Characterization of nodulation defective mutants of *Bradyrhizobium japonicum*

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Abstract

The Rhizobium-legume symbiosis is an opportunistic association between two symbiotic partners that results in the formation of the root nodule. The process depends on the expression of a number of plant and bacterial genes that are considered critical for the establishment and maintainance of the symbiotic state. The merits of a mutational approach to the analysis of symbiosis have been recognized for several years and transposon Tn5 mutagenesis of Rhizobium has led to the identification of several symbiotic genes. This study describes the use of Tn5 mutagenesis for the isolation of symbiotically defective mutants of Bradyrhizobium japonicum. Two classes, auxotrophic and cell surface-altered mutants defective in nodule formation, have been characterized. In B. japonicum USDA 122, histidine auxotrophs that are defective in nodulation have been studied. The mutagenized DNA region has been cloned and the wild-type DNA region isolated by hybridization and complementation. In B. japonicum 61A76, Tn5-induced cell surface-altered mutants have been isolated by selecting for bacteriophage resistance. Several parameters have been used to demonstrate alterations in cell surface components. It has been shown that the Tn5 insertion is not the primary cause of the mutation in two of the characterized mutants. Complementation tests have led to the isolation of a wild-type DNA-containing cosmid, pPS23A, that overcomes the symbiotic defect in one of the mutants. Analysis of the cell

surface showed a partial restoration of surface components in the complemented mutant.

Résumé

La symbiose légumineuse-Rhizobium est une association entre deux partenaires symbiotiques qui résulte en la formation d'un nodule racinaire. Ce processus dépend de l'expression de gènes de la plante et de gènes bactériens considérés critiques pour l'établissement et le maintien de la symbiose. La mutagénèse chez Rhizobium à l'aide du transposon Tn5 a permis l'identification de plusieurs gènes symbiotiques. Plusieurs mutants défectifs pour la symbiose ont été isolés chez Bradyrhizobium japonicum, grâce à la mutagénèse avec Tn5. Deux classes de mutants défectifs pour la formation de nodules racinaires ont été identifiés: des mutants auxotrophes et des mutants dont la surface cellulaire est altérée. Les mutants USDA 122 auxotrophes pour l'histidine chez B. japonicum, défectifs pour la nodulation ont été etudiés. La partie de l'ADN mutagénéisé a été cloné et l'ADN sauvage à été isolé par hybridation et complémentation. Des mutants de B. japonicum 61A76, dont la surface cellulaire est alterée par le transposon Tn5, ont été isolés par sélection à la résistance aux bactériophages. Plusieurs paramètres ont été utilisés pour démontrer une altération de composantes de la surface cellulaire. Nous avons démontré que l'insertion du transposon Tn5 n'est pas la cause principale de la mutation de deux mutants caractérisés. Des expériences de complémentation ont permis l'isolement de l'ADN sauvage contenu dans le cosmide pPS23A,

ce clone restaure les propriétés de symbiose chez un des mutants étudiés. L'analyse des composantes de la surface cellulaire chez ce mutant complémenté a révélé une restauration partielle de ces composantes.

Traduit par Francis Ouellette et Mario Filion.

Preface

This thesis has been assembled in accordance with the regulations of the Faculty of Graduate Studies and Research of McGill University. It consists of an abstract, resume, Chapter I (Introduction and literature review), Chapter II (Methods and Materials), Chapter III (Results), Chapter IV (Discussion), and Chapter V (References).

The results reported in Section III.1 of this thesis on the transposon mutagenesis of Bradyrhizobium japonicum 61A76 were published as part of a paper on "Transposon mutagenesis of "Rhizobium japonicum", by Rostas KR, Sista PR, Stanley J and Verma DPS (1984) in Molecular and General Genetics, volume 197, pages 230-235. All the work involving B. japonicum 61A76 described in the paper was done by the author.

The biochemical characterization of histidine auxotrophs of B. japonicum USDA 122 (Section III.2 in this thesis) was published as a paper entitled "Symbiotically-defective histidine auxotrophs of Bradyrhizobium japonicum USDA 122", by Sadowsky MJ, Rostas KR, Sista PR, Bussey H and Verma DPS (1986) in Archives of Microbiology, volume 144, pages 334-339. The work described in sections III.2.1 and III.2.2 was done in collaboration with Dr. Sadowsky and Dr. Rostas. The work in sections III.2.3 and III.2.4 was done solely by the author.

A paper on the isolation and characterization of B. japonicum 61A76 phage-resistant nodulation mutants is being prepared for publication. The electron micrographs which

comprise figures 14, 15 and 16 of this thesis were the work of Dr. M. Neumirth of the EM center of the Biology Department. Dr. F. Dazzo of Michigan State University carried out the proton NMR spectroscopy of polysaccharides and Dr. R. McQueen ran the gel in Figure 20. All the other results were the work of the author.

Contributions to Original Knowledge

I have established a random Tn5 mutagenesis procedure in Bradyrhizobium japonicum 61A76 that has led to the isolation of several symbiotic mutants.

By isolating Tn5-induced mutants, I have shown that auxotrophy for histidine can lead to a defect in nodulation by B. japonicum USDA 122. I have cloned the DNA region that encompasses the mutation in the histidine biosynthetic pathway, and by hybridization and complementation have isolated the corresponding wild-type DNA.

I have isolated and characterized two Tn5-induced B. japonicum 61A76 mutants that are resistant to a specific bacteriophage and have demonstrated gross alterations in their cell surface. These mutants are also defective in proper nodule development. By complementation, I have isolated a DNA region in a cosmid, that overcomes the symbiotic defect in one mutant, and have shown that the cosmid also partially restores surface defects.

Acknowledgements

I thank Professor Desh Pal Verma for his continued interest and support during the course of this work. I am deeply indebted to Professor Howard Bussey for his constant guidance and encouragement, without which this work would not have been completed. I also wish to acknowledge the advice and support of my supervisory committee members, Dr. E. Käfer and Dr. G. Brown. The generous gifts of Bradyrhizobium japonicum 61A76 specific phage from Dr. Gary Stacey and the suicide vector pGS9 from Dr. V.N. Iyer are highly appreciated.

I am grateful to the many visiting scientists, post-doctoral fellows, fellow-graduate students and research assistants in the laboratory who spent their time and patience to be my teachers at the work bench.

I thank Dr. Ashton Delauney for his invaluable suggestions in the writing of this thesis, and Mr. Robert Lamarche and Mr. Guy L'heureux for their excellent photographic services.

Lastly, I would like to thank Indra Verma for her friendship, Nirupama Deshmane for being a wonderful source of strength and inspiration, and my family for their unflagging support throughout my studies.

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Abbreviations

BSA	bovine serum albumin
EPS	exopolysaccharide
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetraacetic acid
kan	kanamycin
kan^r	kanamycin resistant
kan^s	kanamycin sensitive
kb	kilobase
kd	kilodalton
LB	Luria Bertani broth
LPS	lipopolysaccharide
mg	milligram
OD	optical density
PAGE	polyacrylamide gel electrophoresis
pbm	peribacteroid membrane
PBS	phosphate buffer saline
pfu	plaque forming units
rpm	revolutions per minute
RNA	ribonucleic acid
SCP	sodium chloride phosphate
SDS	sodium dodecyl sulfate
SSC	saline sodium citrate
str	streptomycin
TE	10mM Tris-Cl pH 8, 1mM EDTA
TAE	40mM Tris-acetate pH 8, 2mM EDTA

TBS	Tris buffered saline
tet	tetracycline
tet^r	tetracycline resistant
tet^s	tetracycline sensitive
ug	microgram
YEM	Yeast extract mannitol medium

Chapter I: Introduction

Nitrogen and water are the most frequently encountered limiting factors in agriculture. A crucial problem faced by farmers is to provide plants with nitrogen in an easily assimilable form. The common sources of nitrogen reserves in soil result from: (1) the addition of organic matter such as harvest waste, manure, animal cadavers and waste products; (2) addition of nitrogen fertilizers in the form of nitrates, ammonium salts, urea; (3) nitrogen fixed in the atmosphere such as by electrical storms and (4) biological nitrogen fixation by free living and symbiotic micro-organisms. The Food and Agricultural Organization of the United Nations estimates (Drevon, 1983) that available nitrogen in the form of chemical fertilizers contributes 40 million metric tonnes per year, non-biological nitrogen fixation contributes 10 million tonnes per year and biological nitrogen fixation about 175 million tonnes per year. Clearly, biological nitrogen fixation is the most important source of fixed nitrogen in nature.

I.1 Biological nitrogen fixing systems

Micro-organisms that can fix atmospheric nitrogen do so with the help of the enzyme nitrogenase, which converts atmospheric nitrogen to ammonia under certain conditions. The Food and Agricultural Organization estimates that 50% of all nitrogen fixed on Earth comes from the legume-*Rhizobium*

symbiosis. In areas under legume cultivation (grain legumes such as peas, beans, and soybean, or forage legumes such as alfalfa, clover and sanfoin) average nitrogen fixation rates are on the order of 100 kg N/ha/yr, although twice that level can be achieved by selection of efficient Rhizobium strains, suitable varieties of host plants and optimal growth conditions. The relative rates for uncultivated areas (wild legume-associated fixation, cyanobacteria, and free-living bacteria) are on the order of 30 kg N/ha/yr on the one hand and 200 kg N/ha/yr (forest areas where Alnus rubra is widespread), on the other (Drevon, 1983).

I.2 Nodule formation

The legume-Rhizobium symbiosis is an opportunistic rather than an obligatory association. During the process of root infection, the bacteria undergo rapid developmental changes to make the transition from a complex soil environment to an intimate home within host cells, the root nodule. Likewise, the host response to the symbiont is markedly different than the responses accorded to other soil bacteria and it undergoes extensive cellular, biochemical and molecular changes to accommodate its symbiotic partner. The entire process depends on the expression of a number of plant as well as bacterial genes that are considered critical for the establishment and maintenance of the symbiotic state (reviewed by Bauer, 1982; Verma and Long, 1983; Long et al., 1986).

I.2.1 Early events

The infection of legumes by Rhizobium probably begins with a response by the bacterium to nutrients and signal compounds exuded by the root (Bauer, 1982). Rhizobium cells attach very rapidly to the root surface. The attachment is believed to occur in a polar fashion, end-on to the root surface (Tsien and Schmidt, 1977; Dazzo, 1980). Chemotactic behaviour by Rhizobium towards plant exudate has been shown (Currier and Storbel, 1974); however, it is not an absolute requirement as non-motile strains have been shown to nodulate efficiently (Ames and Bergman, 1981).

Host plant lectins have been implicated in the specific recognition and binding of infective Rhizobium to the root surface and Bohlool and Schmidt (1974) were the first to provide experimental evidence of this effect. The lectin hypothesis suggests that host plant lectins interact selectively with compatible Rhizobium cell surface polysaccharides (Bauer, 1982). Dazzo and his colleagues have subsequently published several studies (reviewed in Dazzo and Truchet, 1983; Dazzo and Gardiol, 1984) that support the lectin model for the R. trifolii-clover interaction. More recently Dazzo et al (1984) have defined a two step attachment mechanism for R. trifolii. The first step involves a nonspecific adherence of Rhizobium to the root. The bacteria are subsequently modified in the rhizosphere leading to their specific attachment in a polar fashion. Lectins play an

essential role only in the second step. Lectins have also been implicated in symbioses involving pea (Kato et al., 1981), alfalfa (Paau et al., 1981) and lotonomis (Law and Strijdom, 1984). The hypothesis is strengthened by the observations that lectin binding capability is transient and can be strongly correlated with nodulation abilities, age of the culture, number of lectin receptors on bacterial capsules and number of infection threads initiated (Bhuvaneswari et al, 1983; Dazzo et al, 1979; Sherwood et al, 1984; Hrabak et al, 1981). However, several reports have challenged the lectin hypothesis. In addition to the observation that some Rhizobium cells can bind to lectins from hosts that they do not nodulate (Dazzo and Hubbel, 1976; Wong, 1980), several legumes are also nodulated by more than one species of Rhizobium. Moreover, lectin pretreatment of Rhizobium does not enhance attachment to roots (Pueppke, 1984) and finally, mutant plants have been isolated that lack the seed lectin and yet are nodulated by Rhizobium (Pull et al, 1978; Su et al., 1980). Thus, the idea that host plant lectins and bacterial surface polysaccharides are the sole determinants of recognition, attachment and specificity is probably too simplistic. It is more likely that these events are just a part of a complex sequence of signal-and-response exchanges between the two symbionts and each step contributes to the overall biological specificity (reviewed in Bauer, 1982, Halverson and Stacey, 1986).

