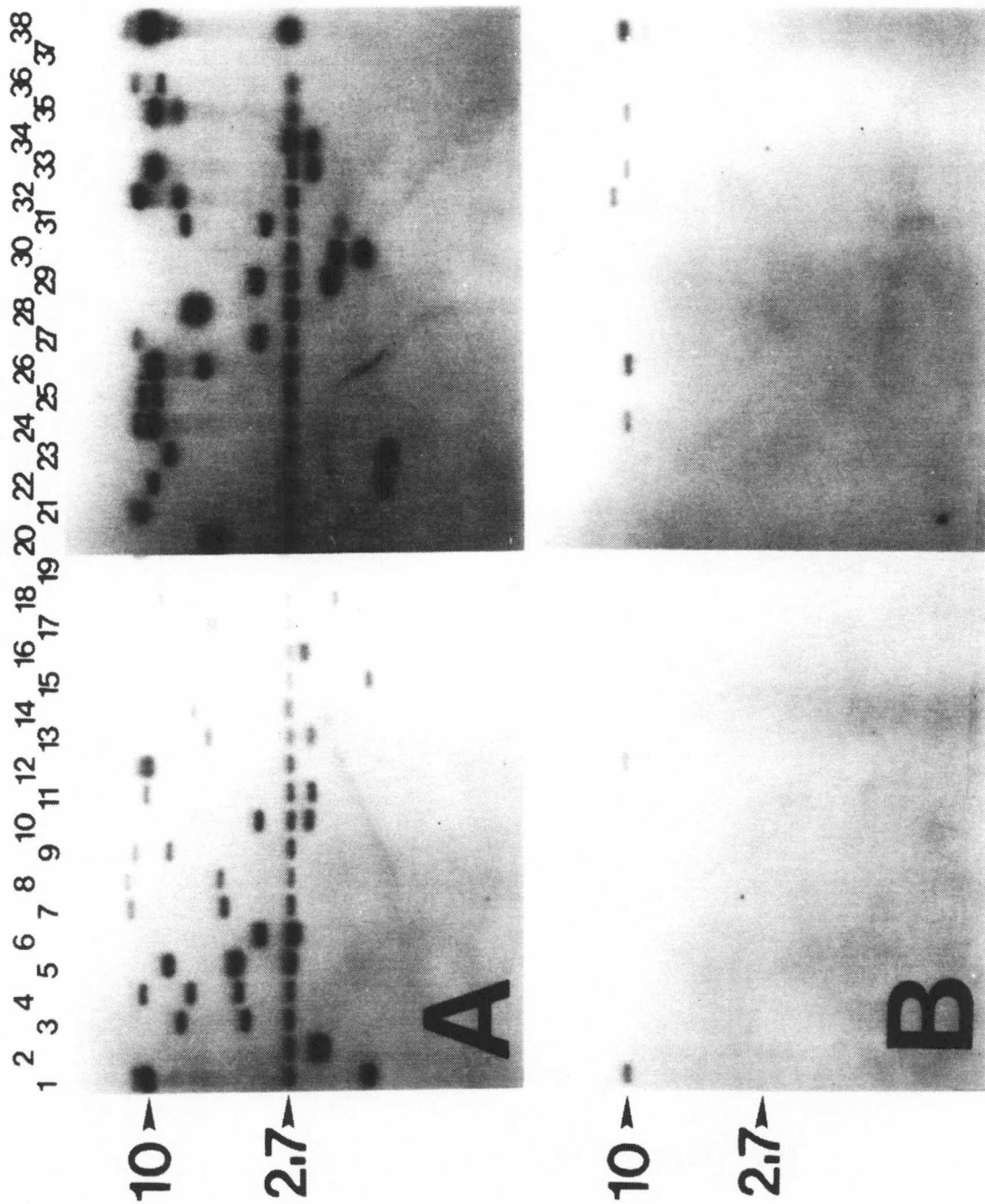


Figure 3

should vary in size from one isolate to the next, depending on the distance in either direction from the Tn5 insertion site to the next adjacent genomic BglIII cleavage site. As shown in Figure 3A, Tn5 appears to have inserted randomly. No detectable homology to pSUP1011 vector DNA was found in the wild-type parental strains. As shown in Figure 3B, however, some of the Km/Sm-resistant isolates contained a fragment that hybridizes intensely with the pSUP101 DNA probe, which lacks Tn5 sequences. The size of the hybridizing band is equal to the 10 kb fragment generated by BglIII digestion of pSUP1011 vector DNA. Such apparent plasmid cointegration was obtained for 12% and 29%, respectively, of the total number of isolates of strains USDA 110 and 61A101C examined.

2. Isolation of Symbiotically Defective Mutants

After repeated screening of all the Tn5 mutants, 25 mutants were found to be defective in nitrogen fixation or nodulation (Table III). Subsequent auxotrophic tests on these mutants revealed 4 histidine auxotrophic nodulation-defective mutants of strain USDA 110. No auxotrophs were identified among the 61A101C mutants. Three non-auxotrophic Nod^- mutants from strain USDA 110 and 4 Nod^- mutants from strain 61A101C were isolated. From strain 61A101C, 14 Nod^+Fix^- mutants were isolated. No Nod^+Fix^- mutants were isolated from strain USDA 110. The reason for this latter result is unknown.

TABLE III. Characterization of 25 Sym⁻ Mutants Isolated from Random Tn5 Mutagenesis.

Mutants	Symbiotic Phenotype		Auxotrophy	Predicted Size of Original <u>EcoRI</u> Fragment (kb) Carrying Tn <u>5</u>
	Nod	Fix		
<u>USDA 110</u>				
ML100	-		His ⁻	ND ^a
ML112	-		His ⁻	ND
ML115	-		no	6.0
ML116	-		His ⁻	6.0 ^b
ML117	-		His ⁻	6.0 ^b
ML118	-		no	13.5 ^c
ML119	-		no	13.5 ^c
<u>61A101C</u>				
ML120	+	-	no	11.1 ^d
ML121	+	-	no	11.1 ^d
ML124	+	-	no	10.5
ML125	+	-	no	12.7
ML126	+	-	no	VC ^e
ML127	-		no	17.3
ML128	+	-	no	8.6
ML129	-		no	11.1
ML142	-		no	5.5
ML143	+	-	no	VC
ML144	-		no	19.3
ML145	+	-	no	10.5 ^f
ML146	+	-	no	5.8 ^g
ML147	+	-	no	10.5 ^f
ML148	+	-	no	10.5 ^f
ML149	+	-	no	5.8 ^g
ML150	+	-	no	VC
ML151	+	-	no	10.5

^aNot determined. Instead BglIII digests of genomic DNA was probed with Tn5.

^{b,c,d,f}Tn5 insertion site is identical.

^eVector co-integration

^gTn5 is inserted into the same EcoRI fragment, but at different positions.

3. Hybridization Experiments

Hybridization of nick-translated pSUP1011 DNA to genomic DNA blots as previously described revealed that all of the symbiotically defective mutants contained Tn₅ in their genome (Table III and Figures 4 and 5). Of 14 Nod⁺Fix⁻ mutants of 61A101C, 3 were found to have co-integrated vector DNA (Table III and Figure 4). These vector co-integrates were not studied further. The results of the hybridization with the 3 Nod⁻ mutants of USDA 110 indicated that at least two independent DNA regions [6.0 kb (ML115 and 117) and 13.5 kb (ML118 and 119) EcoRI fragments] were mutagenized. On the other hand, each of 4 Nod⁻ mutants of 61A101C showed 4 different EcoRI fragments that hybridized to the Tn₅ probe [11.2 kb (ML142), 16.8 kb (ML129), 23 kb (ML127) and 26 kb (ML144)]. It appeared that some mutants could be identical since they showed the same size EcoRI fragment that hybridized to the Tn₅ probe (Table III and Figure 4).

With the aim of identifying mutants that contained Tn₅ at the same site in their genome, BglII-digested genomic DNA blots of several mutants were hybridized with nick translated Tn₅ probe. As an example, two sets of mutants (ML118 and 119) and (ML116 and 117) of USDA 110 showed the same respective hybridization patterns, indicating that they probably contained Tn₅ inserted in the same location (Figure 5). Since each respective set of identical mutants was

Figure 4. Southern hybridization analysis of EcoRI digested genomic DNA from 11 symbiotic mutants of strain 61A101C. DNAs were digested with EcoRI, Southern blotted onto a nitrocellulose filter and probed with the nick translated pSUP101 (lacks Tn5) (B). After washing off the probe, the same filter was hybridized to the nick translated pSUP1011 (contains Tn5) (A). Lanes: 1, ML121; 2, ML120; 3, ML122; 4, ML123; 5, ML125; 6, ML128; 7, ML127; 8, ML129; 9, ML142; 10, ML143; 11, ML144; 12, pSUP1011; 13, pSUP101. Lanes 6 and 10 are examples of pSUP1011 vector DNA co-integrations into the B. japonicum chromosome instead of Tn5 transposition. Size markers on left are in kb. The size of the wild-type fragment containing the Tn5 insert can be obtained by subtracting 5.7 kb (the size of Tn5) from the size of the hybridizing fragments shown. Size markers on left are in kb.

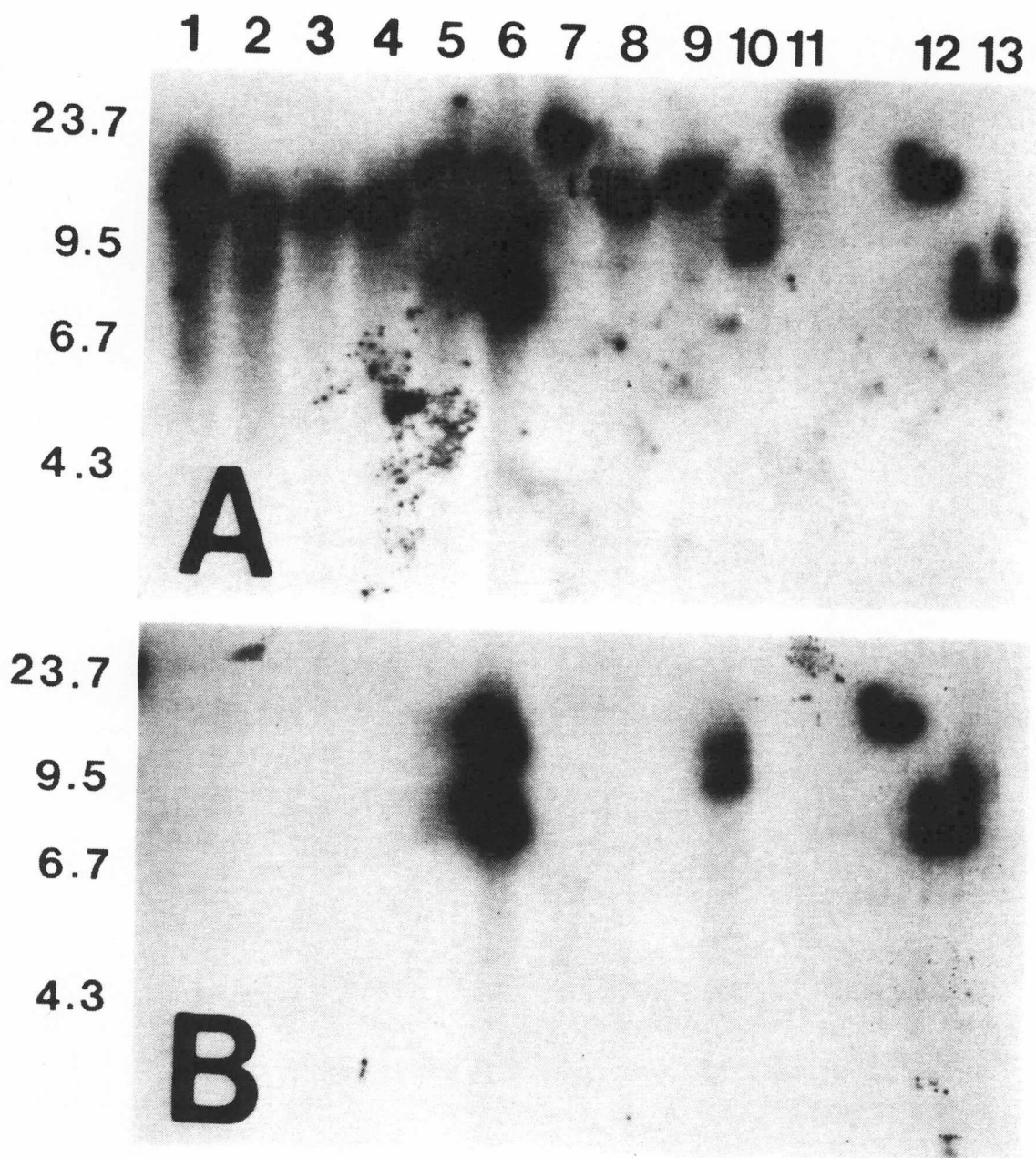


Figure 4

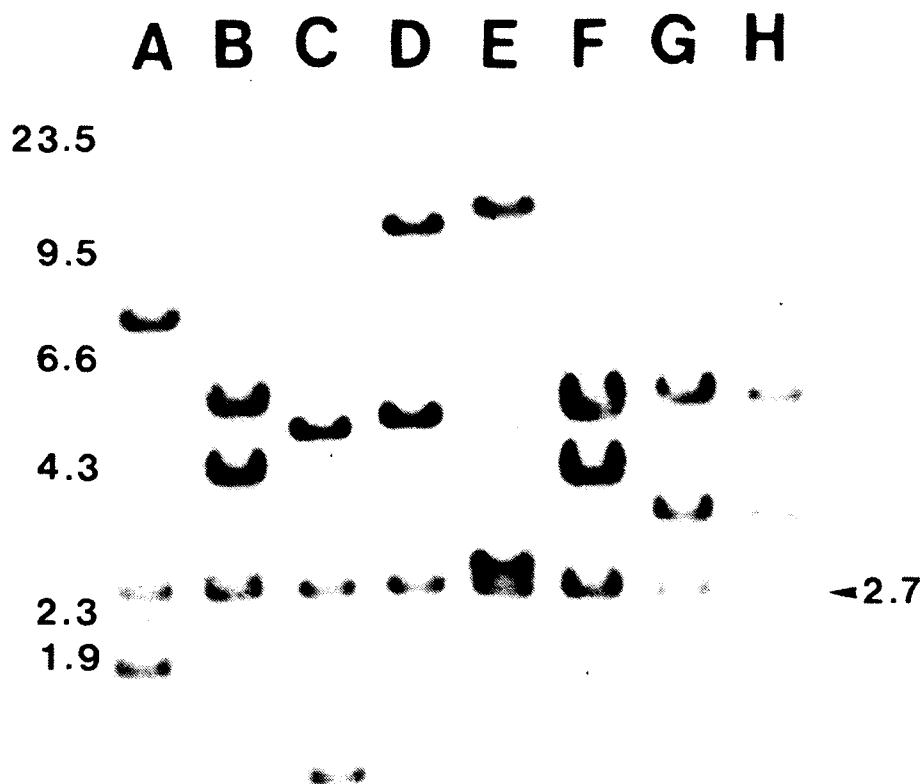


Figure 5. Southern hybridization analysis of BglII digested genomic DNA from 8 symbiotic mutants. DNAs were digested with BglII, Southern blotted onto a nitrocellulose filter and probed with the nick translated pSUP1011 DNA. Lanes: A, ML112; B, ML116; C, ML144; D, ML142; E, ML115; F, ML117; G, ML118; H, ML119. Size marker on left are in kb. The 2.7 kb internal BglII fragment of Tn5 are marked with an arrowhead on right.

obtained from the same mutagenesis, these mutants probably arose from a single mutagenic event.