The most visible event in the infection process is the

induced curling of root hairs (Fahreus, 1957; Nutman, 1959). The bacteria become entrapped in the curl of the root hair which appears important for the initiation of infection. The host deposits new cell wall material internal to the point at which bacteria are entrapped by the curled root hair (Callaham and Torrey, 1981). The new cell wall material is used to form a tubular structure called the infection thread. The bacteria are carried single file into the infection thread which grows towards the base of the root hair cell (Fahreus, 1957; Nutman, 1959). The formation and extension of the infection thread appears to be associated with increased activity of degradative enzymes such as cellulase and pectinase (Verma et al., 1978) and it is possible that a cooperative action of these enzymes facilitates the progression of the infection thread by loosening the plant cell wall framework (Verma et al., 1978; Robertson and Farnden, 1980). During the growth of the infection thread, root cortical cells are induced to divide and differentiate into the nodule meristem (Libbenga and Harkes, 1973). The infection thread passes through the cell wall of the root hair and begins to branch into the adjacent newly divided cortical cells. The bacteria then bud off from the tips of the infection threads surrounded by a membrane of plant origin called the peribacteroid membrane (pbm) (Bergersen and Briggs, 1958). The bacteria divide, enlarge and differentiate within this envelope to form pleomorphic bacteroids. The cytoplasm of infected cells is

filled with bacteroids which can vary from 1-16 per membrane envelope and eventually as many as 24,000-36,000 bacteroids per infected cell may be present (Gresshoff and Rolfe, 1978). It is not known what induces bacteroid development in the nodule. The formation of bacteroids are characterized by frequent polymorphism, disappearance of polyhydroxybutyrate granules, less condensed nucleoids and alterations in DNA content, heme content, ribosome number, cell wall composition and osmotic sensitivity (Bergersen, 1955; Dart and Mercer, 1963; Mckenzie et al., 1973; Newcomb, 1976; Avissar and Nadler, 1978; Shaw and Sutton, 1979; Vance et al., 1980; Sutton et al., 1981). The genes encoding the nitrogenase enzyme are induced in response to secreted plant factors, although they can also be induced at a low oxygen tension in free-living Rhizobium (Kurz and LaRue, 1975; McComber et al, 1975; Pagan et al, 1975; Tjepkma and Evans, 1975). Some other genes also induced by plant factors are hydrogenase, specific cytochromes and proteins destined for the outer membrane (Sutton et al., 1981).

I.2.2 Host cell modification

The nuclei in infected host cells increase in volume several fold and are often polyploid due to endoreduplication. This is apparently the result of the Rhizobium-secreted hormones cytokinin and auxin (Mitchell, 1965; Libbenga et al., 1973; Libbenga and Torrey, 1973). Often more than one

prominent nucleolus can be detected and the cytoplasm becomes dense, filled with numerous free ribosomes (Newcomb et al, 1979). The area of rough endoplasmic reticulum increases and Golgi bodies, ER and pbm are often seen fusing with smaller vesicles (Robertson et al., 1978a,b). In the latter stages these cells become greatly enlarged.

Changes also occur in the uninfected cells of the nodule which are intermingled with infected cells (Dart, 1977; Newcomb, 1981). It has been suggested that the uninfected cells may specialize in metabolic pathways related to nitrogen fixation (Gunning et al., 1974; Newcomb and Tandon, 1981). Methods separating infected cells from uninfected cells (Hanks et al., 1983) allow the demonstration that for soybean, many of the enzymes involved in nitrogen assimilation are either localized or enriched in uninfected cells. Changes also occur to cells in the nodule vascular tissue. They develop a "transfer cell" morphology (Pate et al., 1979). These cells have increased plasma membrane area due to an involution of the secondary cell wall and are thought to function in the movement of photosynthates and the products of nitrogen fixation (Gunning et al., 1974).

The size, shape, morphology and distribution of nodules is host controlled (Kirby and Goodchild, 1966; Dart, 1977). Nodules may be spherical as in soybean, cylindrical or club shaped as in pea or collar shaped as in alfalfa and lupin. Thus, the host and symbiont undergo several stages of

differentiation to form a highly specialized structure, the root nodule, which is the site of nitrogen fixation. Figure 1 summarizes the temporal sequence of events that are believed to occur during the development of the root nodule.

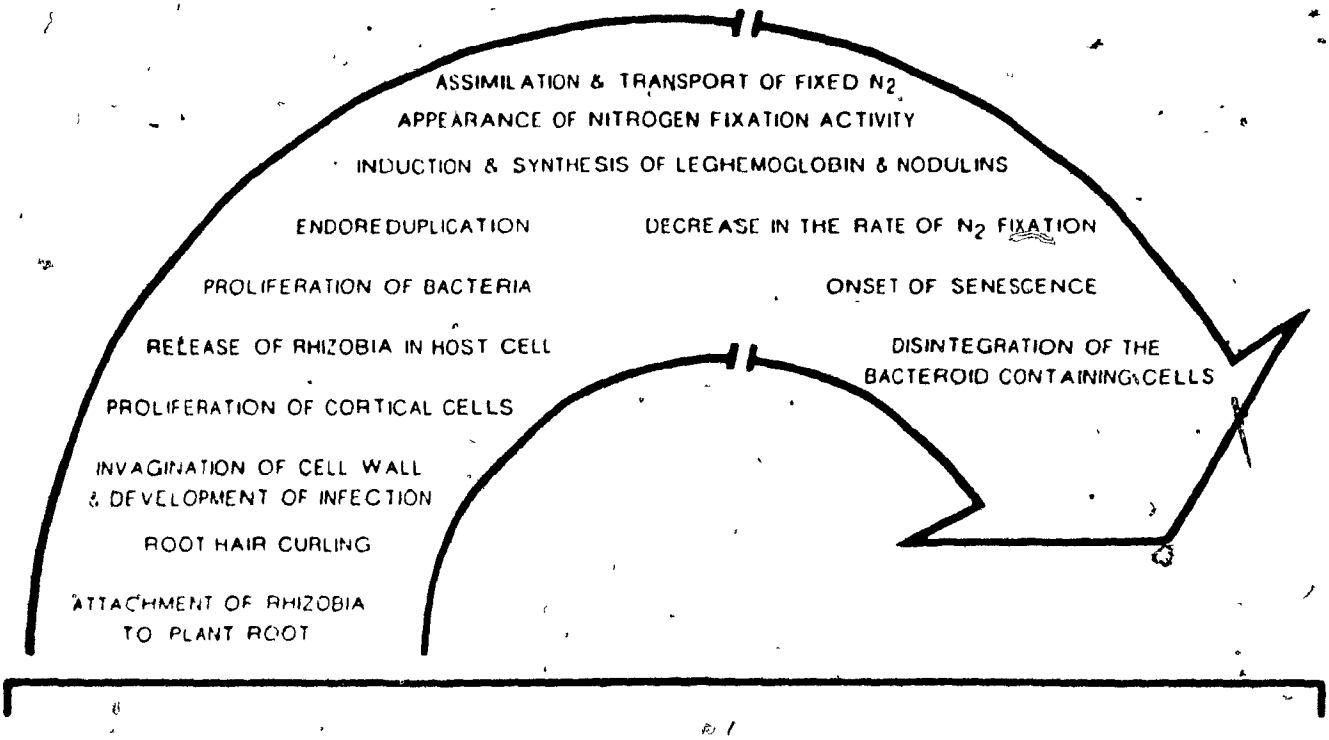
1.3 Nodule function

I.3.1 Biochemistry of nitrogen fixation

The ultimate goal of nodule organogenesis is the complex biochemical process of the reduction of dinitrogen. Rhizobium synthesizes an enzyme complex, "nitrogenase", which is capable of reducing atmospheric nitrogen to ammonia while the host provides energy, carbon compounds and a network of ammonia assimilation pathways. The nitrogenase enzyme consists of 2 proteins : the Mo-Fe protein (dinitrogenase or component I) and the Fe-protein (dinitrogenase reductase or component II). The Mo-Fe protein is a tetramer (a_2b_2) of two dissimilar subunits (mw 50 kd and 60 kd) and contains 2 Mo atoms, 28-34 non-heme iron atoms and 26-28 acid-labile sulfides. The Fe protein is a dimer (y_2 mw 64,000) and contains 4 non-heme iron atoms and 4 acid-labile sulfides (Eady and Postgate, 1974; Hardy and Burns, 1968). In addition to the component proteins, ATP, a reductant, a reducible substrate, Mg^{+2} as an activator and an anaerobic environment are required for nitrogenase activity. The reaction catalyzed by the enzyme is as follows:

$$N_2 + 6H^+ + 6e \longrightarrow 2NH_3$$

Figure 1: Temporal sequence of events following inoculation of a legume plant with Rhizobium leading to symbiotic nitrogen fixation. (Adapted from Verma and Long, 1983).


ASSIMILATION & TRANSPORT OF FIXED N_2
APPEARANCE OF NITROGEN FIXATION ACTIVITY
INDUCTION & SYNTHESIS OF LEGHEMOGLOBIN & NODULINS
ENDOREDUPLICATION
DECREASE IN THE RATE OF N_2 FIXATION
PROLIFERATION OF BACTERIA
ONSET OF SENESCENCE
RELEASE OF RHIZOBIA IN HOST CELL
DISINTEGRATION OF THE BACTEROID CONTAINING CELLS
PROLIFERATION OF CORTICAL CELLS
INVAGINATION OF CELL WALL & DEVELOPMENT OF INFECTION
ROOT HAIR CURLING
ATTACHMENT OF RHIZOBIA TO PLANT ROOT

The flow of electrons through nitrogenase is from reductant (ferredoxin or flavodoxin in cells) to the Fe protein. The electron transfer from the Fe protein to the Mo-Fe protein is coupled to hydrolysis of Mg-ATP. Eventually dinitrogen and protons are reduced at the Mo-Fe protein to produce ammonia (Haaker and Veeger, 1984). The energy required for the reduction of one mole of nitrogen by Rhizobium bacteroids is estimated at 21 moles of Mg-ATP, assuming a hypothetical maximum recovery of ATP from the complete oxidative phosphorylation of glucose (Pate et al., 1981). The enzyme is not substrate-specific as a variety of triple-bond compounds can be reduced. The reduction of acetylene to ethylene is widely used for monitoring the activity of nitrogenase (Hardy et al., 1968). Since both components of nitrogenase are rapidly inactivated by oxygen, various mechanisms have evolved to protect nitrogenase from oxygen damage. These include: an anaerobic habitat as in Clostridium; respiratory protection as in Azotobacter; heterocyst formation as in cyanobacteria; restriction of activity to periods of minimal oxygen-evolving photosynthetic activity as in Gloecapsa; and leghemoglobin protection in legume root nodules (Gallon, 1981). The nitrogenase activity in legume nodules is also associated with hydrogen production, an inevitable consequence of the chemistry of nitrogen fixation (Hoch et al., 1957). The wastage of energy in hydrogen evolution can be as high as 30% (reviewed by Eisbrenner and Evans, 1983). However, certain