We previously cloned approximately 100 kb of DNA that contains the common nod regions of B. japonicum strain USDA 110 (Russell et al. 1985). In addition, the nif genes of B. japonicum strain USDA 110 have also been cloned (Hennecke 1981; Adams et al. 1984). In order to find out whether any of the mutant isolates have Tn₅ inserted into these regions (or in the regions homologous to these regions in the case of 61A101C mutants), Southern hybridization analyses were carried out on the 25 Sym⁻ mutants. The previously isolated nod and nif clones were used to probe EcoRI-digested genomic DNA of all mutants. The four Nod⁻ mutants of strain USDA 110 (ML115, 117, 118 and 119) did not show any alteration in hybridization pattern compared with the wild type when their genomic blots were hybridized with the common nod region (i.e. pRjUT10, pRjUT14 and pRjUT24). Also, in the case of 61A101C Nod⁻ mutants, the hybridization pattern was indistinguishable from the pattern in the wild type when probed in an identical fashion.

Of the Fix⁻ mutants, ML125 appeared to have a deletion in the DNA region homologous to the clone pRjUT25, yet it retains Tn₅ sequences. It has not yet been determined whether the nifH structural gene is deleted. One Fix⁻ mutant, ML146, appeared to have Tn₅ inserted into the nifKD structural genes when probed with nick-translated pRj676

(Hennecke 1981). Genomic Southern blots of each of the Nod⁻ mutants (ML100, 112, 115, 116, 117, 118, 119, 127, 129, 142 and 144) were also hybridized to clones containing the nodABCD and hsnABCD genes of R. meliloti (pPP346 and the bacteriophages listed in Table II, page 35), and the nodIJ genes of R. leguminosarum (pZB15, see Table II, page 35). Where homology was found, there was no apparent alteration of the hybridization pattern that could be due to Tn₅ insertion.

B. Cloning of Two Nodulation Gene Regions from B. japonicum
Strain USDA 110 (Regions I and II)

1. Molecular Cloning of the Tn₅-carrying Cosmids

The Tn₅-containing EcoRI fragments from the three non-auxotrophic nodulation-defective mutants (ML115, 118 and 119) and from ML117 were cloned into the cosmid vector pH_C79. The resulting recombinant clones contained 11.7 kb EcoRI nod::Tn₅ (from ML115 and ML117) or a 19.2 kb EcoRI nod::Tn₅ (from ML118 and ML119) fragments. ML117 was included since hybridization data suggested that it carried a Tn₅ insertion in the same fragment as ML115. Indeed, hybridization studies indicated that the Tn₅ insertions in ML115 and ML117 occurred in different positions within the same 6.0 kb EcoRI fragment. To determine whether the cloned fragments contained Tn₅, the EcoRI digest of the cloned DNA were probed with nick-translated Tn₅ DNA. All the clones

contained hybridizing EcoRI fragments that corresponded in size to the hybridizing fragment in the genomic DNA (Figures 6 and 7). Plasmid profiles of several clones from ML119, 118, 115 and 117, digested with EcoRI are shown in Figure 7. The pHc79 vector DNA share sequence homology with the probe DNA (i.e. pSUP1011 DNA), probably in the tetracycline-resistance determinant. Based on the EcoRI digestion profiles of the overlapping clones, partial EcoRI restriction maps around the Tn5 insertion sites were established (Figures 8 and 9). The approximate location of the Tn5 within the EcoRI fragment was determined by a combination of restriction enzyme digestion and Southern analysis. Several individual EcoRI fragments within the regions were subcloned to be used as probes in colony hybridizations (see below), which are shown in Figures 8 and 9.

2. Isolation of Wild-type Clones Corresponding to the Cloned Tn5-carrying Cosmids

The EcoRI fragments containing Tn5 were isolated from the subclones mentioned above (11.7 kb::Tn5 from pHK759 and pHK51, 19.2 kb::Tn5 from pHK8382, see pages 77 and 78) and used as probes against colony blots of a wild-type gene library or a library produced using a Tn5 mutant having a mutation in a different, unlinked region. Utilizing this approach, wild-type clones corresponding to the Tn5-mutated regions were isolated (i.e. pBj801, 803, 804 and 805 for

Figure 6. Southern hybridization analysis of EcoRI digested DNA cloned from ML118 and ML119. Cosmid DNAs were digested with EcoRI, fractionated on an agarose gel, blotted onto a nitrocellulose filter, and hybridized with nick translated pSUP1011 DNA. (A) Agarose gel of EcoRI digests of cosmid clones. Lane 1, EcoRI digests of ML119 genomic DNA; lane 2, pJS91; lane 3, pJS92; lane 4, pJS93, lane 5, pJS94; lane 6, pJS84; lane 7, pJS86. (B) Autoradiograph of a Southern blot of the above gel. Size markers on left are in kb.

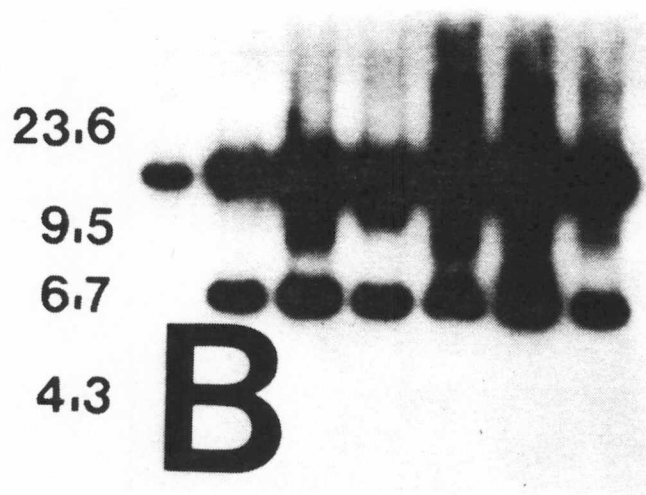
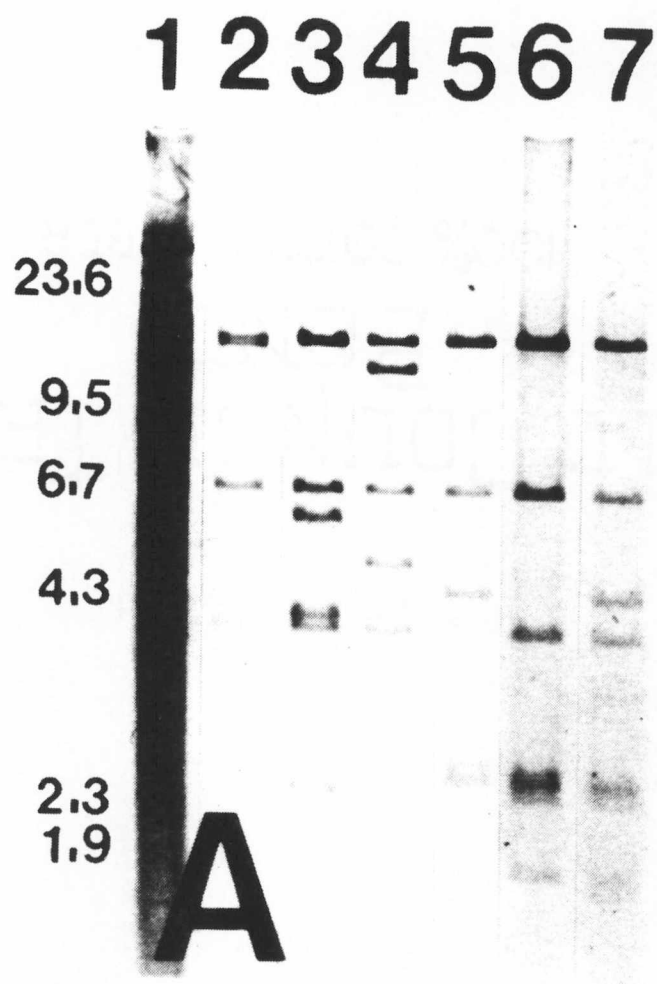


Figure 6

Figure 7. EcoRI digestion profiles of cosmid clones from ML115, 117, 118 and 119. (A and B) Agarose gel electrophoretogram of cosmids clones from ML115, 117, 118 and 119 digested with EcoRI. (C and D) Autoradiograph of a Southern blot of the above gel. Arrowheads point the fragments carrying Tn5 (13.5 kb::Tn5 EcoRI fragment in A and C, 11.7 kb::Tn5 EcoRI fragment in B and D). Vector DNA, pHC79 (6.4 kb) also hybridized to the probe (i.e. pSUP1011 DNA carrying Tn5)

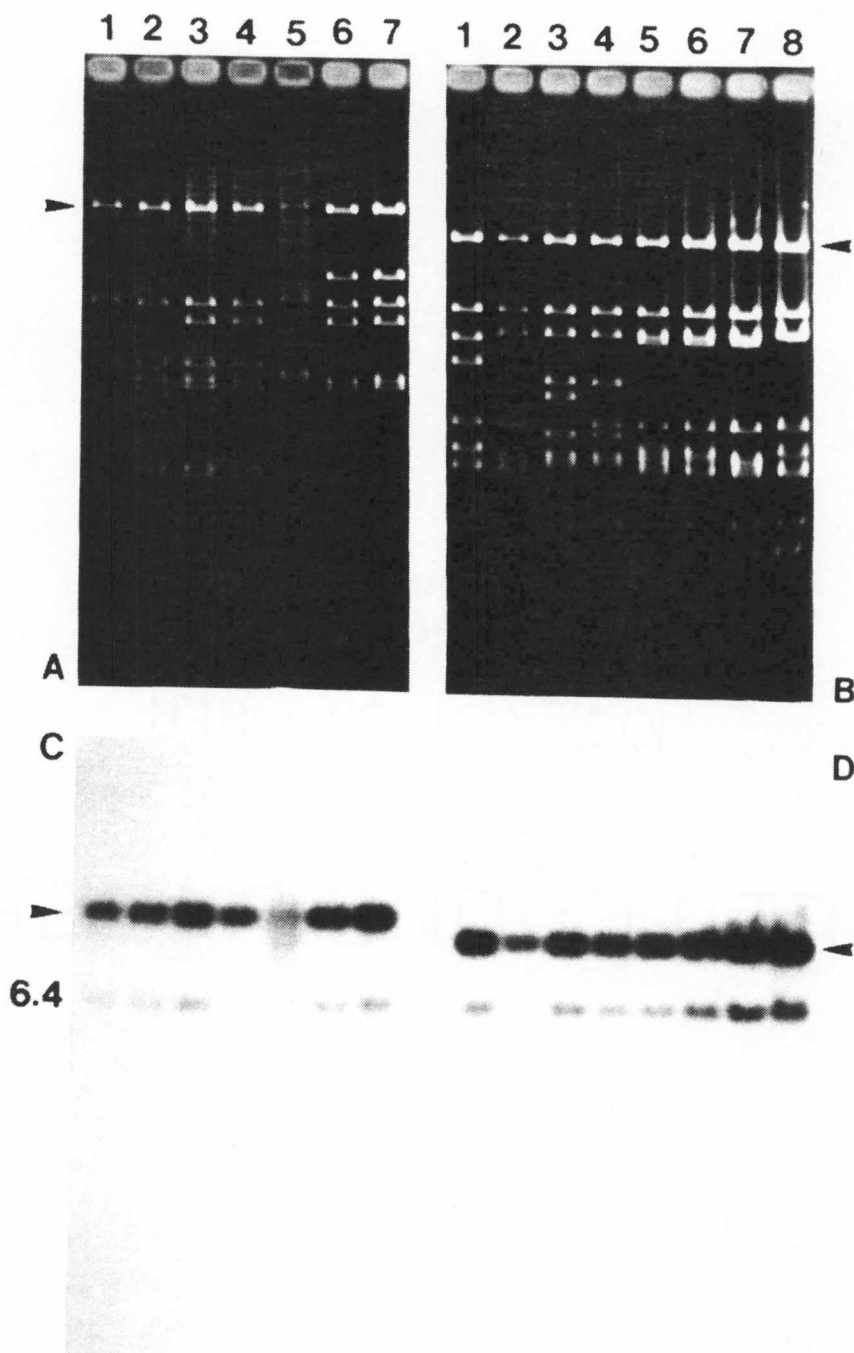


Figure 7

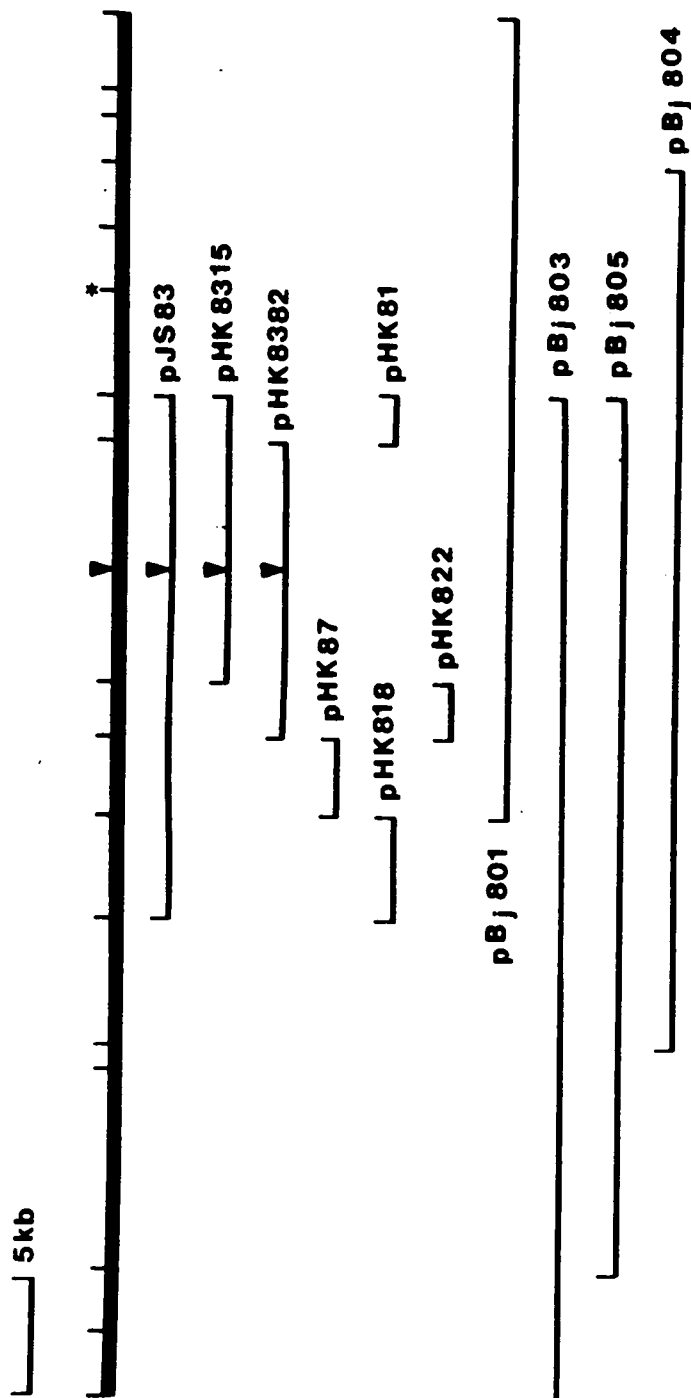


Figure 8. Physical map of the region I DNA. Top line represents the EcoRI restriction enzyme map of the chromosomal gene region cloned from ML118 and 119. Vertical lines are EcoRI restriction sites. Lines with (*) represent EcoRI sites whose exact locations are not known. Tn5 are represented by arrowheads. The approximate positions of the Tn5s were determined by restriction analysis of the subclone, pHK8315. Subclones of a cosmid clone, pJS83 are designated by pHK series. The wild-type cosmid clones (pBj 801, 803, 804 and 805) were obtained from a gene bank by colony hybridization.

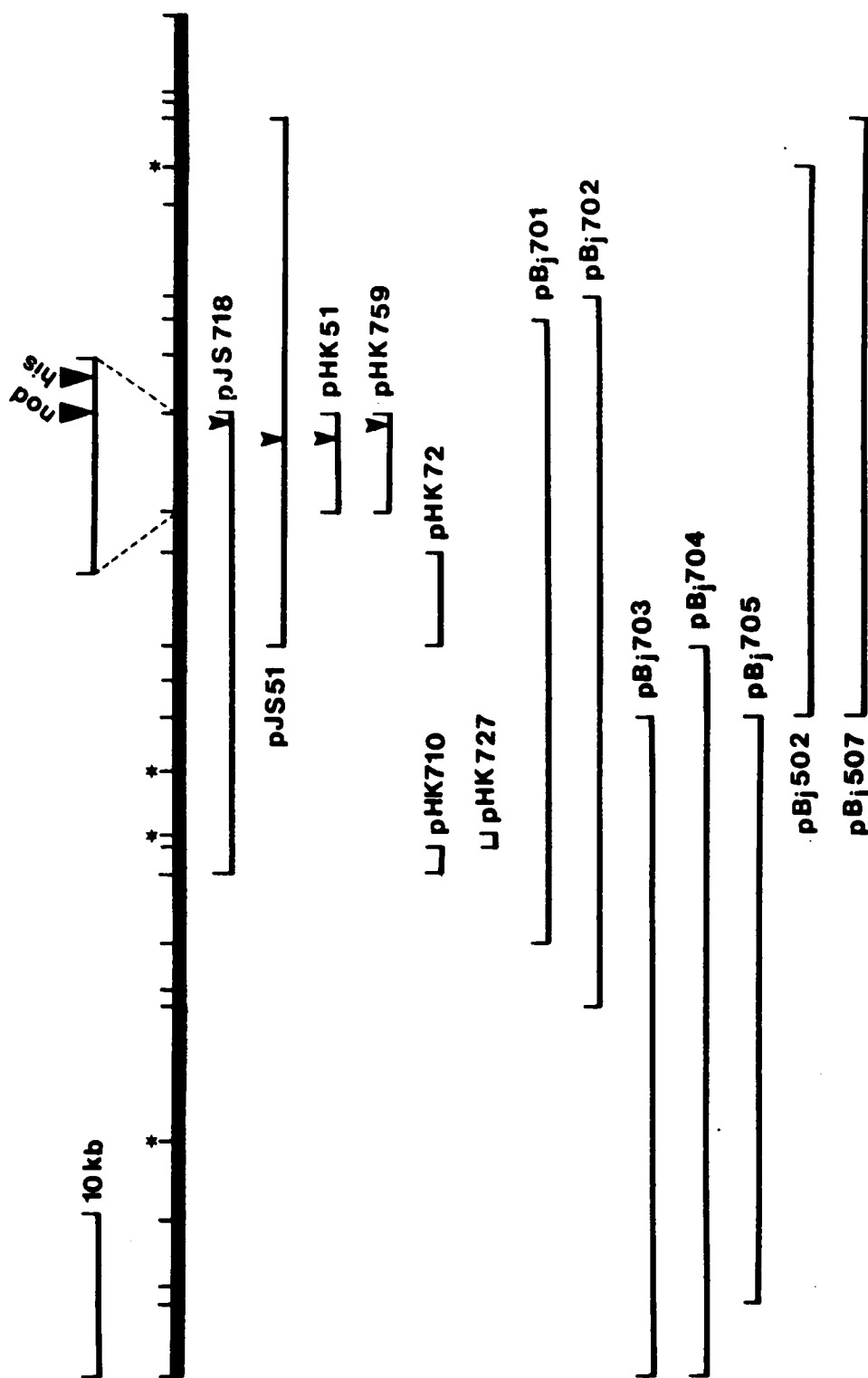


Figure 9. Physical map of the region II DNA. Top line represents EcoRI restriction enzyme map of the chromosomal gene region cloned from ML115 and 117. Exact positions of the EcoRI sites with (*) are not determined. Tn5s are symbolized by arrowheads. The two Tn5 are separated from each other by approximately 1.0 kb.

region I; pBj502, 507, 701, 702, 703, 704 and 705 for region II, Figures 8 and 9). Confirmation that the clones isolated corresponded to the Tn5-mutated regions was obtained by comparison of the EcoRI restriction maps of the regions (Figures 8 and 9), cross-hybridization between clones (Figures 10 and 11), and hybridization of the cloned mutated DNA to wild-type and mutant genomic DNA.

For region I (from ML118 and 119), the total cloned region encompasses approximately 60 kb. Cross hybridization between the left- and right-most cloned DNA (i.e. pBj801 and 803) and the previously known nod genes (pPP346, pZB15, pRjUT10, pRjUT14 and pRjUT24, see Table II, page 35) did not show any detectable homology, confirming the hybridization results obtained from analysis of mutants ML118 and 119 as described earlier. Also, for region II, total length of the cloned DNA was approximately 60 kb DNA. One of the subclones in the region II (pHK710, see Figure 9) was used as a probe in a chromosome walking experiment to obtain an additional 20 kb DNA. Like the region I, hybridization between the region II and the previously known nod genes failed to detect any homology. Positions of two independent Tn5 inserts within the 6.0 kb EcoRI fragment were mapped to be approximately 1.0 kb apart from each other, indicating that the nod locus is situated close to the his locus.

Hybridization of the DNA from two regions adjacent to the mutations to various other rhizobial genomic DNA indi-

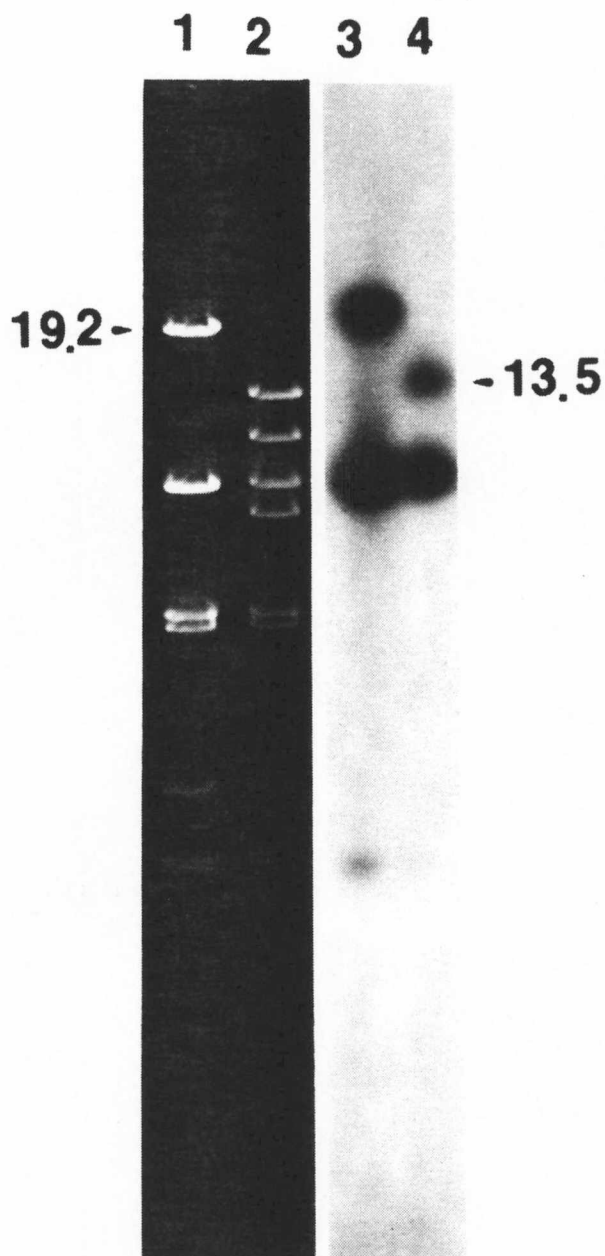


Figure 10. Isolation and verification of the wild-type clone corresponding to the cloned mutated region I DNA. Lanes 1 and 2; agarose gel picture of EcoRI digests of pJS83 (mutant clone) and pBj801 (wild-type clone). Lanes 3 and 4; autoradiogram of the DNA in lanes 1 and 2 following hybridization with nick translated pHK8315 DNA (see Figure 8). Size marker of the two fragments are in kilobase pairs. The size difference between the two fragments is 5.7 kb, which is the exact size of Tn₅.

Figure 11. Isolation and verification of the wild-type clones of the region II. Plasmid DNAs isolated from 4 wild-type clones (lanes B to E) and 14 mutant clones (lanes F to S) were digested with EcoRI, fractionated through an 1.2% agarose gel, and Southern blotted onto a nitrocellulose filter. The filter was hybridized with nick translated pBj701 DNA (see Figure 11). Lanes A and T contain HindIII digested bacteriophage lambda DNA (from the top band; 23.6 kb, 9.4 kb, 6.6 kb, 4.2 kb, 2.3 kb and 2.0 kb). Lanes: B, pBj705; C, pBj704; D, pBj703; E, pBj702; F, pJS724; G, pJS718; H, pJS716, I, pJS715; J, pJS714; K, pJS713; L, pJS712; M, pJS79; N, pJS78; O, pJS76; P, pJS75; Q, pJS74; R, pJS72; S, pJS71.

A B C D E F G H I J K L M N O P Q R S T

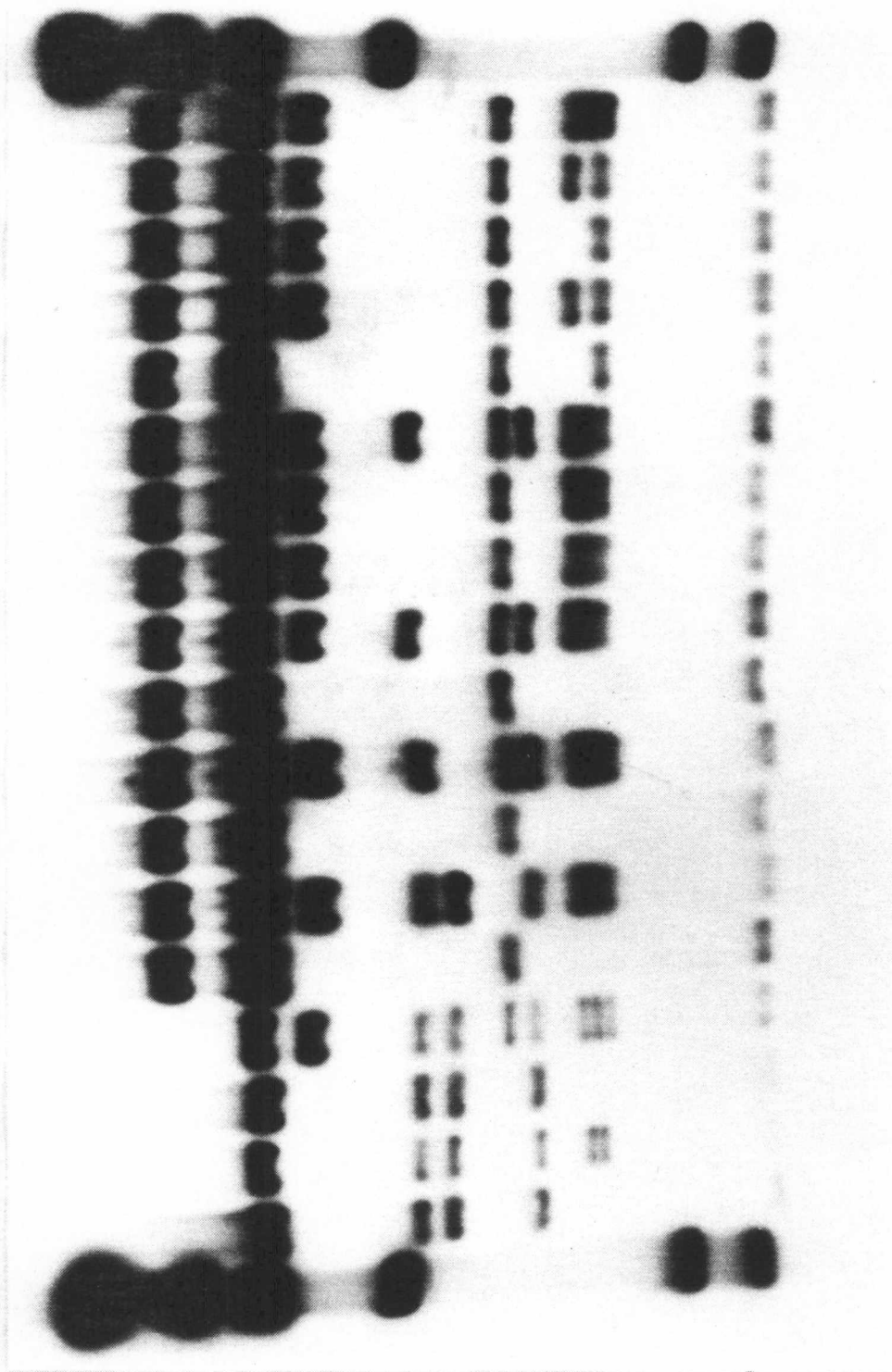


Figure 11

cated that the regions were well conserved only among the slow-growing rhizobia. Region II is well conserved among all the slow-growing rhizobia examined (Figure 12), whereas the region I shows conserved hybridization patterns only with selected strains of B. japonicum (Figure 13). These latter B. japonicum strains (USDA 110 and USDA 122) were previously classified as group I B. japonicum (Stanley et al. 1985). Interestingly, in A. tumefaciens a fragment was found to hybridize to the region I DNA (lane 1 in Figure 13). This apparent homology was not related to the Ti (tumor inducing) plasmid, because the Ti plasmid-cured strain (A136, Table II, page 35) also had the same sized fragment hybridizing to the region I DNA as the parental strain (data not shown).

C. Nodulation Phenotype of Mutation in the Two Regions

In order to determine whether the Tn5 insertions in the two DNA regions were solely responsible for the nodulation-defective phenotype, site directed mutagenesis of the wild-type chromosome was performed using the cloned mutated DNA (see Materials and Methods). B. japonicum transconjugants were selected in which their wild-type DNA region had been exchanged for the Tn5-containing fragment. The two homogenotes, JS831 and JS721, were obtained by using the clones, pJS83 and pJS72, in respective homogenotization experiments. These transconjugants were verified as having resulted from true gene replacement event (Figure 14A).

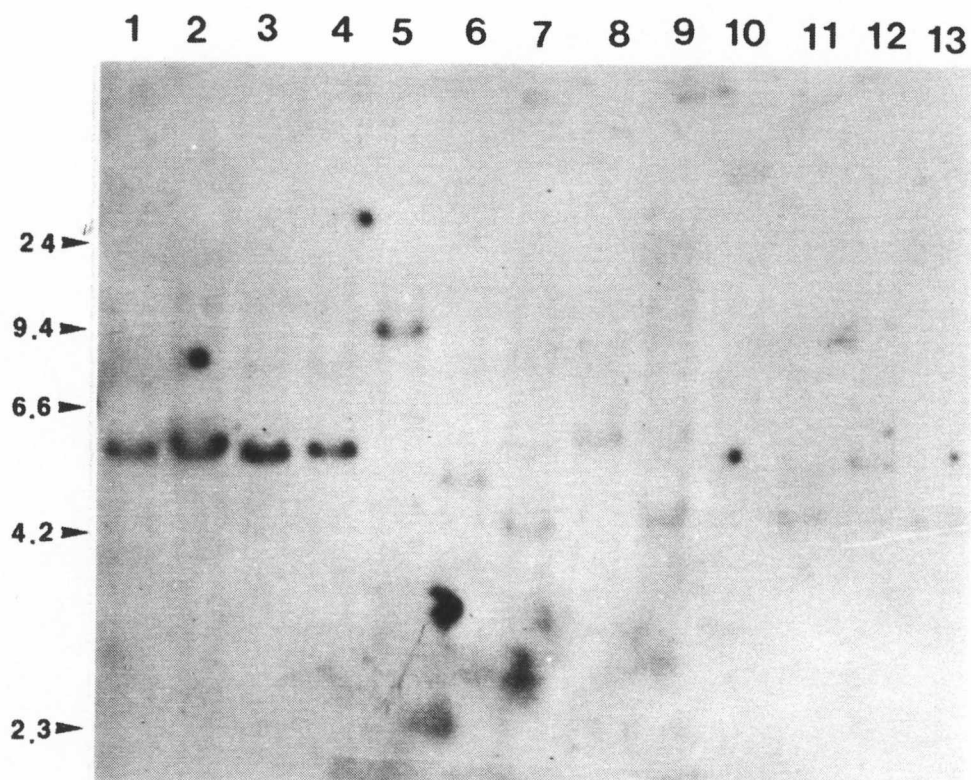


Figure 12. Conserved hybridization pattern of the region II DNA among other rhizobia. Gemomic DNAs from various rhizobia (cut with EcoRI) were probed with the region II DNA (6.0 kb EcoRI fragment) under low stringent hybridization and wash conditions. Lanes 1 through 9; B. japonicum USDA 110 E1-8d1, USDA 110, USDA 122, AN6A, USDA 6, USDA 123, 61A101C, USDA 46, USDA 76. Lane 10, Rhizobium sp. NGR234. Lane 11, B. parasponia. Lane 12, Bradyrhizobium sp. cowpea strain 32H1. Lane 13. Bradyrhizobium sp. peanut strain. Size marker on left are in kb.