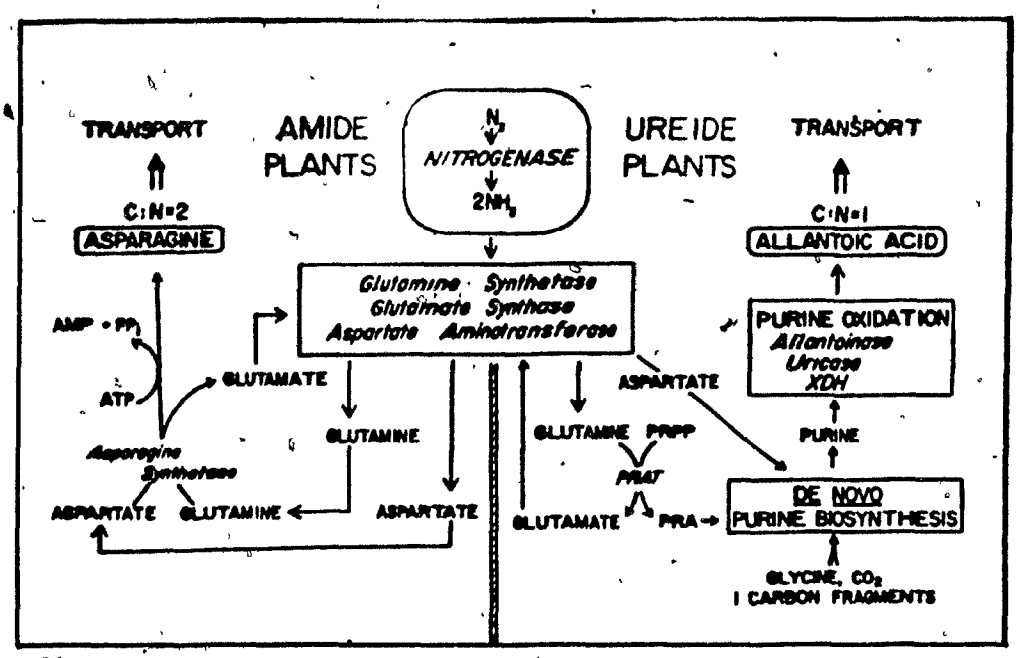
nitrogen fixing systems possess a hydrogen recycling "uptake hydrogenase", an enzyme encoded by the hup gene. Evidence is available to show that the uptake hydrogenase in root nodules helps in the respiratory protection of nitrogenase by scavenging oxygen, in the recovery of energy and also providing electrons for nitrogenase (Eisbrenner and Evans, 1983).

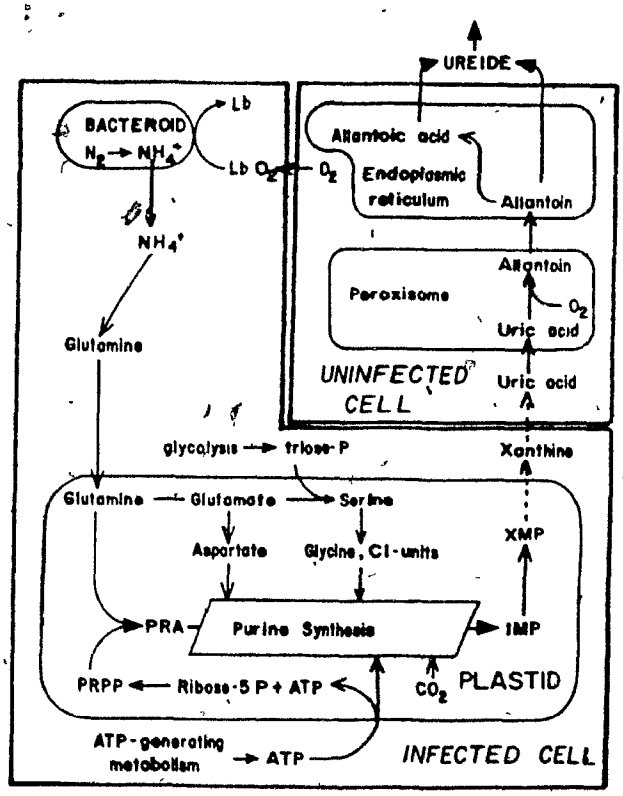
I.3.2 Ammonia assimilation

Ammonia, the first stable product of nitrogen fixation by nitrogenase (Kennedy, 1966), is excreted from the bacteroids and assimilated in the host cytoplasm. It is not clear whether ammonia is actively transported out of the bacteroids or is secreted in response to a pH gradient (Laane et al., 1980; Gober and Kashket, 1983; Blumwald et al., 1985). The ammonia is assimilated quickly, as an excess is toxic to host cells. In the plant cell, glutamine is the initial product of assimilation. Glutamine synthesis could potentially occur in two ways: either by the enzyme glutamate dehydrogenase (GDH) or via the glutamine synthetase/glutamate synthase (GS/GOGAT) pathway. Several studies using labelled precursors and inhibitors, (Ohyama and Kumazawa, 1978; 1980a,b; Meeks et al, 1978) strongly suggest that the GS/GOGAT cycle is the predominant or exclusive pathway used in ammonia assimilation (reviewed by Miflin and Cullimore, 1984). In legumes of tropical and sub-tropical origin such as soybean,

Figure 2A : Ammonia assimilatory pathways in the nodules of amide and ureide transporting plants (Reynolds et al., 1982b). PRPP- 5'-phosphoribosyl pyrophosphate; PRAT-5'-phosphoribosyl pyrophosphate amidotransferase; PRA- phosphoribosylamine; XDH- xanthine dehydrogenase.

Figure 2B : Model for the cellular and subcellular organization of the reactions involved in ureide biogenesis (Schubert and Boland, 1984). IMP- inosine monophosphate; XMP- xanthine monophosphate.


TRANSPORT
AMIDE PLANTS
N2
NITROGENASE
2NH3
UREIDE PLANTS
TRANSPORT
C:N=2
ASPARAGINE
C:N=1
ALLANTOIC ACID
Glutamine Synthetase
Glutamate Synthase
Aspartate Aminotransferase
PURINE OXIDATION
Allantoinase
Uricase
XDH
AMP + PPi
ATP
GLUTAMATE
ASPARTATE
PURINE
Asparagine Synthetase
GLUTAMINE
GLUTAMINE PRPP
PRAT
ASPARTATE
GLUTAMATE
PRA
DE NOVO PURINE BIOSYNTHESIS
GLYCINE, CO2
1 CARBON FRAGMENTS


UREIDE
BACTEROID
N2 → NH4+
Lb
Lb O2
O2
Allantoic acid
Endoplasmic reticulum
Allantoin
NH4+
Allantoin
Peroxisome
O2
Uric acid
Glutamine
UNINFECTED CELL
Uric acid
glycolysis
triose-P
Xanthine
Glutamine
Glutamate
Serine
XMP
Aspartate
Glycine, C1-units
PRA
Purine Synthesis
IMP
PRPP
Ribose-5-P + ATP
CO2
PLASTID
ATP-generating metabolism
ATP
INFECTED CELL

Phaseolus and Vigna, glutamine is incorporated into the ureides allantoin and allantoic acid which are exported from the nodule. On the other hand, temperate legumes such as alfalfa and lupin export nitrogen in the form of the amides glutamine and asparagine (Thomas and Schrader, 1981; Reynolds et al., 1982a,b). Figure 2A summarizes the pathways of ammonia assimilation in root nodules, and Figure 2B outlines a model for the cellular and subcellular organization of biosynthetic reactions involved in the root nodules of tropical legumes synthesizing ureides.

I.4 Host genes involved in symbiosis

The metabolism of the plant is modified in the legume-Rhizobium symbiosis by the expression of a distinct set of genes which direct the synthesis of nodule-specific host proteins which have been termed nodulins (Legocki and Verma 1980; van Kammen, 1984; see Verma et al., 1986 for review). Nodulins are involved in the development, structure, maintenance and overall metabolism of the root nodule and have been characterized from soybean (Legocki and Verma, 1980), pea (Bisseling et al, 1983) and alfalfa (Lang-Unnasch and Ausubel, 1985). About 20 nodulins were detected by in vitro translation of polysomal nodule mRNA in soybean nodules (Legocki and Verma, 1979; 1980) and 20-40 moderately abundant soybean mRNAs were found to be nodule-specific (Auger and Verma, 1981). In addition, the expression of certain other genes is either

stimulated in nodule tissues (Govers et al., 1985), or significantly reduced (Fuller et al., 1983). cDNA cloning of soybean nodule mRNAs revealed that in addition to the superabundant leghemoglobin, four other mRNA species of moderate abundance constitute about 8% of the total transcripts (Fuller et al., 1983).

Soybean contains four major leghemoglobins, encoded by a family of genes whose expression is under the control of Rhizobium-induced nodule differentiation. A number of peribacteroid membrane nodulins have been identified in soybean. Nodulin-24, a pbm nodulin (Katinakis and Verma, 1985), has been detected only in the pbm and not in the plasma membrane of soybean nodules (Fortin et al., 1985). Three pbm nodulins have been studied at the primary and secondary structure levels but no conserved features other than overall high hydrophobicity have been detected (Fortin et al., 1987). Mutants of Bradyrhizobium japonicum that block nodule development at several stages, induce progressively more pbm nodulins as the block is located further in the developmental program (Fortin et al., 1987; Morrison and Verma, 1987). The bacteria also appear to have an influence on the stability of pbm since certain ineffective (Fix^-) strains of B.japonicum e.g. 61A24, fail to induce a stable pbm. This strain elicits a host defense response leading to the production of glyceollin and nodule degeneration occurs rapidly (Werner et al., 1984; 1985). Six other nodulins from soybean sharing sequence

homologies have been described (Mauro et al., 1985; Sengupta-Gopalan et al., 1986; Jacobs et al., 1987; Sandal et al., 1987). Homology among the members is restricted to the C terminal and N terminal ends, with variations in the central region. Their subcellular localizations also vary between the cytosol and pbm (Jacobs et al., 1987). One possible clue to their in vivo function is that the conserved domains of the proteins encode metal-binding sites (Sandal et al, 1987). These genes have apparently evolved from a common ancestor (Jacobs et al., 1987).

The nodulins that mediate metabolic functions of the nodule have been termed "metabolic nodulins" (Verma et al., 1986). The better known members of this class of nodulins are: nodulin-35, a subunit of uricase II in soybean (Bergman et al., 1983; Nguyen et al., 1985); xanthine dehydrogenase in soybean (Triplett, 1985); an isoform of glutamine synthetase in Phaseolus (Lara et al., 1983; Cullimore et al, 1984); nodulin-100, an isoform of sucrose synthetase (Morel and Copeland, 1985; Thummler and Verma, 1987). Conflicting results have been reported for soybean regarding nodule-specific forms of glutamine synthetase (Sengupta-Gopalan and Pitas, 1986; Hirel et al., 1987).

I.5 Biology of Rhizobium

Bacteria which belong to the family Rhizobiaceae comprise the genera Rhizobium and Agrobacterium. They are gram

negative, non-spore forming short rods and are loosely classified based on their growth rates into fast-growers and slow-growers, with average generation times of 2-3 hrs. and 6-8 hrs. respectively. The two classes also differ with respect to metabolic properties such as acid or alkali production on nutritional media (Vincent, 1974). The fast-growers generally nodulate temperate forage legumes such as alfalfa, clover and sanfoin while the slow-growers nodulate tropical legumes such as cowpea, soybean, and Phaseolus. Based on their host range, bacteria are assigned to different cross-inoculation groups (e.g. R.meliloti-alfalfa, R.trifolii-clover, R.leguminosarum-pea, R.japonicum-soybean). It is now known that a very stringent host specificity does not exist and that a single Rhizobium species can infect several host plants. However, the limited species specificity that is seen remains an intriguing aspect of the legume-Rhizobium symbiosis.

The fast-growing Rhizobium such as R.trifolii, R.leguminosarum, and R.meliloti, have been extensively studied for a number of reasons. They are readily amenable to genetic techniques such as transformation, conjugation and transduction. Chemical mutagenesis procedures have also yielded a variety of mutants that have allowed a genetic analysis of their symbiosis. The combination of transposon Tn5 insertion mutagenesis and molecular cloning techniques have led to the identification of several genes involved in the symbiotic process (Beringer, 1980; Rolfe and Shine, 1983).

The slow-growing bacteria, now assigned to the genus Bradyrhizobium to distinguish them from the fast-growers (Jordan, 1982), have proved to be recalcitrant to genetic manipulations (Beringer, 1980). The isolation of biochemical and symbiotic mutants has been difficult and until recently transposon Tn5 mutagenesis has met with little success (Hom et al., 1984; Rostas et al., 1984; So et al., 1987). Additional complexity in dealing with the genus Bradyrhizobium is revealed by the fact that based on DNA homology, serology, restriction site variation, phage typing and protein profiles on SDS gels, the members are highly divergent from each other (Hollis et al, 1981; Haugland and Verma, 1981; Stanley et al, 1985) and can be subdivided into at least 2 symbiotic types (Stanley et al., 1985).