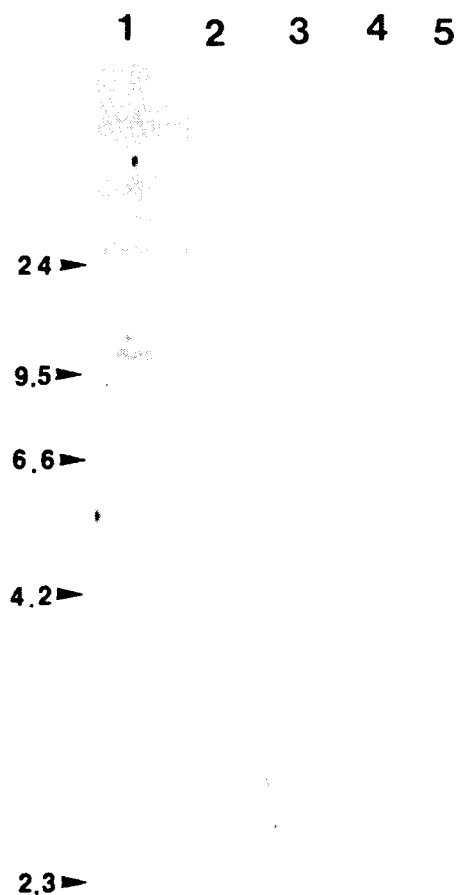


Figure 13. Conserved hybridization pattern of the region I DNA among other rhizobia. Genomic DNAs from various rhizobia and A. tumefaciens (cut with EcoRI) were probed with nick translated 13.5 kb EcoRI fragment of the region I. Lanes: 1, A. tumefaciens strain A6; 2, B. japonicum AN6A; 3, B. japonicum USDA 122; 4, B. japonicum USDA 110; 5, B. japonicum USDA 110 E1-8d1. Size markers on left are in kb.

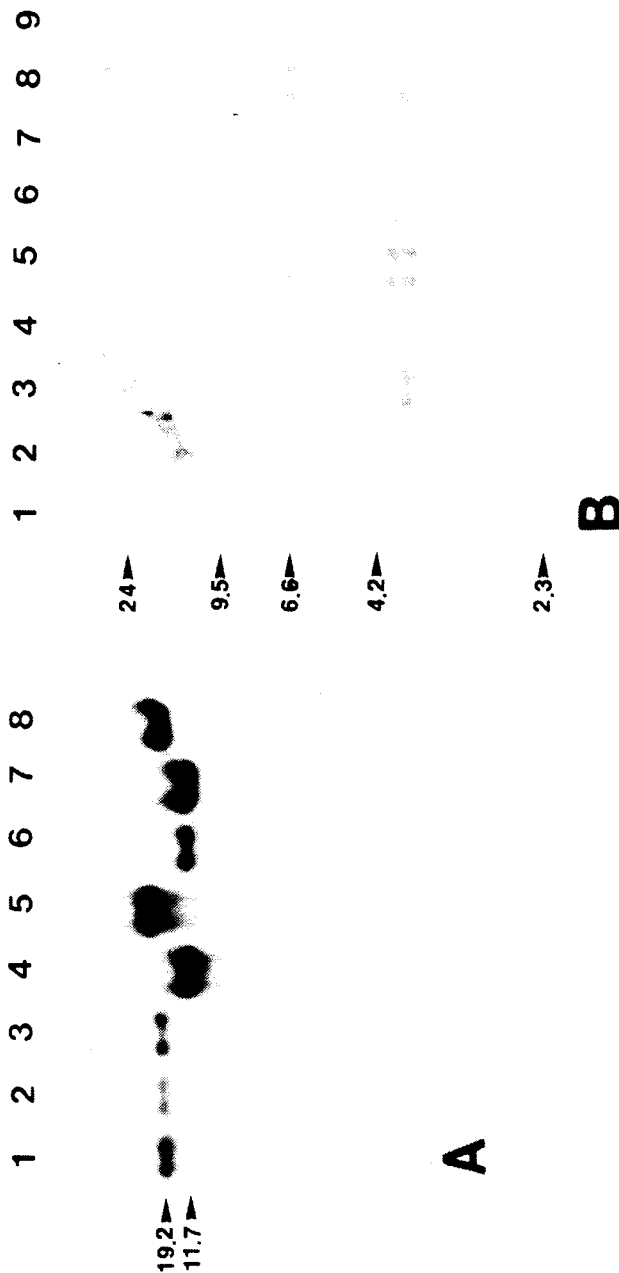


Figure 14.

Southern hybridization analysis of genomic DNA from putative homogenotes. (A) Genomic DNAs were isolated from 5 putative homogenotes and 3 original mutants, digested with EcoRI and probed with Tn5. Lanes 1 to 3, 3 transconjugants from homogenotization involving a ML118 clone, pJS83. Lane 4, transconjugant from homogenotization involving a ML117 clone, pJS72. Lane 5, transconjugant from homogenotization involving a ML115 clone, pJS51. Lanes 6, 7 and 8, ML115, ML117 and ML118, respectively. (B) HindIII digests of genomic DNA from 8 putative homogenotes were probed with Tn5. Lanes 1 to 3, 3 randomly chosen transconjugants from homogenotization involving a ML115 clone, pJS51. Lane 4, wild-type strain USDA 110. Lanes 5 to 9, randomly chosen transconjugants from homogenotization involving a ML117 clone, pJS72. Size markers on left are in kb.

A homogenote was not obtained with the mutated DNA cloned from ML115. Unlike the Km/Sm-resistant transconjugants obtained from the homogenotization experiment involving ML117 clones (e.g. pJS72 and pJS74), the transconjugants from similar experiments involving ML115 clones (e.g. pJS51 and pJS56) appeared to have Tn5 inserted in random positions throughout the genome. This is evident in the Figure 14B as the ML115 transconjugants show irregular hybridization patterns while the ML117 transconjugants show the same pattern as the original mutant ML117.

The two homogenotes thus obtained (JS831 and JS721) were tested for their ability to nodulate soybean and were found to be defective in nodule formation. Therefore, the Tn5 inserted in these regions is responsible for the observed nodulation defective phenotype in these homogenotes. JS721 was also tested for histidine auxotrophy and, like the original mutant ML117, JS721 was found to require histidine for growth on minimal medium.

In nodulation tests both JS831 and JS721 showed a 3 to 4 day delay in nodule appearance when compared to the wild-type parental strain USDA 110 (Figure 15). This nodulation phenotype is different from that described originally, which was Nod^- (So et al. 1987). This discrepancy was likely due to the difference in nodulation assay systems used in the two experiments. When the Tn5-induced mutants from random Tn5 mutagenesis were screened for their nodulation pheno-

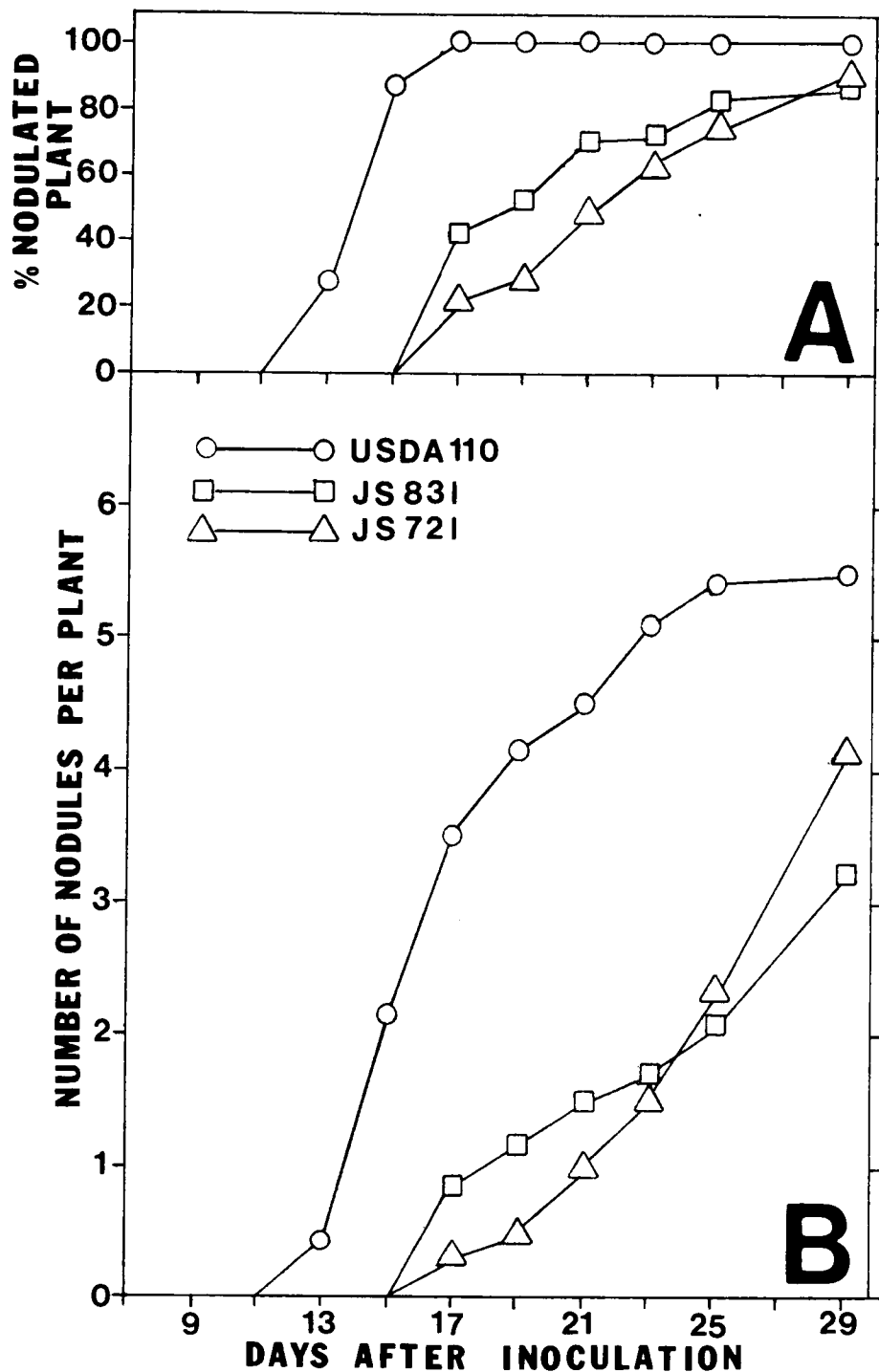


Figure 15. Nodulation phenotype of JS831 and JS721. Two homogenotes and the wild-type strain USDA 110 were tested on *G. max* cv. Essex for their nodulation phenotype. Nodulation kinetics are shown as percentage of plants nodulated (A), and as the average number of nodules per plant (B).

type, they were tested by the bottle assay method (see Chapter II) rather than by the pouch assay method which was used later. Indeed, when the original mutants (ML117 and ML118) were retested on plants by the pouch assay system, nodules appeared with 3- to 4- day delay when compared to wild-type. Even in the bottle assay, strains ML117 and ML118 will nodulate if given sufficient time to do so (data not shown). Nodules produced by JS831 or JS721 were capable of fixing nitrogen when tested by the acetylene reduction assay. The bacteria were isolated from the nodules to determine if they were revertants. One hundred individual nodule isolates were randomly selected and patched onto AMA plates containing kanamycin and streptomycin. All the isolates retained the drug resistance, indicating that the nodules were occupied by the mutants not by revertants. Taken together, it appears that the regions I and II are involved in the nodulation process, but not absolutely essential as a mutation in either of the two regions does not completely block the nodulation process. Due to this, my attention shifted to analyzing the nodulation defective mutants of strain 61A101C.

PART II

A. Molecular Cloning of an Essential Nodulation Gene Region from B. japonicum Strain 61A101C (Region III)

Four Nod⁻ mutants of strain 61A101C were retested for their nodulation phenotype using the two different assay systems described above. All 4 mutants showed a completely Nod⁻ phenotype in the bottle assay at 28 days after inoculation. In the pouch assay, however, only 3 mutants (ML127, 129 and 142) showed a Nod⁻ phenotype (a few nodules were formed by revertants, see below) whereas one mutant (ML144) showed an extensively delayed nodulation phenotype (Figure 16). The bacteria were isolated from the nodules formed by ML144 and found to be Km/Sm-resistant. In contrast, the bacteria isolated from the few nodules formed by ML127, 129 and 142 were Km/Sm-sensitive, suggesting that the nodules were formed by revertants or wild-type contaminants. From the mutant strain ML142, the mutated gene region was isolated (see Materials and Methods). Several overlapping clones containing the Tn⁵ were selected and probed with nick translated Tn⁵ DNA (i.e. pSUP1011) to verify the actual presence of Tn⁵ within the cloned region. As in the ML142 genomic digest, the Tn⁵ probe hybridized to the 11.2 kb EcoRI fragment of all the clones obtained (data not shown).