I.5.1 Bacterial symbiotic genes

Symbiotic genes (sym) are generally defined to be all genes necessary to establish an effective symbiosis. The sym genes can be divided into 3 broad functional categories: nif the structural genes that encode the polypeptides of nitrogenase; fix - all genes that are known to aid in the biochemical process of nitrogen fixation; nod - all genes that are involved in the formation of a nodule structure.

I.5.2 Organization of Symbiotic genes

In several fast-growing Rhizobium species, the

symbiotic genes are located on large indigenous plasmids called sym plasmids (reviewed by Prakash et al, 1980). Several studies have shown that some genes involved in nodulation and nitrogen fixation are located on large, transmissible plasmids which can be cured by cultivation of bacteria at higher than optimal temperatures (37°C). In addition to the plasmid-borne sym genes, chromosomally determined functions are also involved in the establishment of a nitrogen fixing nodule. (Rolfe and Shine, 1983). The biological functions that are known to be associated with sym plasmids in Rhizobium are listed in Table 1.

The nitrogen fixation genes are well characterized in the free-living bacterium Klebsiella pneumoniae (reviewed by Cannon et al., 1985). Seventeen nif genes have been identified and mapped as a contiguous cluster between hisG and shiA. The physical location of all the nif genes have been determined and detailed restriction maps of the 23 kilobase region of DNA which they span, have been established. Transcriptional studies have identified 7-8 operons and nucleotide sequence studies have shown that the nif promoters share characteristics which clearly distinguish them from other promoters of Enterobacteria (Better et al., 1983). Using cloned nif genes from K.pneumoniae as hybridization probes, the corresponding genes from R.meliloti (Ruvkun and Ausubel, 1980), R.leguminosarum (Hombrecher et al., 1981), R.phaseoli (Quinto et al., 1982), R.japonicum (Hennecke, 1981) and

Table 1 Biological Functions Located on Sym Plasmids in Rhizobium Strains

(Adapted from Shine and Rolfe, 1983)

Medium bacteriocin production (Mbp$^+$)	Beringer et al., 1980: Brewin et al., 1980a
Repression of small bacteriocin production (Rsp$^+$)	Djordjevic et al., 1983
Nodulation ability	
Root adhesion (Roa$^+$)	Rolfe et al., 1981; Hooykaas et al., 1981
Hair curling (Hac$^+$)	Johnston et al., 1978; Rolfe et al., 1981 Djordjevic et al., 1983; Forrai et al., 1983
Host range specificity	Truchet et al., 1983; Rosenberg et al., 1981
Nodule function (Fix$^+$)	Brewin et al., 1980b
Nitrogenase enzyme complex	
nif H (Fe-protein)	Nuti et al., 1979; Hombrecher et al., 1981
nif D (-subunit of Mo Fe-protein)	Morrison et al., 1983
Hydrogenase (Hup$^+$) production	Brewin et al., 1980c
Genes influencing cell surface polysaccharide synthesis	Carlson et al., 1983; Kuempel et al., 1983
Pigment production (Pig$^+$)	Beringer et al., 1980; Lamb et al., 1982
Transfer functions (Tra$^+$)	Beringer et al., 1980
Incompatibility	Beringer et al., 1980
Chromosome mobilization ability (Cma$^+$)	Beringer et al., 1980

R.parasponia (Shine et al., 1983) have been identified. Two key observations led to the suggestion that certain aspects of nif control in Rhizobium are very similar to nif control in K.pneumoniae (Ausubel et al., 1985). Firstly, nif promoter structure is highly conserved (Better et al., 1983) between various Rhizobium species and K.pneumoniae suggesting that the Rhizobium nif genes are regulated by homologues of the K.pneumoniae regulatory genes nifA and/or ntrC. Secondly, a R.meliloti nif regulatory gene which maps 5 kb upstream of the nifHDK operon is approximately 50% homologous to both K.pneumoniae nifA and ntrC in the central 200 amino acid region that is also conserved between the two K.pneumoniae proteins (Buikema et al., 1985). Moreover, the identification of more genes that are K.pneumoniae nif like (nifABE, ntrC, fixABC) in Rhizobium has given credence to the suggestion of similar pathways of nitrogen regulation in asymbiotic and symbiotic nitrogen fixing bacteria (DeBruijn et al., 1984).

I.5.3 Nodulation genes

The first nodulation genes to be identified were those that encode the root hair curling (hac) ability in R.meliloti (Long et al., 1982). They are present on the sym plasmid in the fast-growers and fall within a 14 kb region in R.leguminosarum (Downie et al., 1983), R.trifolii (Schofield et al., 1984) and in two regions separated by about 12 kb in R.meliloti (Long et al., 1982; Kondorosi et al., 1984). Based

on their phenotypic properties the nod mutants can be grouped into 2 broad categories: (1) those that completely lose their ability to induce root hair curling; these mutations map to the nodABC operon and totally block nodulation; and (2) those that exhibit impaired nodulation ability, the level of which may vary on different hosts (Downie and Johnston, 1986). Nod$^-$ mutants deficient in root hair curling can be complemented for nodulation on their normal host plant with corresponding genes from a heterologous Rhizobium species (usually referred to as "common nod"), suggesting that these highly conserved root hair curling genes (nodABC) are not involved in host-specific recognition. Another gene nodD which is transcribed divergently from nodABC was until recently believed to be a part of the common nod cluster, but recent evidence suggests otherwise (see next section). The biochemical role of the nodABC products are not known. The nodC product has a hydrophobic carboxy terminal end and is associated with the bacterial outer membrane (John et al, 1985). The nodABC genes are in one transcriptional unit, which in R.leguminosarum also contains two other genes, nodI,J. The predicted amino acid sequence of nodI protein shows a marked homology to that of a group of bacterial ATP-binding proteins involved in active transport. The nodJ gene encodes a very hydrophobic integral membrane protein that may function in conjunction with the nodI protein in the uptake of some plant-made metabolite(s) (Evans and Downie, 1986).

Other nodulation genes have been implicated in host recognition on the basis that mutations in these genes are not complemented by genes from a heterologous species of Rhizobium. Two of these genes, nodF and nodE (also called hsnA and hsnB for host specific nodulation), are homologous among R.leguminosarum, R.trifolii, and R.meliloti and are presumably allelic variations (Downie and Johnston, 1986). Interestingly, a strain of R.trifolii carrying a mutation within the nodE gene was shown to acquire the ability to nodulate peas, albeit at a very low level (Djordjevic et al., 1985) suggesting that host range could be negatively controlled. The predicted amino acid sequence of the nodF gene product resembles that of acyl-carrier-proteins from bacteria, plants and animals (Shearman et al., 1986), especially around a serine residue that binds the phosphopantethine cofactor. Since deletion of nodF in R.leguminosarum does not affect its growth, it is clearly not essential to Rhizobium fatty acid biosynthesis; however, it may have a role in lipopolysaccharide (LPS) or exopolysaccharide (EPS) biosynthesis. Two other host specific nodulation genes, hsnC and hsnD (also called nodG and nodH), have been identified in R.meliloti (Horvath et al., 1986). The hsnD determines the host-specific root hair curling event and mutations within the hsnABC genes affect infection thread development. The putative nodG polypeptide shows homology to ribitol dehydrogenase of K.pneumoniae and could therefore be involved in sugar metabolism (Debeile et al., 1986). It is

possible that such host-specific nodulation genes encode products involved in the synthesis of a new type of bacterial outer coat that is recognized by the plant (Downie and Johnston, 1986).

In B.japonicum, a combination of random transposon Tn5 mutagenesis and site directed mutagenesis has identified several genes. Regensburger et al (1986) have defined at least 10 independant fix genes in B.japonicum 110. Guerinot and Chelm (1986) have reported a gene, hemA, that is involved in heme synthesis but is not essential for symbiosis. Nieuwkoop et al (1987) defined a locus encoding host range specificity of B.japonicum 110 and Marvel et al (1987) described a locus in B.japonicum Rp501, encoding nodulation functions for non legumes. Both loci were found to be closely linked to the common nod genes in these strains. Morrison and Verma (1987) have described a gene in B.japonicum 61A76 which appears to be required for the release of bacteroids from infection threads.

I.5.4 Regulation of nod gene expression

The regulation of nod gene expression has been analyzed in different Rhizobium species by placing the Escherichia coli lacZ gene under the control of the nod gene promoter (Rossen et al., 1985; Mulligan and Long, 1985; Innes et al., 1985). The nodD gene is expressed constitutively but there is little or no expression of other nod genes unless the bacteria are exposed to a plant factor that is present in legume root

exudates; this implies that in the rhizosphere there is a strong induction of nod gene expression. This induction does not occur in strains mutated in nodD, suggesting that this gene regulates the induction of other nod gene transcriptional units. In R.meliloti 3 copies of nodD have been detected on the sym plasmid, 2 of which (nodD1 and nodD2) are necessary for efficient nodulation of alfalfa (Gottfert et al., 1986; Long et al., 1986). Moreover in R.leguminosarum, nodD may be autoregulatory since it inhibits the expression of a nodD-lacZ fusion (Rossen et al., 1985). A comparison of DNA sequences of nodD from several Rhizobium species suggests a nucleotide sequence homology of 50%, with the amino terminal end being the most conserved domain (Horvath et al., 1987). This conserved domain exhibits significant homology to the E.coli regulatory protein lysR (Applebaum et al., 1985; Horvath et al., 1987). Interestingly, a nodD gene from Rhizobium strain MPIK3030 can not only suppress nodD mutations in R.meliloti but can also extend the host range of R.meliloti transconjugants to include Macroptilium atropurpureum (siratro). Furthermore, the MPIK3030 nodD is able to interact with plant factors from its native host siratro as well as from alfalfa. These results show that the nodD protein host-specifically mediates the regulation of nod genes and therefore can no longer be termed a common nodulation gene (Horvath et al., 1987).

The plant factors that regulate nod genes (stimulators

or inhibitors) have also been identified using gene fusions to lacZ. The inducing molecules turn on nod genes at very low concentrations and they include flavonoids such as luteolin (from alfalfa, Peters et al, 1986), 7,4'-dihydroxyflavone (from clover, Redmond et al, 1986), and naringenin (from pea, Firmin et al, 1986; Zaat et al, 1987). Moreover, transcriptional activation of nod gene promoters by 7-4' dihydroxyflavone was found to be growth phase dependant with maximum stimulation in early log phase cells. This was found to be true for the constitutive nodD gene as well. Inhibitory compounds present in white clover root exudates were identified as umbelliferone (a coumarin) and formononetin (an isoflavone). An interesting observation that all strong stimulatory and inhibitory compounds possessed a 7-hydroxy moiety suggested that there was one binding site for these compounds. When the inhibitors were tested at a 10-fold molar excess over the stimulatory compounds a complete inhibition of nod gene induction was observed (Djordjevic et al., 1987a). An intriguing feature of the regulatory molecules is that an inhibitor of nod genes in one Rhizobium strain can act as a stimulator of other genes in another Rhizobium strain. Diadzein, a repressor of nod genes in R.leguminosarum (Firmin et al., 1986) and R.trifolii (Djordjevic et al., 1987) has been reported to be a stimulator in R.japonicum (Sadowsky et al., 1987) of a another set of bacterial genes (Olson et al, 1985), not related to nod. As the response to regulating

molecules is very rapid, it is believed that the regulators either traverse the bacterial cell wall or perhaps there is some involvement of an active membrane-bound uptake or transmembrane signalling system. It is also possible that the phenolic compounds act at a common membrane site and that a secondary signal is responsible for the induction of nod genes, either in combination with the nodD product or perhaps by activating the nodD product itself. Stimulatory compounds may mediate the conversion of nodD product to a positive regulator while the inhibitory compounds may compete for and occupy the binding site resulting in poor or no activation of the nodD product. The activated nodD product may stimulate transcription from the "nod box", a highly conserved 40 nucleotide sequence that has been found upstream of all inducible nod operons (Rostas et al., 1986; Schofield and Watson, 1986; Shearman et al., 1986).

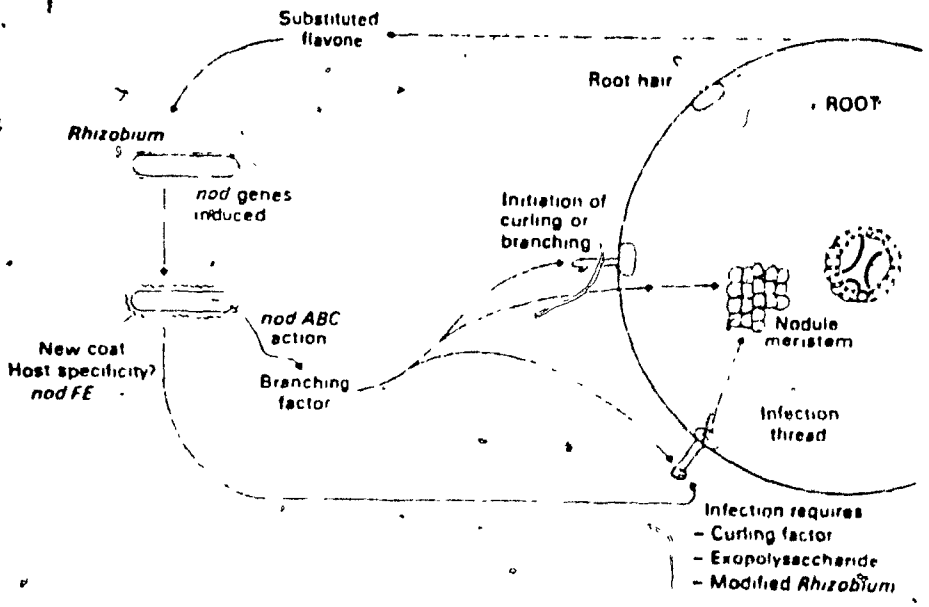
True to the nature of symbiosis, Rhizobium also appears to send signals to plant roots. The effects include root hair deformation, root growth inhibition and the thick and short root phenotype (Bhuvaneswari and Solheim, 1985; van Brussel et al., 1986; Canters-Crammers et al., 1986). Genetic loci on the sym plasmid as well as on the chromosome are required for the synthesis of these small molecular weight compounds. The exact role of these compounds for the establishment of an effective symbiosis and their relationship to indoleacetic acid (IAA) and cytokinins produced by Rhizobium is unclear (Badenoch-

Jones et al., 1983; Phillips and Torrey, 1970). A model outlining the events of nodulation is shown in Fig. 3. In response to nod gene induction the bacteria appear to synthesize low molecular weight factors that induce root hair deformation and branching on clover (Bhuvaneswari and Solheim, 1985) and in case of a small seeded legume, inhibit overall root growth (van Brussel et al., 1986). These factors may be molecules of plant origin that are modified by the nodABC gene products. Such signal molecules may also be involved in the maintenance of infection thread development and the induction of early nodule meristematic development (Downie and Johnston, 1986).

I.5.5 Importance of cell surface

The first point of contact between the symbiotic partners, is the bacterial surface. Hence, it is reasonable to predict that the bacterial mutants altered in surface components will somehow be altered in symbiosis. Mutants resistant to bacteriophage have been shown to have altered symbiotic properties (Kleczkowska, 1965). A correlation between antibiotic resistance and symbiosis has also been described (Schwinghamer, 1975). In B. japonicum, ineffective and non-nodulating mutants were shown to lack a surface antigen associated with the O antigen of the LPS (Maier and Brill, 1976; 1978). Another report makes the correlation between phage resistance and nodulation (Stacey et al, 1982).

Figure 3 : A model outlining the events of nodulation.
(Adapted from Downie and Johnston, 1986).


Substituted flavone
Root hair
ROOT
Rhizobium
nod genes induced
Initiation of curling or branching
nod ABC action
New coat Host specificity? nod FE
Branching factor
Nodule meristem
Infection thread
Infection requires
- Curling factor
- Exopolysaccharide
- Modified Rhizobium

The role of polysaccharides in nodulation has been controversial (Sanders et al., 1978; Sanders et al., 1981), however, convincing evidence is now available with the isolation of Tn5-induced mutants which show that exopolysaccharide (EPS) is essential for nitrogen fixation in the nodule (Chakravorty et al., 1982). In R.meliloti, mutations in genes involved in EPS synthesis (exo loci) resulted in the formation of nodules without infection threads and few bacteria could be seen within intercellular spaces rather than within nodule cells (Finan et al., 1985; Leigh et al., 1985). Thus, it appears that EPS functions in the initiation of the infection thread and/or in growth of bacteria within. In Agrobacterium tumefaciens, the loci chvA and chvB are known to be essential for attachment of bacteria to plant cells and oncogenic transformation of dicotyledonous plants (Douglas et al., 1985). The chvB gene has been implicated in the synthesis of an unique EPS, B-1,2 glucan (Puvanesrajah et al., 1985). An analogous locus (called ndv) has been studied in R.meliloti and mutations in this locus have been shown to be important for nodule development (Dylan et al., 1986). Borthakur et al. (1985) identified a gene, psi (for polysaccharide inhibition), located close to the nod genes on the sym plasmid of R.phaseoli. This gene product which is possibly membrane-associated (Borthakur and Johnston, 1987) inhibited EPS synthesis and nodulation ability when cloned in R. phaseoli on a multicopy plasmid. A second gene,

restoration), also located on the sym plasmid, overcame the EPS and nodulation defects of psi, when it was cloned on a multicopy plasmid. Using lacZ fusions it has been further demonstrated that transcription of psi was inhibited by psr (Borthakur and Johnston, 1987).

I.6 A mutational approach to the analysis of symbiosis

The primary reason for isolating and characterizing a mutation is to assess its consequence, or, in genetic terminology, its phenotype. In an ideal mutation experiment, two organisms are submitted to careful comparison, one being mutated at a known site and the other lacking the mutation. Any observed difference between the two is then attributed to the mutation; by characterizing this difference, inferences can be made about the function of the corresponding wild-type gene. One approach to designing such an experiment is to begin with a phenotype of interest (such as a failure to initiate symbiosis) and after mutagenesis, search for mutant organisms that exhibit this phenotype. The mutation can then be characterized by genetic mapping, cloning and sequencing the wild-type and mutant genes. This method, where the phenotype determines the selection of the mutation for analysis, has the advantage that each new mutation has a high probability of contributing to an understanding of the phenotype of interest (Botstein and Shortle, 1985).

Several studies have used the mutational approach in

Rhizobium to help in understanding symbiosis. Classical mutagenesis procedures using a variety of physical (ultraviolet rays) and chemical agents (nitrosoguanidine, nitrous acid, etc.) have yielded symbiotically defective mutants. In many instances the symbiotic defects were also linked to auxotrophy or alterations in the cell surface. However, the limitation of using such mutagenizing agents was the inability to restrict or eliminate the secondary mutations also caused by the agents. A revolutionary development in mutagenesis came with the realization that naturally occurring transposable elements could be used to generate insertion mutations. Unlike chemical or radiation mutagenesis, transposon mutagenesis usually results in single, unique physical alterations in the gene that has been mutated. The single most important advantage of transposon-tagging any gene of interest is the possibility of cloning the mutated gene by molecular techniques (Botstein and Shortle, 1985). Transposon Tn5 mutagenesis has proved to be an effective tool in the identification of symbiotic functions, especially in Rhizobium species (Buchanan-Wollaston et al., 1980; Meade et al., 1982; Lamb et al., 1982; Schofield et al., 1983; Kondorosi et al., 1984) and it has been successfully used for the physical and genetic characterization of symbiotic genes at the molecular level.

I.7 Rationale for present work

Whereas knowledge about sym genes has progressed rapidly in the genus Rhizobium, relatively little progress has been made in Bradyrhizobium. Although a few highly conserved fix and nod genes have been cloned using hybridization probes from other previously characterized Rhizobium species (Hahn and Hennecke, 1985; Russel et al., 1985; Fuhrman et al., 1985), this approach is limited in that it only allows the definition of genes that are common to all root nodule bacteria. It is, therefore, important to establish a random mutagenesis procedure, so that a range of mutants blocked at different stages of symbiosis can be obtained. Characterization of such mutants and the genes involved may allow the dissection of the legume-Rhizobium symbiosis at the molecular level.

In this work, transposon Tn5 was used to generate several symbiotically defective mutants in B.japonicum (Rostas et al., 1984). Two classes of symbiotic mutants have been characterized: mutants with an altered cell surface as seen by resistance to a strain-specific bacteriophage; and mutants that are auxotrophic for histidine (Sadowsky et al., 1986). The DNA regions encompassing the mutations have been cloned and the wild-type regions isolated from a genomic cosmid library. The wild-type fragments have been shown to be authentic by genetic complementation of the mutations.

II.1 Materials

II.1.1 Bacterial strains

Bradyrhizobium japonicum 61A76 was obtained from the Nitragin Company. B. japonicum USDA 122 was obtained from Dr. H.J. Evans, Oregon State University, Corvalis. The bacteriophage specific for B. japonicum 61A76 was from Dr. G. Stacey, University of Tennessee, Knoxville. Escherichia coli WA 803 containing pGS9 was from Dr. V.N. Iyer, Carleton University, Ottawa. All other plasmids were obtained from the authors cited in the text.

II.1.2 Media and growth conditions

All E. coli strains were grown in Luria Bertani (LB) broth (Maniatis et al., 1982). Slow-growing B. japonicum strains were grown aerobically at 25 to 28°C in either 20E medium (Werner et al., 1978), or Yeast extract mannitol (YEM) medium (Vincent, 1970). Auxotrophic tests were done in the minimal medium described by Bishop et al. (1976).

II.1.3 Sources of chemicals and enzymes

All chemicals were of high purity grade and were obtained from BDH, Boehringer Mannheim, Sigma, Difco laboratories, or Bethesda Research Laboratories (B.R.L.). Restriction and modification enzymes were either from B.R.L. or Boehringer Mannheim.

II.2 Methods

II.2.1 Bacterial matings

Bacterial conjugations were done by the triparental mating system of Ditta et al. (1980). The Escherichia coli cultures were grown overnight in LB at 37° C containing appropriate antibiotics. The strain containing the helper plasmid, pRK2013, was similarly grown in LB containing 50ug/ml kanamycin. Recipient cultures were grown either in 20E medium or YM at 28° C, to mid-logarithmic phase. Cells were harvested by centrifugation in a Sorvall centrifuge at 12,000xg for 10 minutes at room temperature. The cell pellets were washed once with sterile saline, resuspended in either water or fresh medium. Equal volumes of donor, helper and recipient cultures were mixed and forced onto a sterile Millipore membrane filter (0.45u pore size) The filter membrane was transferred to a 20E or YEM agar plate and incubated at 28° C for 3 days. The filter paper was then aseptically transferred to tubes containing 2 ml sterile water vortexed vigorously and aliquots were plated on appropriate selective plates. In control experiments the donor and recipient cultures alone were plated on selective plates.

II.2.2 Screening of transconjugants

Transconjugants that grew on selective plates were streak-purified on the same selective plates and then stored in

microtiter plates in 50% glycerol at -70° C. Auxotrophic tests were done as per the method of Holliday (1956). The transconjugants were transferred from the microtiter dishes onto Bishops minimal medium with 0.1% arabinose as carbon source. After incubation at 28° C for 10 days colonies that did not grow or grew very poorly were identified and retested on minimal plates containing various combinations of organic nitrogen sources as described in the Holliday (1956) auxotrophy scheme. The identified auxotrophs were retested with and without the specific compound to confirm the phenotype. Reversion to prototrophy was tested by growing the auxotrophs in liquid cultures without antibiotics and plating them on Bishops minimal medium without antibiotics.