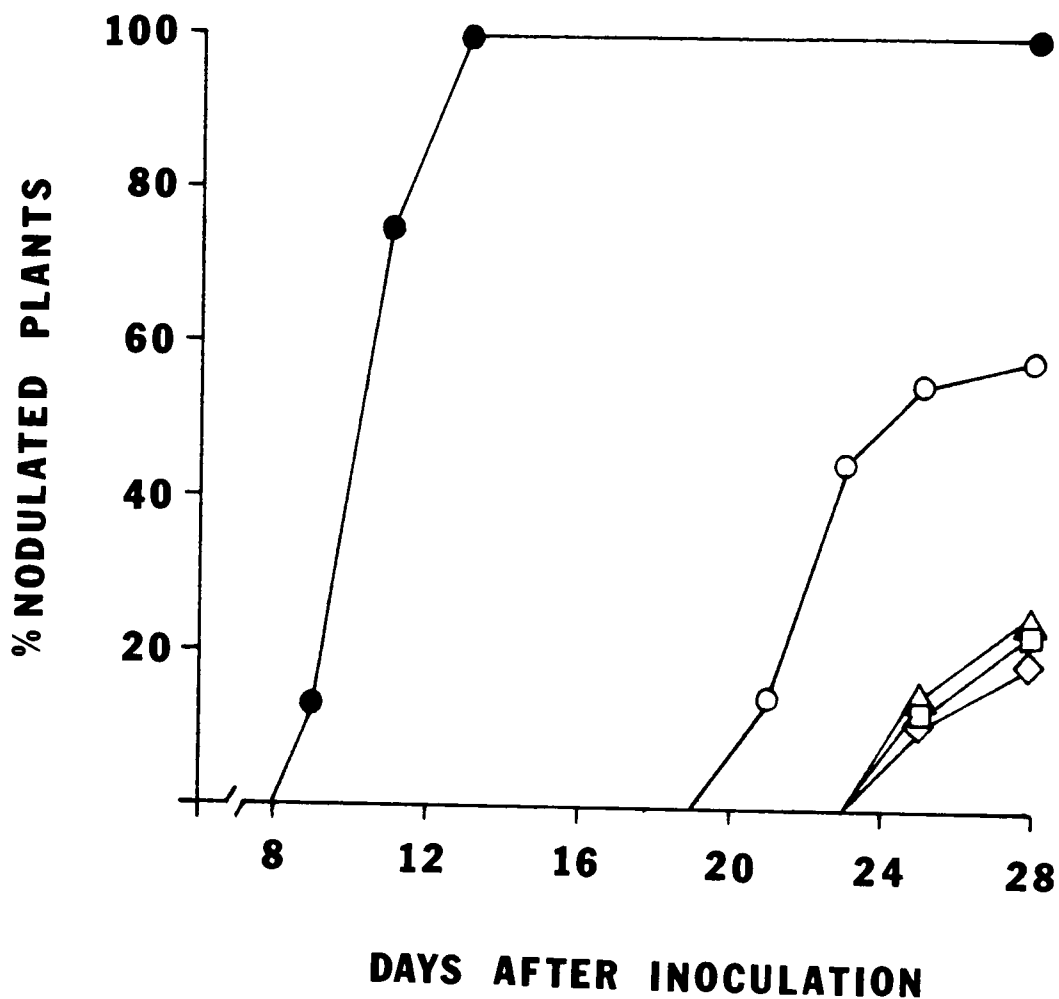


Figure 16. Nodulation phenotype of 4 Nod^- mutants of strain 61A101C. The wild-type strain 61A101C (●) and 4 nodulation defective derivatives; ML127 (◇), 129 (△), 142 (□) and 144 (○). Thirty plants (*G. max* cv. Essex) were inoculated with each strain and grown in plastic pouches (see Chapter III). The nodules were scored at regular time intervals. Nodulation kinetics are shown as percentage of plants nodulated.

In order to determine whether the Tn5 insertion in the region was the direct cause of the Nod^- phenotype shown by the original mutant, the cloned DNA fragment carrying Tn5 (pJS314) was introduced into the wild-type strain 61A101C to induce double-crossover, homologous recombination, resulting in a marker exchange to produce strain JS314. A true replacement event was confirmed by hybridization of EcoRI and BamHI digests of the putative homogenote's genomic DNA with the 11.2 kb EcoRI fragment carrying Tn5 as a probe (Figure 17). Both strains, ML142 (original mutant) and JS314 (homogenote), contained a single EcoRI fragment of 11.2 kb in size that hybridized to the probe. In the wild type strain 61A101C, a 5.5 kb EcoRI fragment hybridized to the probe. Because Tn5 is 5.7 kb long and contains no EcoRI site, these results indicates that the two mutant strains contain a single Tn5 insert located within a 5.5 kb EcoRI fragment. When BamHI digests were analyzed in the same manner, two fragments of 5.7 kb and 6.0 kb were detected in ML142 and JS314, while one fragment of 5.4 Kb was detected in 61A101C. As the Tn5 and the 5.5 kb EcoRI fragment each contains a single BamHI site (see page 99), three fragments were expected to hybridize to the probe in the two mutant strains. Indeed, after a prolonged exposure of the X-ray film, an additional faint band of 2.8 kb was observed in all three strains, which contained the leftmost 200 bp BamHI-EcoRI fragment plus 2.5 kb EcoRI-BamHI fragment outside the

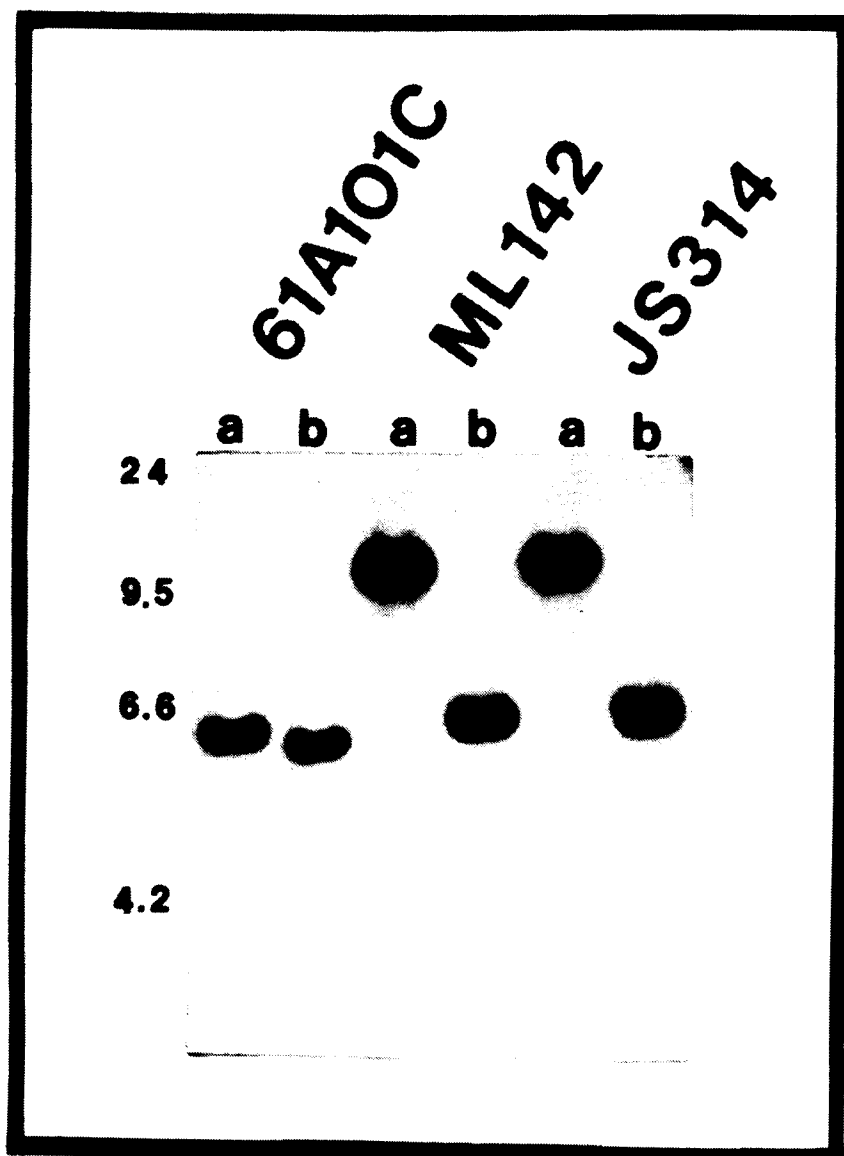


Figure 17. Southern hybridization analysis of genomic DNA from wild-type strain 61A101C, original mutant ML142 and the homogenote JS314. EcoRI digests (lane a) and BamHI digests (lane b) of genomic DNA were probed with the nick translated 11.2 kb EcoRI fragment containing Tn₅. The hybridizing band in lane b of ML142 and JS314 is doublet.

5.5 kb EcoRI fragment. Also, results obtained from additional hybridization experiments with HindIII and ClaI digests of the three strains were consistent with the above results (data not shown)

The symbiotic phenotype of the homogenote (JS314) was determined by plant infection tests. As shown in Figure 18, the specific mutation in the region results in a Nod^- phenotype. This Nod^- phenotype of JS314 was demonstrated irrespective of whether it was tested by the bottle assay or the pouch assay. This clearly indicated that the cloned region was essential for nodulation.

To study this region further, the wild type sequence, corresponding to the cloned mutated region was isolated by screening a genomic library by colony hybridization using the 11.2 kb EcoRI fragment carrying Tn5 as a probe. The 11.2 kb::Tn5 EcoRI fragment DNA was isolated by gel purification, nick translated, and used in colony hybridization. One positive clone was isolated and designated as pBj101. Its authenticity was confirmed by agarose gel electrophoresis and hybridization. The EcoRI digestion pattern of the mutant clone (pJS314) and the wild-type clone (pBj101) were similar to each other (Figure 19). The 5.5 kb EcoRI fragment of pBj101 was enlarged to 11.2 kb by the insertion of the 5.7 kb Tn5 DNA. The insertion event was confirmed by hybridization by using the 11.2 kb EcoRI

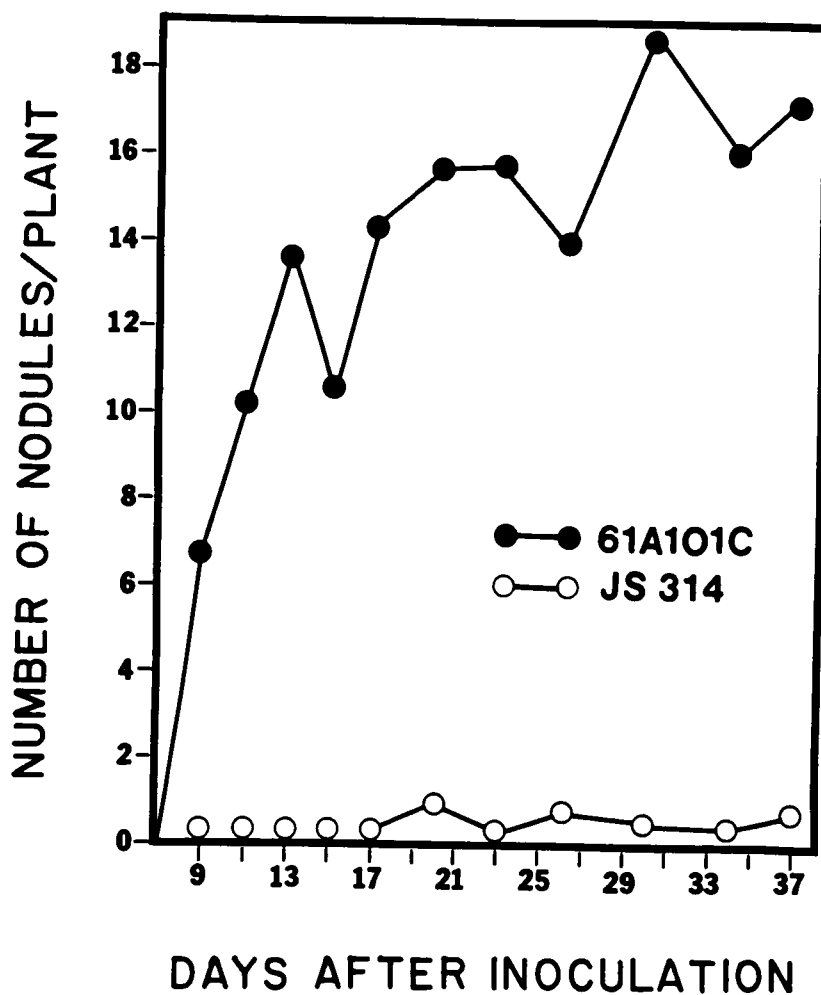


Figure 18. Nodulation phenotype of the homogenote JS314 and the wild-type strain 61A101C. Two sets of 110 plants were inoculated with each of two strains (1 ml of early stationary phase culture). At time intervals, 10 plants were sacrificed to determine the nodulation phenotype.

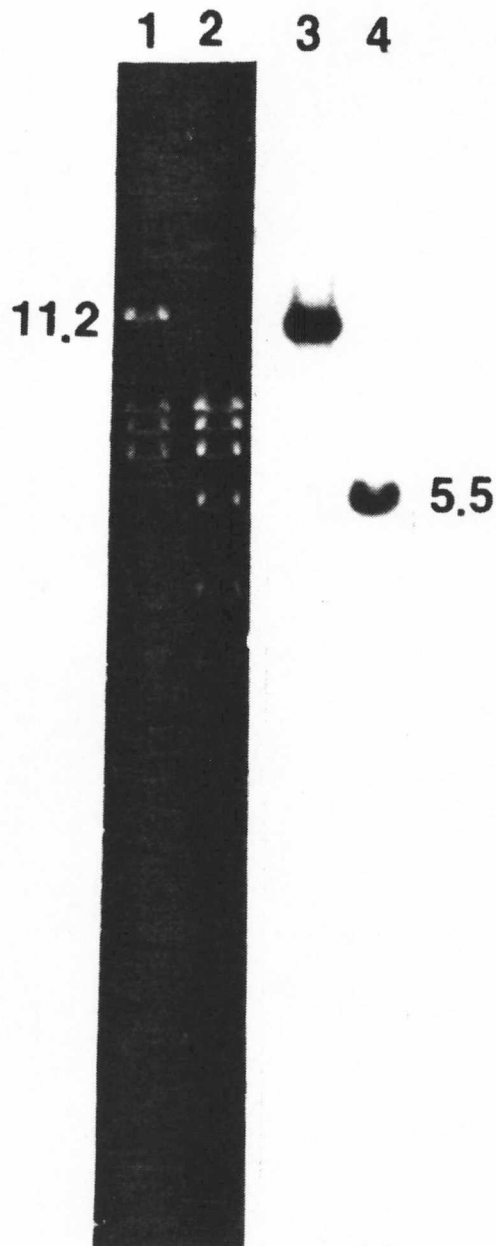


Figure 19. Southern hybridization analysis of wild-type and mutant clones of the region III. A wild type clone, pBj101 (lanes 2 and 4), and a mutant clone, pJS314 (lanes 1 and 3) were digested with EcoRI, fractionated on an agarose gel (lanes 1 and 2). After Southern transfer, the DNAs on filter were hybridized with the nick translated 11.2 kb EcoRI fragment containing Tn₅ (lanes 3 and 4). The size difference between the two fragments in lane 3 and lane 4 is the exact size of the Tn₅ (5.7 Kb).

fragment (5.5 kb EcoRI fragment plus Tn5) as a probe, which hybridized to the 5.5 kb fragment of pBj101.

B. Molecular Characterization of the Region III

1. Localization of Tn5 Insertion Site

Knowing that there is no HindIII site within the 5.5 kb EcoRI fragment and that HindIII cleaves Tn5 twice in both inverted repeat sequences, it was quickly determined that Tn5 was inserted in the middle of the 5.5 kb EcoRI fragment. This fragment was subsequently subcloned into the expression vector ptac11 (Amann et al. 1983) in two orientations (pHK32 and pHK34). Later, by hybridization the more precise location of Tn5 was narrowed down to a 0.3 kb PvuI-PstI fragment (see page 101).

2. Is the Region III Conserved Among Other Rhizobia?

To determine the extent of conservation of region III among a variety of rhizobia, genomic digests of various Rhizobium and Bradyrhizobium species were hybridized with the nick translated 5.5 kb EcoRI fragment (pHK34 DNA) under low stringency hybridization conditions (see standard DNA manipulation section in Chapter II). The results show that the region is well conserved among various Bradyrhizobium species, while no hybridization is detected to Rhizobium species, including R. fredii and Rhizobium sp. NGR234, other soybean endosymbionts (Figure 20). In the case of B. japonicum strain USDA 6, a single 1.7 kb EcoRI fragment

Figure 20. Conserved hybridization pattern of the region III DNA among various rhizobia. Southern analysis of genomic digests (cut with EcoRI) from various rhizobia was performed using nick translated 5.5 Kb EcoRI fragment as a probe. Lanes A to I; B. japonicum USDA 110 E1-8d1, USDA 110, USDA 122, AN6A, USDA 6, USDA 123, 61A101C, USDA 46, USDA 76: lane J, Rhizobium sp. NGR234: lanes L and M; Bradyrhizobium sp. cowpea strain 32H1 and peanut strain: lanes N and O; Rhizobium fredii USDA 193 and USDA 194: lanes P to R; Rhizobium leguminosarum, R. meliloti and R. trifolii, respectively: lane S; Agrobacterium tumefaciens. Size markers on left are in kb.