II.2.3 Plant infection tests

The symbiotic phenotypes of exconjugants were tested by the split-root assembly system of Kosslak and Bohlool (1984). Preliminary screenings were done on Glycine soja. The seeds were surface-sterilized by soaking in 4% calcium hypochlorite for 20 minutes, followed by several cycles of rinsing with sterile water. The seeds were scarified with a sterile scalpel and germinated on water-agar plates. Seedlings were transferred aseptically to sterile plant-test assemblies. The assemblies were made as follows. Into a large test-tube were placed a few pieces of small rocks, followed by a short length

of absorbent string. The tube was filled with pre-wetted vermiculite or a 3:1 mixture of vermiculite and perlite. About 10-15 ml of 0.4x Hoaglands nutrient solution (Hoaglands and Arnon, 1938) were added, the tube was covered with aluminum foil and autoclaved. Germinated seedlings were transferred to the tubes, inoculated with the appropriate bacterial culture and the seedling covered with a 1 cm layer of paraffin-coated sand. The tubes were placed in wooden racks and transferred to plant growth chambers. The plants were watered each week with sterile Hoaglands nutrient solution and scored for their symbiotic phenotype after 3 weeks. Potential symbiotic mutants were retested in triplicate for 2 cycles on G.soja followed by 1 cycle on Glycine max cv Prize or cv Lee. The nitrogen-fixing activity of root nodules was tested by the acetylene reduction assay (Hardy et al, 1968). Root systems of the entire plant were transferred to 75 ml serum bottles and capped with serum stoppers. 7.5 ml of acetylene were injected through the septum and the bottles incubated at room temperature. 1 ml samples of the gas mixture were withdrawn at 15 minute intervals in 1ml disposable syringes and the gas mixture stored by piercing the needle on rubber stoppers. 0.5 ml of the gas mixture were then injected into a Pye-Unicam gas chromatograph with a Poropak N column. The oven temperature was set to 75° C, and the injector and detector were set to 150 C. A standard gas mixture of 10% acetylene and 1000ppm ethylene were run at all settings of the attenuator to determine standard peak heights.

The peak heights of samples were used to calculate the acetylene-reducing activity of each plant.

II.2.4 Root hair curling studies

The root hair curling assays were performed in Fahreus slide chambers (Fahraeus, 1957). Sterile seeds of G. soja which were pregerminated on water agar plates were placed on the open end of the microscope slide chamber and inoculated with 0.1 ml of a logarithmic culture of the mutants and wild-type. Three replicates of each culture were examined for several days at regular intervals, under a Zeiss photomicroscope with bright field illumination. An equivalent number of uninoculated control plants as well as plants inoculated with a heterologous bacterium R. meliloti were also examined.

II.2.5 Light and Electron microscopy

Nodule tissue from wild-type and nodule-like tissue from mutant-inoculated plants were cut into 1 mm pieces and fixed in glutaraldehyde-paraformaldehyde at 4°C overnight. The fixed tissue was washed thoroughly in 0.1M sodium cacodylate buffer (pH 7.2) and treated with 1% OsO_4 in sodium cacodylate buffer for one hour. Following extensive washings with 0.1M sodium cacodylate buffer, the tissue was dehydrated in an ethanol series over ten to twelve hours and embedded in Spurr resin by slow infiltration over two days and polymerization at 65°C overnight. Thick sections were prepared for light microscopy,

stained with toluidine blue and photographed using a Zeiss photomicroscope. Thin sections were mounted on a nickel grid with carbon coated Formvar films, stained with uranyl acetate and phosphotungstic acid and examined using a Philips-200 EM.

II.2.6 Isolation of bacteriophage-resistant mutants

A 0.2 ml aliquot of a 61A76 specific phage stock (10^{10} pfu/ml) was spread on YEM plates. The Tn5-transconjugants from microtiter wells were transferred onto phage agar plates and incubated at 28° C for 5-7 days. Colonies that appeared resistant were retested by streaking the bacteria across a previously streaked phage suspension.

II.2.7 Phage adsorption

Adsorption of phage to the resistant cells was determined as follows: Logarithmic cultures of the test strains were infected with 0.1ml of a 10^5 pfu/ml phage suspension and incubated at 28 C for 30 minutes. The infected bacteria were centrifuged in an Eppendorf centrifuge for 10 minutes and appropriate dilutions of the supernatant were plated on a lawn of sensitive host cells to estimate free phage particles. Controls of the phage suspension alone, wild-type cells and a heterologous host were done at the same time.

II.2.8 Physical analysis

Genomic DNA from Bradyrhizobium japonicum cultures was

isolated as per Haugland and Verma (1981). Late log phase cultures were washed once in TE (10mM Tris-Cl pH 8, 1mM EDTA) and resuspended in a half-volume of TE. Predigested pronase and SDS were added to a final concentration of 1mg/ml and 1% respectively, and incubated at 37°C. Lysis was usually observed within one hour. The lysed cells were extracted with an equal volume of phenol, phenol-chloroform and chloroform and the aqueous layer precipitated with 2 volumes of ethanol. The DNA was recovered by centrifugation at 12,000xg for 10 minutes at 4°C, the pellet washed with 70% ethanol, dried briefly and resuspended in a suitable volume of TE. The DNA was dialyzed with several changes of TE buffer for small-scale preparations and purified on CsCl gradients for large-scale preparations.

Restriction enzyme digestion was carried out in the appropriate buffer recommended by the manufacturer. The digested DNA was electrophoresed on 0.7% agarose gels in Tris acetate buffer (1xTAE, 40mM Tris acetate, pH 8, 2mM EDTA) at 2v/cm, stained in 1x TAE containing 1ug/ml ethidium bromide and visualized on a short wave UV transilluminator.

II.2.9 Southern blots and hybridizations

Transfer of electrophoresed DNA to Genescreen (New England Nuclear) was done as described by Southern (1975). The agarose gel was denatured in 0.5M NaOH, 1,5M NaCl for 30 minutes followed by neutralization in 1M Tris-Cl, 0.5M NaCl for 30

minutes. The DNA was transferred overnight using 2x SSC (0.3M NaCl, 0.03M Na citrate) as transfer buffer and immobilized onto the support by baking at 80°C for 2 hours.

The DNA filters were pre-hybridized in heat-sealable bags at 65°C, in a solution containing 6x SCP (1x SCP is 0.1M NaCl, 0.03 M Na_2HPO_4 and 0.001M EDTA), 0.1% sodium sarkosyl, 100 ug/ml calf thymus DNA and 5% dextran sulfate. ^{32}P-labelled radioactive probes were prepared by the nick-translation procedure of Rigby et al (1977) and about 10^6 counts were added to each bag. Hybridizations were carried out at 65°C with continous shaking for 8-12 hours. The filters were washed for 5 minutes with 2x SSC, 0.1% SDS followed by two 20 minute washes at 65°C with 2x SSC, 0.1% SDS and finally for 10 minutes with 0.2x SSC, 0.1% SDS at room tempekature. The filters were air-dried and autoradiographed with Kodak X-Omat AR film at -70°C with an intensifying screen.

II.2.10 RNA isolation and Northern blot analysis

RNA was isolated by the method of Salerno and Lampen (1986). Briefly, 200 ml logarithmic phase cells were collected by centrifugation and resuspended in 2.7 ml 0f lysis buffer which contained 10mM Tris-Cl pH7.5, 1mM EDTA, 4mM sodium pyrophosphate and 50 ug/ml chloramphenicol. The cells were lysed by a freeze thaw cycle after which 0.3 ml of 10 mg/ml lysozyme were added and incubated at 37°C for ten minutes. To this solution, a solution of 7M guanidium hydrochloride-1%

sarkosyl, 1mM EDTA, 10mM Tris-Cl pH 7.5 was added and the RNA pelleted by centrifuging through a cushion of 5.7 M CsCl at 26,000 rpm for 16 hours at 15°C. The RNA pellet was dissolved and extracted once with phenol:chloroform and precipitated with 2.2 volumes of cold ethanol. The RNA was dissolved in 0.1 ml TE buffer and quantitated spectrophotometrically. For Northern blot analysis, 10 ug of RNA was glyoxylated and electrophoresed in a 1.2% agarose gel in 10mM phosphate buffer pH 7.0 with buffer recirculation. Transfer to Genescreen paper, hybridization conditions and washing of filters were carried out as recommended in the Genescreen instruction manual.

I.2.11 Cloning of Tn5-containing fragments

Total genomic DNA was prepared from the Tn5-induced mutants as described before. The DNA was restricted with the enzyme EcoR1, extracted with phenol-chloroform, precipitated with ethanol, and redissolved in TE. To 1 ug of this DNA was added 0.1 ug of EcoRI cut and calf intestinal phosphatase treated-pBR322 DNA and ligation carried out at 14°C for 12 hours. One third of the ligation reaction was transformed into Escherichia coli RR1 cells made competent by the procedure described by Maniatis et al. (1982) and plated on LB plates containing 50ug/ml of kanamycin and 15ug/ml of tetracycline. Colonies that appeared on plates were analysed by the miniprep method of Priefer (1984) for the presence of recombinant

clones containing the expected insert fragment.

II.2.12 Construction of genomic libraries

Total genomic DNA isolated from wild-type B.japonicum 61A76 was treated with varying concentrations of the appropriate restriction enzyme to establish the optimum concentration required to partially digest DNA to give 20-25 kb fragments. 200 ug of genomic DNA was partially digested under the previously determined conditions and size-fractionated either on a 10-20% sucrose gradient or a 1.25-5M NaCl gradient by centrifuging in a Beckman SW 41 rotor at 26,000rpm for 16 hours or 35,000rpm for 4 hours respectively. 1 ml fractions were collected from the gradient tubes and 30 ul of alternate fractions were electrophoresed on a 0.4% agarose gel. The fractions containing 20-30 kb sized DNA were pooled, diluted with water, precipitated with ethanol, and redissolved in 100ul TE. The cosmid vector was digested with the relevant enzyme and treated with calf intestinal phosphatase. Different molar ratios of the vector and insert DNA were ligated separately and packaged into lambda phage particles using the *in vitro* packaging procedure (Hohn and Murray, 1981), as described by Maniatis et al. (1982). An aliquot of the packaged particles was used to infect E.coli HB101 at 37°C and plated on LB plates containing 15ug/ml of tet. The ligation mix that gave the maximum number of colonies was repackaged on a large scale, infected into E.coli HB101

and plated on LB tet plates. Individual colonies were picked with sterile tooth-picks into microtiter wells containing 200ul of HM freezing medium with tetracycline (tet). The cells were grown for 8-10 hours in the wells and stored at -70°C. Minipreparations of cosmids from 10-20 random colonies were checked for the presence of inserts and the average insert size was found to be 20 kb.

The genomic library was replicated from the microtiter wells onto Genescreen filters placed on LB agar plates containing 15ug/ml of tet. The colonies were grown for 8 hours and lysed on the filters by placing the filter in 2-3 ml of denaturing solution on a Saranwrap sheet, followed by neutralizing solution. The filters were air-dried, baked at 80°C for 2 hours and hybridized with radioactive probes as described before.

II.2.13 Isolation of cosmid DNA from nodule bacteria

The nodules were surface sterilized by immersing in a mixture of 70% ethanol and 2.5% hydrogen peroxide for 2-3 minutes followed by several washes with sterile water. The nodules were crushed and the bacteria plated on YEM plates containing 100 ug/ml each of str, kan and tet. Single colonies that appeared were inoculated into 5 ml aliquots of YEM liquid medium containing the same concentrations of antibiotics. A logarithmic phase culture was diluted to an OD_{600} of 0.1 and allowed to grow to an OD_{600} of 0.2. About 3 ml of cells were

centrifuged and the cell pellet washed once with 1M NaCl. The cells were treated for cosmid DNA preparation by the alkaline lysis method of Priefer (1984). An aliquot of the cosmid DNA preparation was transformed into competent E. coli (Maniatis et al., 1982) and the transformation mixture plated on LB plates containing 10 ug/ml of tet. Single colonies were analysed by the alkaline lysis method for the presence of cosmid DNA.

II.2.14 Preparation of antiserum

Wild-type B.japonicum cells were grown to log phase in YM liquid medium at 28°C. The cells were centrifuged, washed in sterile phosphate buffered saline (PBS) and resuspended in 5 ml of sterile PBS. 0.5 ml of the suspension were injected intraperitoneally into New Zealand white female rabbits (weighing 6-8 lbs.) followed by intramuscular injection the next day. Booster doses of the antigen were given at weeks 1 and 2 and the serum collected from the rabbits after 4 weeks.

II.2.15 Quantitation of nucleic acids and proteins

Protein samples were quantitated by the method of Bradford (1976) using the Biorad protein assay dye reagent. Standard absorbance curves were derived from samples containing 10 to 100 ug of BSA (Fraction V, Sigma Chemicals). For each sample to be quantitated, at least two different dilutions were analyzed. The colour reaction was quantitated at 595 nm using

a visible range spectrophotometer.

RNA and DNA samples were quantitated at 260 nm in a UV spectrophotometer using the relationship 1.0 optical density = 40 and 50 ug/ml of RNA and DNA respectively.

II.2.16 Polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis (PAGE) of protein samples was performed as described by Laemmli (1970). Routine analyses were performed on 12% polyacrylamide gels.

For analysis of oligosaccharides the following modification was done to reduce LPS aggregation (Puvanesarajah et al., 1987). 4 M urea was incorporated into the gels and the SDS concentration was increased from 0.1% to 1% in the gels and tank buffer. Oligosaccharide bands were visualized by the periodic acid-modified procedure of Dubray and Bezard (1982).

II.2.17 Western blotting of proteins and immunodetection

Proteins were transferred from polyacrylamide gels to nitrocellulose (0.45 um, Millipore) at 200 mA for 16 hours in 20 mM Tris pH 8.3, 150 mM glycine and 20% methanol. Following transfer, the blot was incubated with 3% BSA in TBS (10 mM Tris-Cl pH 7.3, 0.9% NaCl) for 2 to 3 hours at room temperature. Antiserum was added to 1% BSA in TBS at dilutions of 1:100 to 1:400, and the blot was incubated for 2 hours with agitation. After one hour of washing with several changes of TBS, the blot was incubated for a similar period of time with

approximately 10^6 cpm/ml of ^{125}I-labelled protein-A in 1% BSA in TBS. The blot was washed as above for one hour and exposed to X-ray film.

II.2.18 Isolation of polysaccharides

Cells were grown aerobically in Bishops minimal liquid medium (Bishop et al., 1976) containing 4 uM diadzein. After five days, the cells were centrifuged and resuspended in sterile water. Approximately 10^6 cells/plate were spread on Bishops minimal agar plates containing 4 uM Diadzein. The plates were incubated at 28° C. The cells were scraped off the plates after five days and suspended in 20 ml PBS. The suspension was vortexed and the cells collected by centrifugation. The supernatant was saved as crude EPS. The cell pellet was resuspended in 0.5M NaCl and stirred for 30 minutes. The cells were centrifuged at 12,000xg for 10 minutes at room temperature and the supernatant saved as crude capsular polysaccharide. To both crude preparations, 3 volumes of 90% ethanol were added and stirred overnight at 4° C. The polysaccharides were collected by centrifugation at 12,000xg for 10 minutes, washed with 95% ethanol and dissolved in 5 ml of water. This solution was dialyzed extensively against water. The crude extracts were recovered by lyophilization and sent to Dr. F. Dazzo, Michigan State University, for chemical analysis. Crude total polysaccharide extracts for PAGE analysis were prepared by boiling logarithmic phase cells in 0.05M phosphate buffer, containing 1% SDS.

II.2.19 Plasmid and cosmid DNA isolation

E. coli cells containing recombinant plasmids were grown overnight in the presence of appropriate antibiotic(s) without chloramphenicol amplification of plasmid replication. DNA was extracted from cells by either the boiling method described by Holmes and Quigley (1981) or the alkaline lysis method (Maniatis et al:, 1982). DNA was purified on CsCl-ethidium bromide density gradients by the method described by Garger et al. (1983). Centrifugation was performed at 200,000xg for 16 hours in a Beckman Ti 70.1 rotor. Ethidium bromide was removed as described by Maniatis et al. (1982), the CsCl removed by dialysis against TE buffer and the DNA precipitated with ethanol.

Chapter III : Results

III.1 Tn5 Mutagenesis of B.japonicum

The suicide vector commonly used for introduction of Tn5 into Rhizobium is pJB4JI, which consists of a promiscuous P-1 type R factor (pPH1JI) carrying a copy of bacteriophage Mu and Tn5 (Beringer et al., 1978). However, the presence of Mu has been shown to cause secondary genetic changes (Meade et al., 1982). Preliminary experiments using this vector indicated that it was not suitable for the generation of random Tn5 insertion mutants in the slow-growing Bradyrhizobium species as the frequency of vector cointegration in the genome was high. To avoid extraneous introduction of Mu sequences with Tn5, Selvaraj and Iyer (1983) constructed a new suicide plasmid, pGS9, composed of a p15A type replicon and a N-type transfer system. This suicide vector transposed Tn5 into Bradyrhizobium at frequencies sufficient to allow the isolation of a large number of insertion mutants (Rostas et al., 1984).

III.1.1 Isolation of kan^r and str^r exconjugants

E.coli WA803 containing pGS9 was used as the conjugal donor and pRK2013 as a helper in the triparental mating system of Ditta et al. (1980). Approximately 4000 B.japonicum 61A76 transconjugants were isolated on 20E complex medium containing streptomycin (str) and kanamycin (kan) each at 200 ug/ml. The

background level of resistance to these individual antibiotics was 50 ug/ml and 25 ug/ml respectively. In all mating experiments, the donor, helper and recipient cells were also plated on selective media and no colonies were seen even after 14 days. The frequency of transfer of kan and str resistance into B.japonicum 61A76 was 10^{-6} per recipient cell whereas the spontaneous mutation frequency was lower than 10^{-9}.

III.1.2 Physical analysis of putative Tn5 mutants

The presence of Tn5 was confirmed by colony hybridization of the putative mutants with a Tn5 probe. Approximately 1700 colonies were transferred from microtiter wells onto nitrocellulose filters placed on selective 20E medium and hybridized to a radioactively labelled lambda::Tn5 probe. More than 95% of the colonies hybridized to the probe. To check for the presence of vector sequences, the Tn5 probe was stripped from the filter and the same filter was hybridized to radioactively labelled pACYC184, which constitutes part of pGS9. The results indicated that pGS9 vector sequences were absent from the vast majority of the mutants. This was corroborated by microbiological tests showing that less than 1% of the mutants had the pGS9-encoded chloramphenicol resistance.

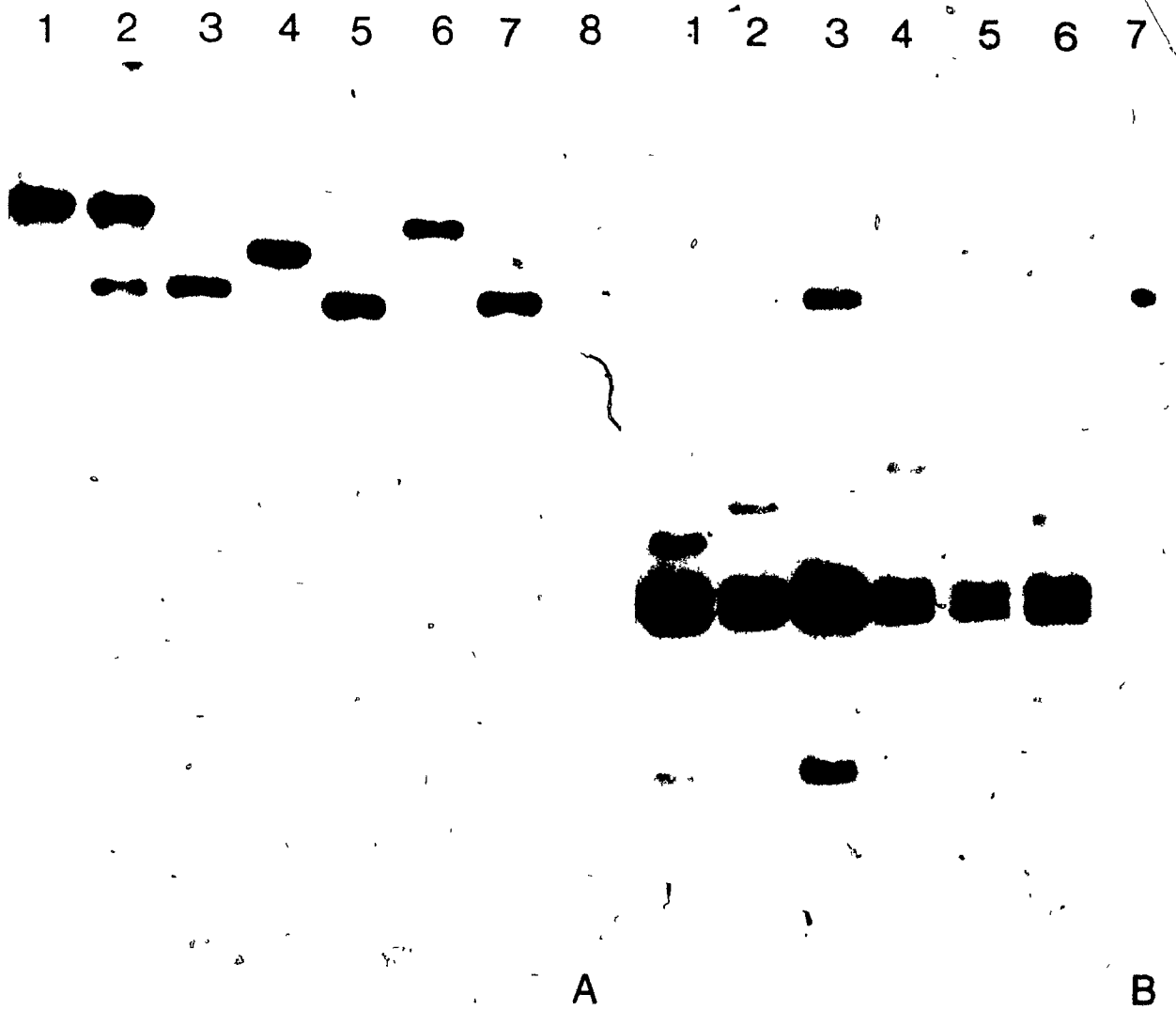
The specificity of Tn5 insertion was investigated as follows. Since Tn5 does not contain an EcoRI site, EcoRI digests of genomic DNA carrying a single Tn5 insertion should

show a unique fragment hybridizing to Tn5. Genomic DNA from 7 randomly isolated colonies was prepared, digested with EcoRI, electrophoresed in a 0.8% agarose gel, transferred to Genescreen paper and hybridized to ^{32}P-labelled lambda::Tn5. Fig 4A shows an autoradiogram where each mutant has a single EcoRI fragment containing Tn5. To confirm this observation, a second digest was done with XhoI. This enzyme generates four Tn5 fragments two of which are internal fragments and of the predicted size. The other two fragments of various lengths contain two identical 0.5 kb Tn5 segments attached to the bacterial flanking sequences at the site of insertion. Fig. 4B shows the border fragments were of different sizes, suggesting a lack of site specificity for insertion of Tn5.

III.1.3 Isolation of Tn5-induced auxotrophs

The physical analysis of Tn5 mutants showed apparent random insertions in the Bradyrhizobium genome. To show that these insertions could be correlated with phenotypic changes, 3550 mutants were screened for auxotrophy. The screening was done on Bishops minimal medium as described in the methods section. Twenty potential auxotrophs were identified in the initial screening. However, further characterization of these mutants by the method of Holliday (1956) showed that only 2 mutants were strict auxotrophs, 1 requiring cysteine and the other requiring glycine. The frequency of auxotrophy was estimated to be 0.05%. Revertants of the auxotrophs were found

Figure 4 : Hybridization analysis of Tn5 transconjugants of Bradyrhizobium japonicum 61A76 (A) Hybridization of ^{32}P-labelled lambda::Tn5 DNA to EcoRI digests of DNA from seven random mutants (lanes 1-7). Lane 2 shows a partial digest and lane 8 is wild-type DNA. (B) The same probe was hybridized to XhoI digests of the strains shown in lanes 3-8 in (A).