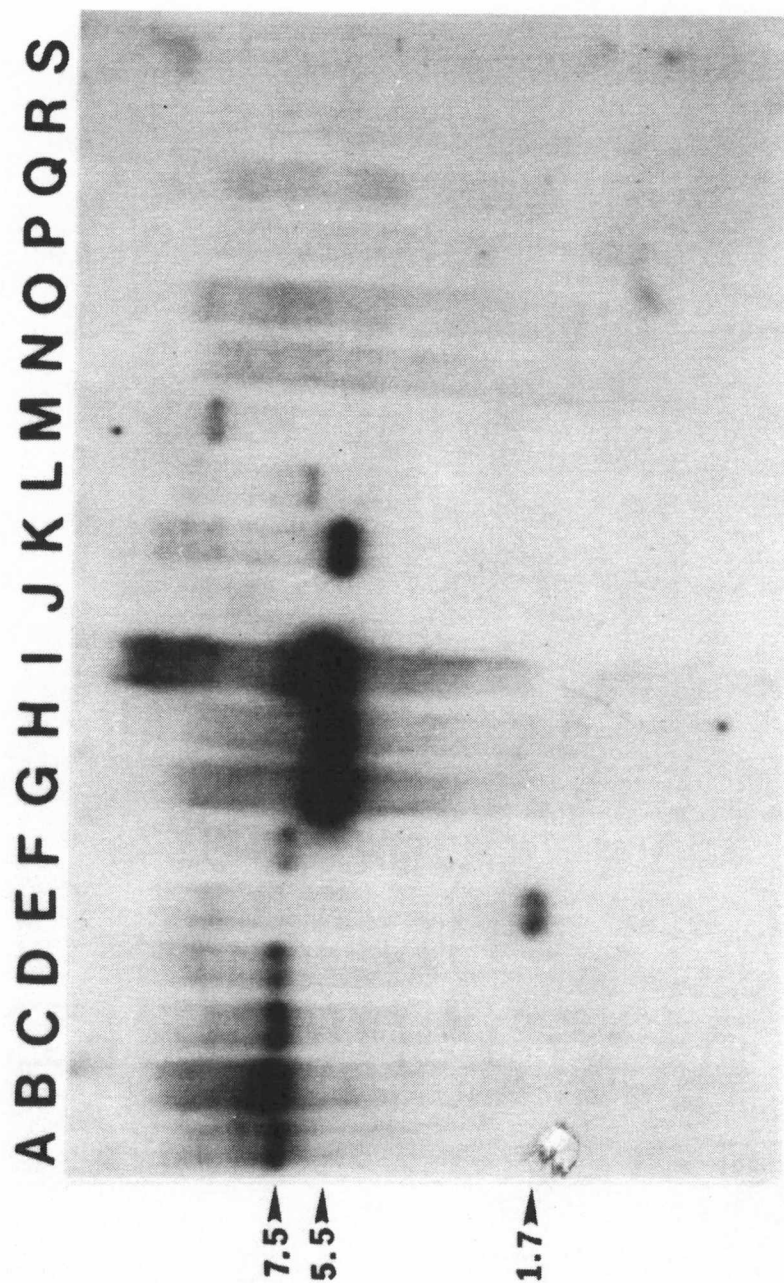


Figure 20

hybridized to the probe (5.5 kb EcoRI fragment). This particular hybridization pattern has been extremely useful in tentatively delimiting the nod gene within the 5.5 kb EcoRI fragment (see below).

Our laboratory has a nodulation-defective mutant (strain E1-8d1) of B. japonicum USDA110 which has a large deletion (ca. 300 Kb) around the nif genes (kindly provided by Dr. H. Hennecke). This deletion mutant was found to be generated by recombinational fusion of distant reiterated sequences (RS-alpha) present in several copies around the nitrogenase structural genes (Hahn and Hennecke 1987). The deletion in the mutant strain E1-8d1 did not appear to encompass the region III because the mutant contained the same DNA fragment (7.5 kb EcoRI fragment) hybridizing to the probe as the wild-type strain USDA 110 (see lane 1, Figure 20).

3. Physical Mapping of the 5.5 kb EcoRI Fragment

Further characterization of the nod region III required a detailed restriction map, which was constructed by analyzing the fragments produced by digestion with various restriction enzymes in single, double or triple combinations. When necessary, DNA was blotted and hybridized with various fragments to verify the restriction sites (Figure 21). Hybridization between strain USDA 6 genomic DNA and the individual subfragments of the 5.5 kb EcoRI fragment allowed narrowing the essential region to a

2.9 kb XhoI fragment, which spans the Tn5 insertion site. This 2.9 kb XhoI fragment, but not the flanking regions, hybridized to the 1.7 kb EcoRI fragment of USDA 6 genomic DNA (Figure 20). Thus, it is likely that the conserved nod locus is contained in this 2.9 kb fragment.

4. Hybridization between the Region III and the Known Nod Genes Isolated from Rhizobium and Bradyrhizobium Species

As described earlier, no alteration in the hybridization pattern between the wild-type and ML142 was detected when the previously cloned nod genes were used as hybridization probes to genomic DNA from these two strains. Moreover, no cross-hybridization was detected between regions I, II and region III. This suggests that each of these regions define new nodulation gene loci in B. japonicum.

5. DNA Sequence of Parts of the 2.9 kb XhoI Fragment

To understand in more detail the structure of the nod gene(s) in the region III, attempts were made to determine the nucleotide sequence of a portion of the region around the Tn5 insert site. Based on the location of the mutation within the region and the conserved hybridization pattern among other Bradyrhizobium species (Figure 20), a 2.9 kb XhoI fragment was believed to contain the functional nod gene(s). So far, nucleotide sequences of parts of the 2.9 kb fragment (three non-contiguous stretches, ca. 500 bp

each) were determined by the dideoxy chain termination method of Sanger et al. (1977). Further sequencing work is in progress; however, search for homology of the sequences obtained thus far against the DNA sequence data bank failed to find any significant homology to the previously known gene sequences, except for the nodC gene of R. leguminosarum. The homology to the nodC gene consists of a single stretch of 32 bp and is of unknown significance.

C. Phenotypic Properties of Mutation in the Region III

1. Cultural Characteristics of JS314

In an attempt to determine the function of the newly isolated nod gene, a careful characterization of the phenotype of mutant JS314 was performed. The mutant strain JS314 exhibited no observable difference in colony morphology or mucoidy from the wild-type parental strain when tested on RDM or defined medium agar plates. Also, growth of JS314 in minimal medium broth is indistinguishable from that of its wild type parent in terms of doubling time, gross cultural characteristics, and turbidity, indicating that the Nod⁻ phenotype of JS314 did not result from a simple auxotrophic defect. Also, JS314 is actively motile and its subpolar flagellum is observed under the electron microscope (Figure 22A). Taken together, the mutation in JS314 does not seem to be in any essential genes for prototrophic growth on laboratory medium. Interestingly, however, a large

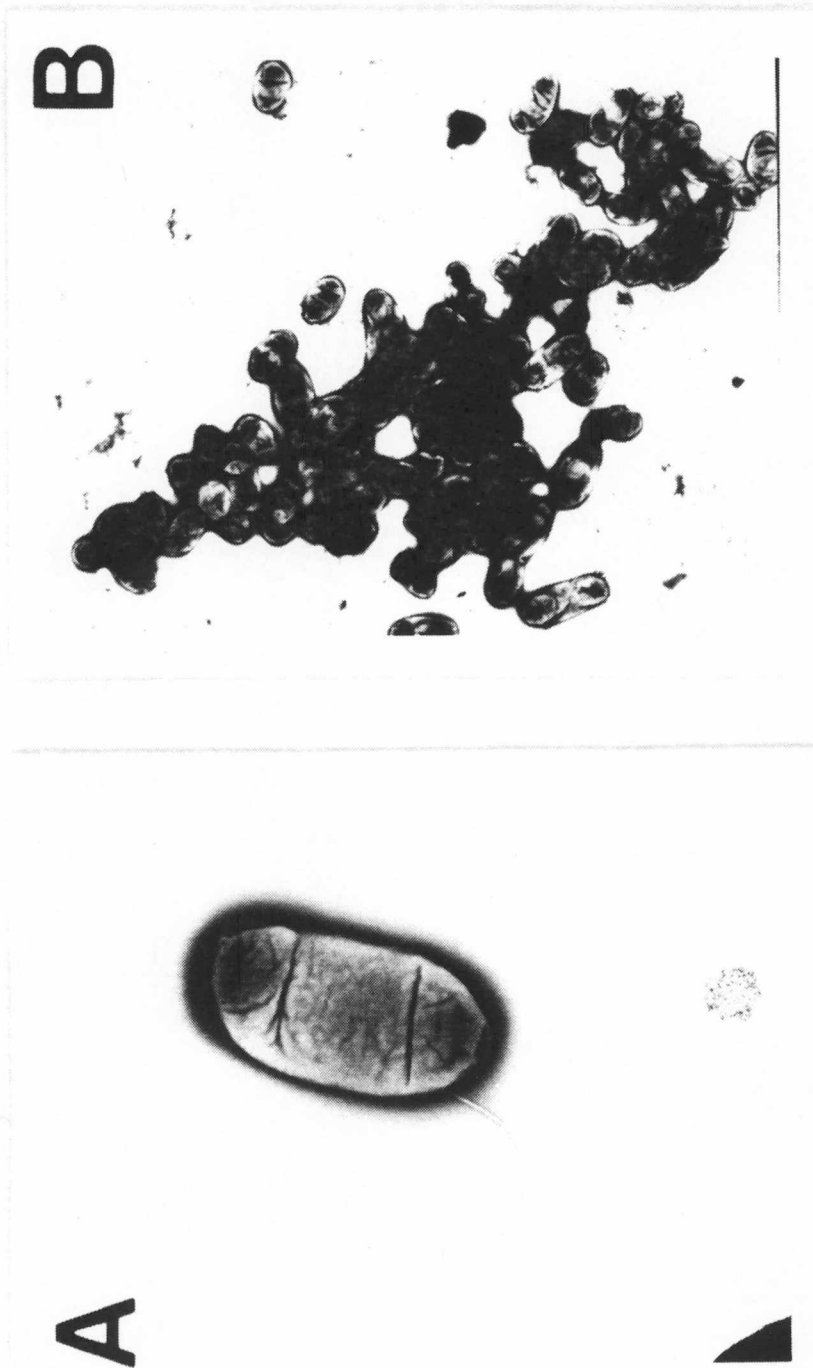


Figure 22. Electron microscopic examination of JS314 cell.
A: Electron micrograph of JS314 cell. Single subpolar flagellum is present. Magnification is 28,000 X. B: Electron micrograph of a clump formed by JS314. Magnification is 7,000 X.

proportion of JS314 cells in liquid medium aggregate to form sizable clumps (Figure 22B), which are not observed in wild type culture. This clumping is observed at all phases of growth. Moreover, when the cells were collected by centrifugation, JS314 cells formed a "rocket"-shaped pellet rather than the compact round pellet formed by wild type cells (Figure 23). Interestingly, when JS314 cells were recentrifuged after being washed in PBS, a round compact pellet was formed. Taken together, these data suggest that the mutation in JS314 causes considerable modification of the cell surface or an extracellular component(s).

2. EPS Analysis of JS314

In order to investigate the differences in the cell surface polysaccharides of JS314, the EPS was isolated and sent to V. Puvanesarajah for analysis. After lyophilization, the EPS fraction isolated from JS314 differed from that of wild type; the wild type EPS was flaky while JS314 EPS looked like a cotton-ball. Chemical analysis of the EPS samples from the two strains by gas chromatography showed a significant difference in sugar composition. The two sugars, rhamnose and glucuronic acid, which have been shown to be a major component of EPS of B. japonicum (Dudman 1978), were present in almost identical ratios in the two samples but the glucose content was considerably higher in JS314 than in the wild type. The percentage ratios of the three sugars content

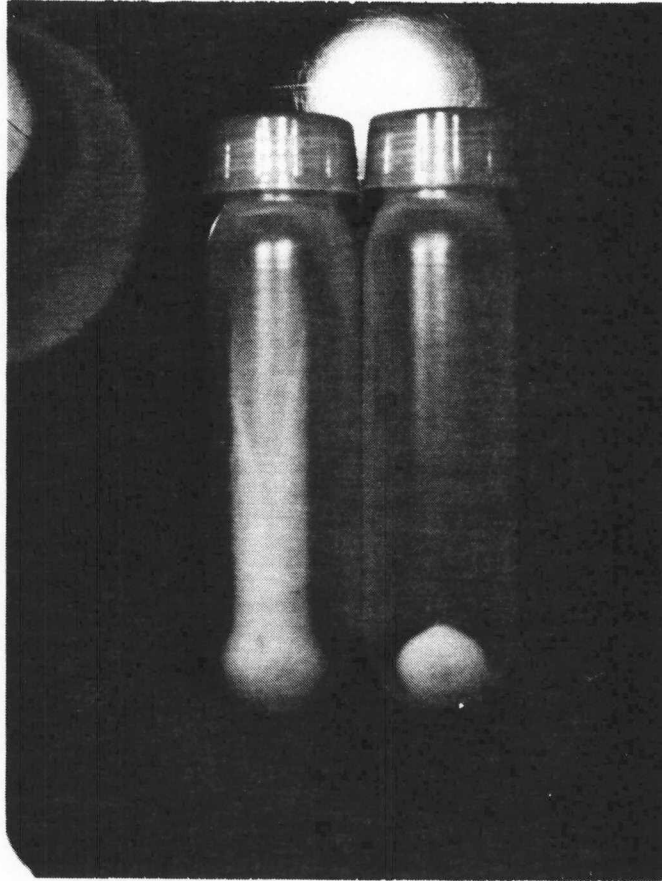


Figure 23. Abnormal cell pellet formed by JS314 cells after centrifugation. Photograph of 61A101C (right) and JS314 (left) cell pellets formed by centrifugation. Thirty milliliter of 6-day old cultures were harvested by centrifugation at 10,000 rpm with Beckman JA-17 rotor.

(rhamnose:glucuronic acid:glucose) were 54:13:8 and 27:7:50 for wild-type strain 61A101C and JS314 respectively. These differences are currently under investigation, but preliminarily suggest that JS314 may overproduce beta-2-glucan.

3. Protein analysis of JS314

The cytoplasmic and membrane protein profile of JS314 were compared with that of the wild-type parental strain by one-dimensional SDS-PAGE. The cytoplasmic protein profiles were indistinguishable between the two strains. However, when the membrane-associated proteins from the two strains were compared, JS314 was found to lack a protein band of 14 kd in size, that was present in wild-type strain (Figure 24).

4. Nodule-like Structures Formed by JS314

As described earlier, JS314 does not induce nodule formation, although the curling of root hair cells (first visible sign of successful infection) is observed by light microscopy of soybean seedling roots following inoculation with JS314. However, during further nodulation tests it was noticed that at a low frequency JS314 could induce 1 to 2 anomalous protrusions on the primary root of certain cultivars of soybean after a long delay (ca. 17 days after inoculation). These structures had a callus-like appearance

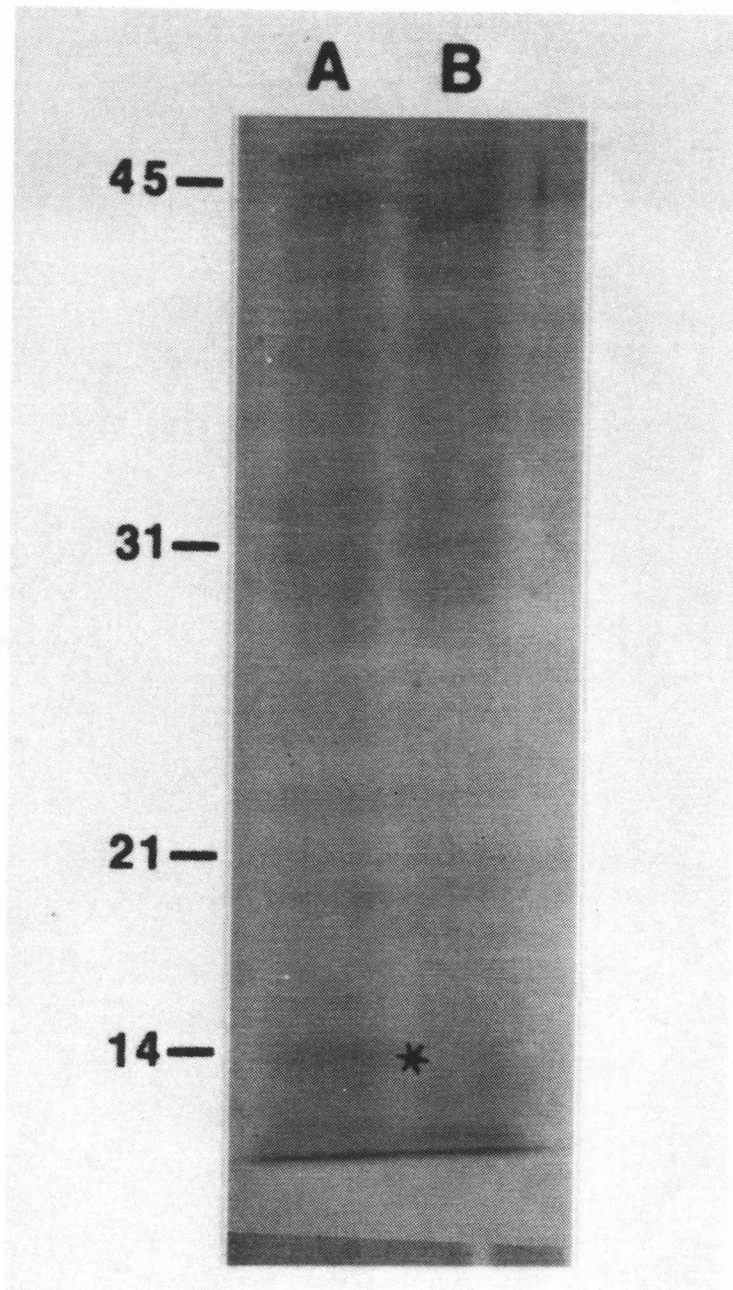


Figure 24. Profiles of membrane fraction proteins of JS314. Membrane-associated proteins were isolated from wild-type (A) and JS314 (B), fractionated through SDS-PAGE, and stained with Coomassie blue. The missing polypeptide band is marked with the star. The polypeptide band (ca. 31 kd) seen in the mutant sample is neomycine phosphotransferase encoded by Tn₅. Size markers on left are in kd.

with a white fuzzy covering and were given a trivial, descriptive name, "popcorn" nodule. Interestingly, the frequency of popcorn nodule formation varied among different cultivars of soybean (Table IV), suggesting a possible interaction between the mutation in JS314 and the host plant's genotype.

These "popcorn" nodules were analyzed by light and electron microscopy (performed in conjunction with Dr. Evans Roth). By light microscopy, it was determined that the popcorn nodule has a single meristematic area that tends to be somewhat depressed in its center (Figure 25B). Unlike normal nodules, a single vascular strand traverses the structure, and is surrounded by several sclereids and many other cells (Figure 25A). Electron microscopic examination of the popcorn nodules showed no evidence of infection threads or bacterial presence. Most of the plant cells contain a central vacuole typical of root cells and have a thin layer of peripheral cytoplasm that contains small numbers of the usual cell organelles (Figure 26). In no cases are cells seen that are typical of normal nodule cells that have no central vacuole and large numbers of Golgi bodies, mitochondria, and amyloplasts and large amounts of endoplasmic reticulum.

The appearance of the popcorn nodules may be due to the cytoplasmic invasion and degradation of the cell walls at intervals (Figure 26), causing cell-wall remnant leaflets to

TABLE IV. Nodulation Phenotype of JS314 on Different Cultivars of Glycine max

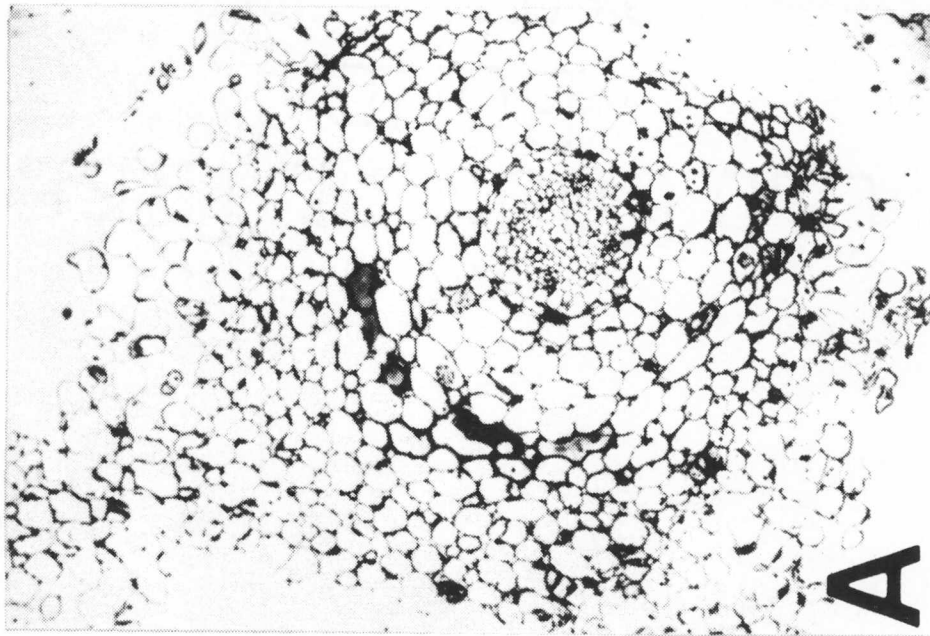
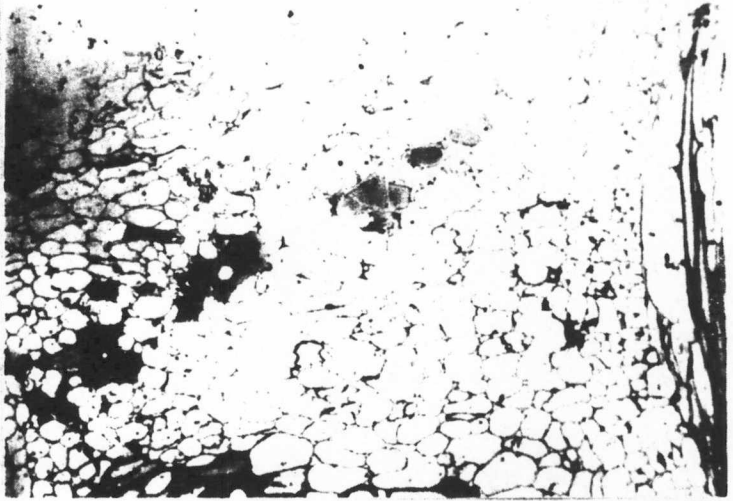
Cultivar	Maturity Group	% Nodulated Plants*	Description of nodules
BSR 101	I	50	white, empty
Centry 84	II	30	white, empty
Clark 63	IV	54	small, empty
Clay	0	25	small, white
CN 290	II	0	
Dawson	0	0	
Douglas	IV	10	popcorn
Elf	III	0	
Essex	-	7	popcorn
Fayette	III	0	
Gnome 85	II	50	white, fuzzy
Hack	II	25	small, white
Harosoy 63	II	0	
Hodgson 78	I	33	white, empty
Hardee	-	0	
Pella	III	22	popcorn
Peking	-	0	
Pershing	IV	25	popcorn
Pyramid	IV	0	
Union	IV	0	
Williams	III	0	

* Ten to fifteen plants were inoculated with 1 ml of early stationary phase culture (ca. 10^8 cells/ml). After 28 days, nodulation phenotype was examined, and N_2 -fixing ability was checked by acetylene reduction assay. All the nodule-like structures examined did not fix nitrogen.

Figure 25. Light micrographs of nodule-like structure formed by JS314. Azure-stained sections of a plastic-embedded nodule-like structure were examined. (Courtesy of Dr. Evans Roth)

(A). Cross section of the sample. In the center of the structure, there are primitive vascular systems. Magnification is 220 X.

(B). Longitudinal section of the sample. Many cells have been induced to proliferate but do not organize into a typical nodule organization. Cells eventually die, leaving behind pieces of cell walls. Upper: The center area is composed of cells that have only a thin layer of cytoplasm. Cell wall remnants from dead cells are being cluffed off around the edges of the "nodule". Magnification is 200 X. Lower: At the root, which is located at the bottom of the field, a double palisade of cells is typically seen. Cells throughout the popcorn nodule show very little cytoplasm. Magnification is 200 X.



B

A

Figure 25

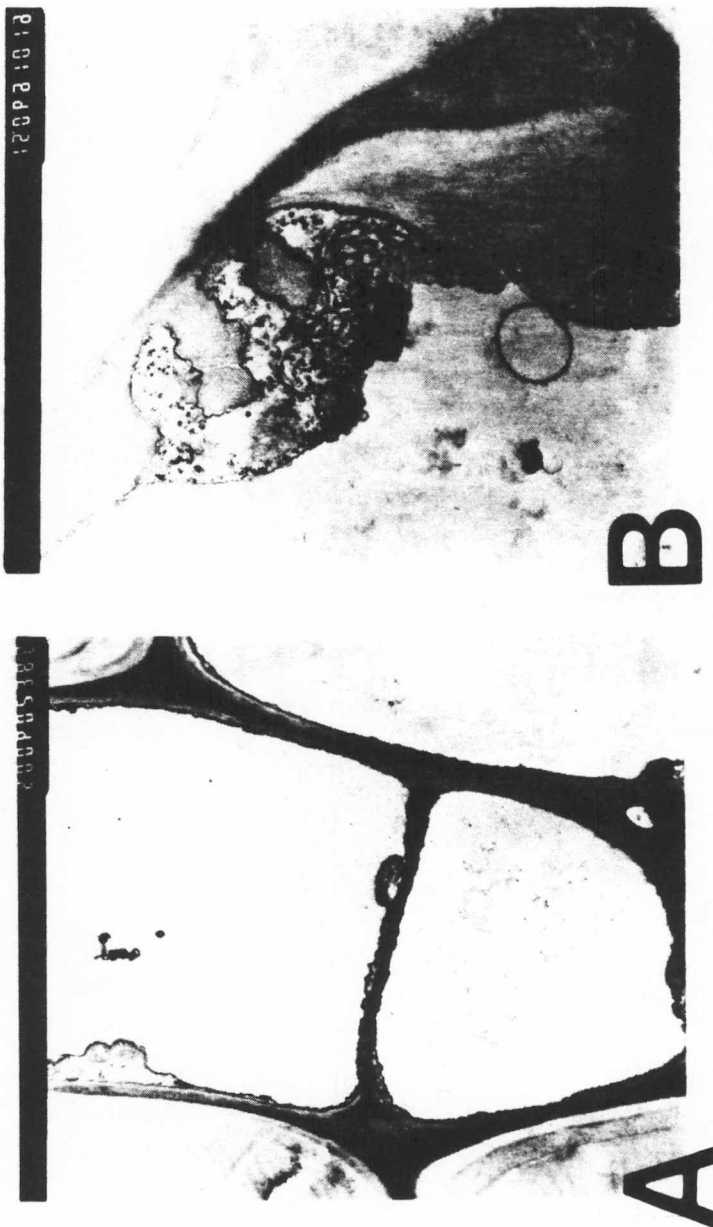


Figure 26. Electron micrographs of nodule-like structure formed by JS314. Plant cell has a very thin cytoplasm (A). Magnification is 12,500 X. In some part of the cell, cell wall is engulfed by cytoplasm and appear to be degraded (B). Magnification is 37,500 X. This might be responsible for the detachment of outerlayer cells from the structure. (Courtesy of Dr. Evans Roth)

be formed. The cells then die, leaving a layer of such leaflets covering the popcorn nodule (Figure 26). At the tips, the popcorn nodule has a protruding ring of such leaflets, but in its center, the meristem precludes this action, so that the top has a depressed darkened center, hence the popcorn terminology seems quite apt.

CHAPTER IV

DISCUSSION

PART I

A. Isolation and Characterization of Tn5-induced Mutants of B. japonicum

Twenty five Sym⁻ mutants of two strains of B. japonicum USDA 110 and 61A101C were isolated by transposon Tn5 insertional mutagenesis and subsequent nodulation tests of 6926 Km/Sm-resistant transconjugants on soybean plants. Transposon mutagenesis provided several advantages over conventional U.V. or chemical mutagenesis: 1) Most mutants were generated from a single mutagenic event. 2) Mutants were tagged with antibiotic resistance markers of the transposon such that one could follow the mutants by the drug resistance. 3) Some of the mutated regions were successfully cloned by taking advantage of the phenotypic marker of the transposon. The transposon Tn5 has been used to generate random symbiotic mutants in a number of Rhizobium and Bradyrhizobium species (Beringer et al. 1978; Cen et al. 1982; Scott et al. 1982; Meade et al. 1982; Forrai et al. 1983; Hom et al. 1984; Rostas et al. 1984; Applebaum et al. 1985) and the resulting mutants were used to clone the DNA sequences essential for nodulation (Scott et al. 1982; Chakravorty et al. 1982; Downie et al. 1983; Scott et al.

1985). Hybridization analysis of the 25 Sym⁻ mutants indicated the presence of Tn₅ within their genome. Vector DNA was found co-integrated into the genome in 3 Fix⁻ mutants of strain 61A101C. These latter mutants could also be used to clone adjacent genomic DNA as described by Donald et al. (1985), although this was not pursued in this study.

The Nod⁻ mutants isolated (ML115, 118, 119, 127, 129, 142, 144) in this study are the first Tn₅-induced non-auxotrophic Nod⁻ mutants of B. japonicum to be reported. The low frequency of Nod⁻ mutants (7/6924) in this study is consistent with the results of others (Maier and Brill 1976; Stacey et al. 1982). Hom et al (1984) isolated Fix⁻ mutants of B. japonicum USDA 110 at a frequency of 3%, which is higher than the level at which Fix⁻ mutants of 61A101C were obtained (14/3,300 = 0.4%). The reason why we were unable to obtain Fix⁻ mutants of USDA 110 is unknown although a number of explanations are possible. No controlled studies were performed, however, we did notice a tendency for Tn₅ insertions in B. japonicum to be unstable. Since the plant exerts a selection for a Sym⁺ phenotype, any Fix⁺ revertants would be favored in plant tests. Due to the potential instability of Tn₅ insertions in B. japonicum, the significant level of co-integrates formed, and the apparent generation of secondary mutagenesis events (for example ML125), Tn₅ mutants of B. japonicum should be analyzed carefully.

Sadowsky et al. (1986) obtained His⁻Nod⁻ mutants of B. japonicum strain USDA 122 by random Tn5 mutagenesis. We also isolated such mutants (ML116 and 117) suggesting that, at least in some B. japonicum strains, histidine auxotrophy may result in a Nod⁻ phenotype. When histidine was supplied to the plant growth medium, the defective nodulation phenotype of ML117 was not restored. This is in contrast to the result reported by Sadowsky et al. (1986) which indicated that histidine-amended plants formed as many effective nodules as wild-type strain.

B. Cloning of Two Nodulation Gene Regions from B. japonicum strain USDA 110 (regions I and II)

Since strain USDA 110 is the B. japonicum strain most widely used for genetic studies (Hennecke 1981; Fischer and Hennecke 1984; Russell et al. 1985; Adams et al. 1984; Nieuwkoop et al. 1987; Hahn and Hennecke 1988), the 3 Nod⁻ mutants and one His⁻Nod⁻ mutants of strain USDA 110 were the initial focus of research. Hybridization of Tn5 sequences to genomic DNA regions carrying Tn5 suggested that two unique gene regions were mutagenized; that is region I defined by ML118 and 119, and region II defined by ML115 and 117. These two DNA regions were successfully cloned into E. coli by taking advantage of the drug resistance marker encoded by Tn5. Individual regions were marker exchanged into the wild type strain to generate site-specific

mutations. Homogenotes corresponding to ML117 and ML118/119 showed a Nod⁻ phenotype on both Glycine max and G. soja. With the cloned mutated region from ML115, a homogenote was not obtained. The reason for this failure is not clear, but is interesting in that the two transposons positioned 1.0 kb apart behaved quite differently.

The cloned region II from ML115 and ML117 is of particular interest since ML117 is His⁻Nod⁻ while strain ML115 is His⁺Nod⁻. Strain ML115 was found to be a prototroph by repeated tests for growth on minimal medium. Also, growth by ML115 was not stimulated by histidine addition. Therefore, the mutation in ML115 identifies a nod locus linked to a his locus. However, the possibility cannot be eliminated that the ML115 mutation affected amino acid metabolism in such a way that the defect was only manifested during the symbiosis.

The cloned regions I and II do not appear to be linked to the common nod genes and appear unique for the following reasons. Firstly, light microscopic examinations of soybean root hairs inoculated with these mutants showed marked curling (data not shown). Secondly, when the common nod regions of USDA 110, which were isolated previously in our laboratory (Russell et al. 1985), or cloned nif DNA (Hennecke 1981; Adams et al. 1984) were used as hybridization probes to genomic DNA, no alterations in the hybridization pattern of the 4 mutants were observed

compared with the wild-type. Thirdly, the EcoRI restriction map of the cloned regions (Figures 8 and 9, pages 77 and 78) shows no apparent similarity to the previously cloned regions. Lastly, hybridization of the R. meliloti nodABCD and hsnABCD and R. leguminosarum nodIJ genes to genomic DNA of the mutants indicated that the Tn5 mutation was not in a region homologous to these probes. Therefore, the two DNA regions probably define new gene loci involved in soybean nodulation. From the EcoRI restriction maps of the two regions, it can be assumed that these two regions are separated by at least 50 kb. Using the cloned Tn5 mutagenized regions as probes, the wild-type counterparts were isolated from a wild-type gene library. Further investigation of the wild-type DNA sequences will provide more information on the genes involved in the nodulation process between soybean and B. japonicum.

C. Nodulation Phenotype of Mutation in the Two Regions

Initial nodulation tests revealed that the site-specific mutation in two regions resulted in a Nod⁻ phenotype. However, in further nodulation experiments using a different nodulation assay method (the pouch assay, see Chapter III), the two homogenotes JS831 and JS721 exhibited only a 3-to-4 day delay in the onset of nodulation and formed fewer nodules than wild-type, rather than a complete Nod⁻ phenotype. This discrepancy was due to the different

plant growth conditions in the two assay systems because the original mutant strains (ML118 and 117) also showed a 3-to-4 day delay in nodule appearance when tested by the pouch assay. It is conceivable that the plant is under stress when it is grown in a bottle with a covering plastic Wirlpak bag. There should be a reduced light intensity and a reduced air circulation due to the plastic bag. Indeed, the same plants grown in bottles without a Wirlpak bag showed an increased nodulation phenotype. As an example, 10 plants inoculated with JS721 were grown in bottles with the plastic bags and the other 10 plants without bags. With the plastic bag, only 3 out of 10 were nodulated, whereas 7 out of 10 plants were nodulated without bags. Therefore, it is advisable that results obtained from a plant infection test should be carefully examined.

The slow-to-nodulate phenotype shown by JS831 and JS721 appeared to be due to defects in nodule initiation rather than nodule development because the nodules formed were normal in terms of their morphology and nitrogen-fixing ability. Halverson and Stacey (1985) examined a slow-to-nodulate phenotype of B. japonicum (strain HS111) and found that the nodules eventually formed were significantly below the root tip marked at the time of inoculation. Preliminary experiments showed that the average distance of the uppermost nodule from the root tip mark made at the time of inoculation was 2.7 cm and 2.4 cm below the mark for JS831

and JS721, respectively, compared with 0.5 cm for the wild-type strain. Although this nodulation phenotype is similar to the one reported by Halverson and Stacey (1985), the following observations are in contrast to this assumption. Firstly, unlike HS111, the slow-to-nodulate phenotype of JS831 and 721 was not reversed by pretreatment of the inocula with soybean lectin. Secondly, when restriction enzyme digests of genomic DNA of HS111 and wild-type strain were hybridized to the cloned region I or II DNA, no differences were observed between the two strains, suggesting that the mutations in JS831 and 721 were not in the same region as the mutation in HS111. However, the possibility cannot be excluded that HS111 has a mutation which is not discernable by this hybridization experiment (e.g. a point mutation or a small deletion) because it was isolated after U.V mutagenesis (Stacey et al. 1982)

Recently, Hahn and Hennecke (1988) reported the cloning of a new nodulation gene region from B. japonicum by genetic complementation of a deletion mutant derivative of USDA 110, E1-8d1 which had a large deletion (ca. 300 kb) around the nif genes. The nodulation phenotype of the strain E1-8d1 was similar to that of JS831 and JS721 in that the nitrogen-fixing nodules were formed on soybean with a delay of 4 days (see below). However, the regions I or II did not appear to be located within the region deleted in the strain E1-8d1 because the deletion mutant retained the EcoRI fragments of

the wild-type strain USDA 110 that hybridized to the region I and II DNAs as shown in Figures 12 and 13 (pages 84 and 85), respectively. This hybridization also confirmed that the regions I and II were not closely linked to the nif genes.

Putnoky and Kondorosi (1986) identified a DNA region analogous to the regions I and II on the R. meliloti Sym plasmid closely linked to the common nod and hsn genes that influenced the kinetics and efficiency of nodulation. This region was termed efn (efficiency of nodulation). Deletion of this region in R. meliloti resulted in a 10-day delay in nodule appearance and fewer number of nodules when compared to the wild-type strain. The differences in days of delay (10 days for efn and 3 to 4 days for regions I and II mutations) may be explained by differences between alfalfa and soybean seeds. The R. meliloti efn region contains the conserved nod box sequence, but the regions I and II do not appear to carry nod box-like sequence by hybridization. It remains to be seen whether the efn region is the R. meliloti counterpart of the regions I or II.

PART II

A. Molecular Cloning of an Essential Nodulation Gene Region from B. japonicum Strain 61A101C (region III)

The objective of this study was to search for further nodulation genes in the slow-growing B. japonicum which are

essential for the nodulation process. Since the above two gene regions cloned from strain USDA 110 were found not to be absolutely essential for the nodulation process, the other 4 Nod⁻ mutants of strain 61A101C (ML127, 129, 142 and 144) were examined. The experience in studies described in part I showed that the nodulation phenotype of mutants observed in the bottle assay must be interpreted with caution, and an additional assay needed to be conducted to support the results. Therefore, the nodulation phenotype of these mutants were carefully reexamined by two different nodulation assay systems, the bottle assay and the pouch assay. Three mutants were selected which showed a Nod⁻ phenotype irrespective of the plant growth condition.

The mutated gene region was isolated from ML142 and confirmed to be essential for the nodulation process by analysis of a site-specific mutation obtained using the cloned, mutated DNA region. The result indicated that nod region III was unique and appeared to define a new nodulation locus; hybridization of the region with other, previously known, nod genes revealed no detectable sequence similarity under the conditions used.

B. Molecular Characterization of the Region III

Using the cloned, mutated DNA as a probe, a wild-type clone was isolated from a gene bank by colony hybridization. This cloned, wild-type region was characterized in further

detail. All B. japonicum strains examined contained a single hybridizing fragment to the region III probe, suggesting that the nod gene in the region III is present in a single copy in these bacteria. The lack of homology of the region III to genomic DNA from R. fredii or Rhizobium sp. strain NGR234, other soybean endosymbionts, suggests that the gene affected by Tn5 insertion has a general function in nodulation and is not likely to be involved in host specificity. However, the possibility cannot be excluded that the two rhizobia have a sequence corresponding to the nod region III which is too divergent to be detected under the hybridization conditions used.

The conserved hybridization pattern of the region III among the slow-growing rhizobia could be useful in strain identification as different rhizobia can be distinguished by their unique hybridization pattern. This is also the case for the region II DNA (Figure 12, page 84). The limited sequence conservation shown by the region I can be used to distinguish group I and II B. japonicum (Stanley et al. 1985) because it hybridized only with the group I B. japonicum (Figure 13, page 85). Together, the three cloned regions could be used for taxonomic, as well as ecological, studies of slow-growing Bradyrhizobium strains (manuscript in preparation).

The function of the nod gene(s) in the region III and the precise nature of the molecular defect caused by the

mutation are not known. Although the molecular genetics of nodulation is progressing rapidly, the biochemical function of the nod genes identified to date are unknown. This is mainly because the nodulation genes have been defined only by their phenotypic characteristics. Therefore, most of the approaches employed in attempts to determine their biochemical functions have been indirect (for example, DNA sequence determination and comparison with other known gene sequences or careful phenotypic characterization of the mutation in the nod genes). Hence, it was hoped that the sequence data of region III, particularly around the mutation site, would suggest more direct research avenues for defining gene function.

At the time of this writing, the sequence data are not complete. However, the partial sequence data appear to provide some interesting clues as to the region. Firstly, the joined sequences of three non-contiguous stretches (ca. 500 bp each) were compared with gene sequences in Genbank, but no significant sequence conservation was found. Secondly, there are no sequences significantly similar to the nod box sequence. Thirdly, there is a potential open reading frame (ORF) which might span the mutation site. The ORF has a prospective ribosome binding site (Shine-Dalgarno sequence) and the typical prokaryotic promoter sequences [-10 (Pribnow box) and -35 sequences] 5' to a possible ATG start codon. The exact length of this ORF awaits further

sequencing of the region around the PvuI site close to the Tn5 insertion site (Figure 21, page 101). Interestingly, upstream of this ORF there is a 32 bp stretch showing 81% sequence conservation with the nodC gene of R. leguminosarum. The significance of this homology remains to be established.

C. Phenotypic Properties of Mutation in the Region III

The region III mutation does not appear to affect housekeeping genes because the mutant strain JS314 is a prototroph and its general growth characteristics are the same as the wild-type parental strain. However, the mutation may affect nodulation due to pleiotrophic effects on subtle cell structures or functions which become more important in the symbiotic process.

Based on the preliminary observations made concerning the cell surface of JS314 (clumping and rocket-shaped cell pellet), it was believed that JS314 has an altered cell surface. The disappearance of the rocket-shaped cell pellet in the second centrifugation after being resuspended in PBS suggested that the different behavior of the JS314 cells in the centrifugal field was due to some extracellular component(s) present in the culture medium (or some component bound loosely to the cell surface). In order to identify the alteration, JS314 EPS was compared with that of the wild-type parental strain using gas liquid

chromatography. The mutant EPS contained considerably higher content of glucose. The exact cause of this increased glucose content is not known. Presumably, the increased glucose content in the JS314 EPS fraction was due to overproduction of beta-2-glucan (personal communication with Dr. Puvanesarajah). Further studies on the EPS fraction of JS314 are underway to quantitatively measure the beta-2-glucan content. The overproduced beta-2-glucan might explain the rocket-shaped pellet formed by JS314 because it could make culture medium more viscous so that the movement of cells lagged under centrifugal force.

How might the mutation in JS314 cause an overproduction of beta-2-glucan? One possible explanation came from the observation made on the JS314 cells grown in liquid medium, that is, clumping of cells may have resulted in overproduction of beta-2-glucan. The relationship between reduced oxygen pressure and beta-2-glucan production is not unprecedented. The R. trifolii EPS fraction from a culture grown at low oxygen pressure contained a higher amount of beta-2-glucan (Zuvenhuizen 1986). Alternatively, the mutated gene might code for a protein involved in the regulation of the beta-2-glucan synthesis or secretion (see below). It would be interesting to see whether the glucose content in JS314 EPS decreases with increased oxygen supply to the culture medium.

Protein analysis revealed that JS314 lacked a protein that was closely associated with the cell membrane. The simplest interpretation of this result, together with the Nod⁻ phenotype of JS314, is that the Nod⁻ phenotype of JS314 is a consequence of its defect in synthesis of the 14 kd membrane protein. The finding that the protein is closely associated with membrane is consistent with the observation that JS314 has considerable cell surface modifications. The function of this protein is unknown. It is tempting to suggest that the protein plays a role in bacterial surface modification during the infection process.

The observation that JS314 could induce root hair curling indicates that the mutation in JS314 blocks a step(s) in nodule formation beyond root hair curling. The anomalous structures (so called popcorn nodules) formed by JS314 on certain cultivars of soybean were studied by light and electron microscopy. It was hoped that this structural analysis of the popcorn nodules would provide clues as to the possible function of the nod gene in the region III. The popcorn nodule differed from the wild type nodule in several important features. Firstly, there was no sign of infection thread proliferation within the structure, although it was not determined whether limited infection thread growth might be occurring during the earliest stages of infection. Therefore, a simple explanation of the nodulation defect in JS314 would be that the ability of

JS314 to induce infection thread formation or development is blocked. Secondly, the plant cells were completely devoid of bacteria. The failure to isolate bacteria from the popcorn nodules supports the absence of bacteria. Thirdly, instead of having peripheral vascular bundles and a central symbiotic zone as in wild-type nodules, the popcorn nodules had a poorly developed vascular system in a central position. The result was a structure that had a single meristematic region, had a very simple vascular system, and contained only empty cells. Lastly, only 1 to 2 popcorn nodules developed per plant (approximately 1 out of 15 plants with Glycine max cv Essex) and these were always on the primary root. The differences in the frequencies of popcorn nodule formation among cultivars may be explained by the different responsiveness to infectivity of JS314.

There are numerous possible explanations for the block in infection thread formation. For example, JS314 may be recognized by the host plant as being incompatible (i.e. as a pathogen), probably due to altered cell surface features (or a lack of proper modifications) and elicit a plant defense response. Recently, a plant response similar to that seen with incompatible host-pathogen interaction was reported from ultrastructural studies on the infection process between host plant siratro and a mutant of Rhizobium strain NGR234 (Djordjevic et al. 1988). The mutant induced a rapid accumulation of osmiophilic droplets in the cells

around the inoculation sites between 15 and 24 hr after inoculation. The mutant appears similar to JS314 in that its acidic EPS components are identical to that of the wild-type strain, but its beta-2-glucan content is different. However, we have not detected any osmiophilic granules with the structures induced by JS314. Another possibility is that JS314 may lack enzymes necessary for the penetration of root hairs. Evidence for host cell wall degradation at the infection site has been reported for the soybean-B. japonicum symbiosis (Calvert et al. 1984). The production of hydrolytic enzymes (e.g. pectinolytic and cellulolytic enzymes) by rhizobia is known (Hubbell et al. 1978), and has been postulated to play a role in host plant cell wall degradation at the infection site (Callaham and Torrey 1981). Alternatively, the aggregated JS314 cells could be a direct physical cause of blockage of penetration. However, this seems to be less probable since untreated JS314 cells or those completely dispersed by vortexing induced a similar nodulation phenotype (data not shown). Also, the clumping of JS314 cells in liquid medium does not necessarily predict the behavior of the bacteria in the rhizosphere.

To my knowledge, this is the first report on the cloning and characterization of a gene region from B. japonicum that affects cell surface components and concomitantly affects nodulation of soybean. The results gained in this study have firmly established that the cloned

gene defines a unique and new nodulation gene. In order to determine the exact role the cell surface components play in the B. japonicum-soybean symbiosis, an understanding of the organization and regulation of genes responsible for the synthesis of surface components is required. Further studies on the cloned region III will provide better understanding of the cell-cell communication between plant and microbe during the nodulation process.

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