1 2 3 4 5 6 7 8 1 2 3 4 5 6 7
A
B

on minimal plates at a frequency of less than 10^{-8}

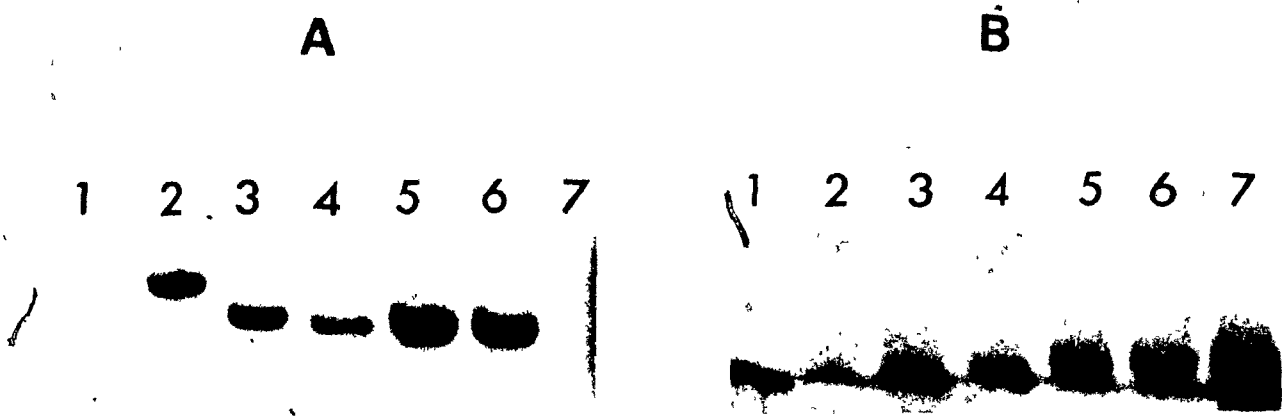
III.1.4 Isolation of symbiotically-defective Tn5 mutants

Plant tests to identify symbiotically defective mutants were carried out on Glycine soja, an ancestral soybean species which permitted the testing of a large number of mutants. 2,500 mutants were tested for their symbiotic phenotypes, out of which 6 mutants were identified as defective in nitrogen fixation. The plants appeared yellow and starved for nitrogen. Subsequent verification of these mutants by the acetylene reduction assay on the North American soybean cultivar G. max cv Prize showed that five mutants (5/95, 6/2, 8/1, 8/41, 8/57) were completely ineffective while one (7/50) was partially effective. Hybridization of Southern blots of EcoRI-digested genomic DNA from these mutants to radioactively labelled Tn5 (Fig. 5A) and pRmR2 (which contains part of nifDH) (Fig. 5B) showed that the Tn5 insertion was not in the structural genes nifDH of the nitrogenase enzyme complex.

III.2 Characterization of symbiotically-defective histidine auxotrophs of B.japonicum USDA 122

An independant study of B.japonicum USDA 122 identified four histidine auxotrophs, two of which were defective in symbiosis (Sadowsky et al., 1986). These mutants were characterized with respect to their biochemical and symbiotic defects as follows:

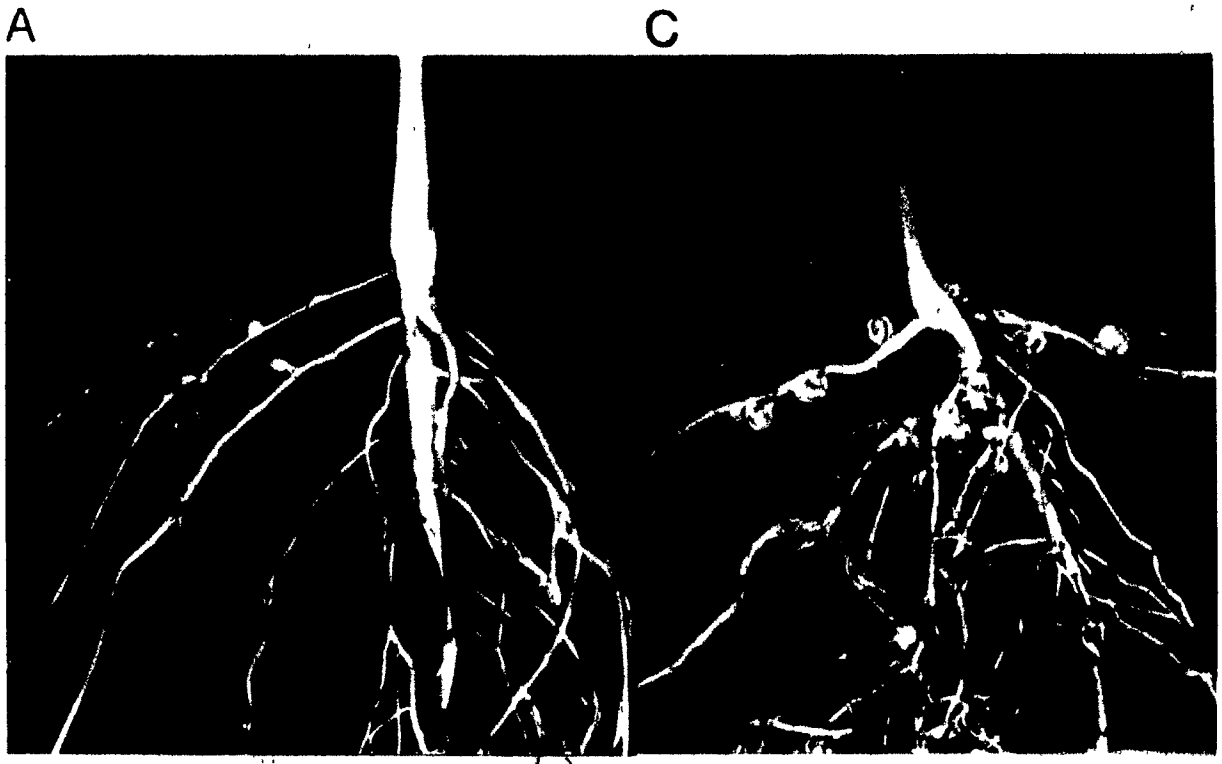
Figure 5 : Hybridization analysis of Tn5-induced fix$^-$ mutants of B. japonicum 61A76 (A) EcoRI digested genomic DNA from mutants 5-95 (lane 1), 6-2 (lane 2), 7-50 (lane 3), 8-1 (lane 4), 8-41 (lane 5), 8-57 (lane 6) and wild-type (lane 7) hybridized with ^{32}P-labelled lambda::Tn5 DNA. (B) The same filter was hybridized to ^{32}P-labelled pRmR2. Note all lanes contain a band which comigrates with a band in the wild-type lane 7.


A
1 2 3 4 5 6 7
B
1 2 3 4 5 6 7

III.2.1 Biochemical and symbiotic characterization of His mutants

The histidine-requiring mutants, His1 and His3, were found to be unable to induce root nodules on soybean plants whereas mutants His2 and His4 were symbiotically competent. When two intermediates of the histidine biosynthetic pathway were added to minimal plates, it was observed that all four mutants grew in the presence of the intermediate L-histidinol but did not grow in the presence of L-histidinol phosphate. Prototrophic revertants could be obtained from all mutants at frequencies of less than 10^{-7} when grown on non-selective Bishops minimal plates. However, prototrophic revertants could not be isolated when grown in the presence of 200 ug/ml str and kan. All prototrophic revertants examined were sensitive to str and kan as well as being symbiotically competent, and possibly arose from the excision and loss of Tn5. Minimal medium supplemented with 0.1% soybean root extract could support the growth of all four mutants. When the growth of the mutants was monitored in the presence of intact soybean roots, cell numbers of mutants His1 and His3 did not increase from an initial inoculum level of 10^5 cells/ml over a nine day period. However, when the growth medium was supplemented with 100 ug/ml of L-histidine, cell counts for mutants His1 and His3 increased to 10^7-10^8 cells/ml in four days. While mutants His1 and His3 did not form nodules on G.soja or G.max, root deformations were occasionally seen (Figure 6). In a few

Figure 6 : Root deformations and nodulation with respective cross-sections 28 days after inoculation of Glycine max cv Lee with (A) and (B) Bradyrhizobium japonicum USDA 122:Tn5 mutant His1, (C) and (D) B. japonicum USDA 122:Tn5 His4.


A
C
B
D

instances, nodules were formed and were found to contain kan^s prototrophic revertants. In separate experiments, other phenotypes were also seen, as detailed below. The mutants His2 and His4 on the other hand, formed nodules and fixed nitrogen at wild-type levels. The bacteria isolated from such nodules were either kan^r auxotrophs (mutant type) or kan^s prototrophs (revertant type). The biochemical and symbiotic properties of the His mutants are summarized in Table 2.

III.2.2 Biochemical complementation of symbiotic defects of mutants His1 and His3

To determine the effect of added L-histidine on the nodulation ability of His1 and His3, the mutants were inoculated onto G. max cv Lee and the plant growth medium was supplemented with 100 ug/ml of L-histidine. Results presented in Table 3 show that with exogenously added histidine, the nodulation defects of both mutants were completely overcome. When inoculated onto plants (5 plants per treatment) without added histidine, mutant His1 formed no nodules (with the exception of three nodules on one plant), while with added histidine the mutant showed wild-type behaviour (Table 3). Similarly the nodulation pattern of mutant His3 resembled the wild-type in the presence of added histidine. The bacteroids in the nodules formed by His1 and His3 mutants on non-histidine-supplemented plants had several phenotypes. Nodules produced by His1 contained kan^r auxotrophs, whereas the

Table 2 Symbiotic and biochemical properties of USDA 122::Tn5 histidine-requiring mutants

Mutant designation	Nodulation[a] on		Growth[b] on				Prototrophic revertants			
							Symbiotic phenotypes		Frequency	
	G. soja	G. max	Bis medium	L-Histidine	L-Histidinol	L-histidinol phosphate	min med	nodules	min(-kan)	min(+kan)
His-1	No	No[c]	No	Yes	Yes	No	Nod^+	Fix^+	$< 10^{-7}$	$< 10^{-9}$
His-2	Yes	Yes	No	Yes	Yes	No	-	-	$< 10^{-7}$	$< 10^{-9}$
His-3	No	No[c]	No	Yes	Yes	No	Nod^+	Fix^+	$< 10^{-7}$	$< 10^{-9}$
His-4	Yes	Yes	No	Yes	Yes	No	-	-	$< 10^{-7}$	$< 10^{-9}$

[a] Plant tests were done using 5-7 plants/mutant (Glycine soja) or (Glycine max, cultivar Lee and Peking)

[b] Growth determined after 14 days on Bishop's minimal medium (Bis) or supplemented with 50 µg/ml of the indicated substance

[c] When inoculated on G. max, occasional root deformation was observed

Table 3 Effect of exogenously supplied L-histidine on the nodulating ability of His-1 and His-3 mutants on G. max cv. Lee

Strain	Treatment[a]	Mean[b] nodule number	Mean[b] nodule wet-weight (mg)	Nodule isolate phenotype class[e]	Symbiotic phenotype of nodule reisolates[g]
USDA 122	None	33	14	II(8)	Nod^+, Fix^+
(Parent)	L-histidine	27	12	II(8)	Not determined
His-1	None	0.6[c]	8	I(3)	Nod^+, Fix^+
	L-histidine	44	5	I(30)	Nod^-
His-3	None	1.2[d]	13	I,II,III[f](4)	Nod^+, Fix^+
	L-histidine	12	17	I(33)	Nod^-

[a] Histidine added to plant growth medium at concentration of 100 µg/ml

[b] Average of 5 replicates

[c] One plant had 3 nodules

[d] One plant had 6 nodules

[e] Phenotype classes: I, kan resistant-auxotrophs; II, kan sensitive-prototrophs; and III, kan sensitive-auxotrophs. Numbers in parentheses refer to number of nodules examined

[f] One nodule contained kan resistant-auxotrophs, bacteria from the other three were kan sensitive and were either auxotrophic or prototrophic

[g] Two isolates of each class examined

bacteroids in His3-elicited nodules could be divided into 3 different classes: class 1 consisted of kan^r auxotrophs, class 2 consisted of kan^s prototrophs and class 3 consisted of kan^s auxotrophs.

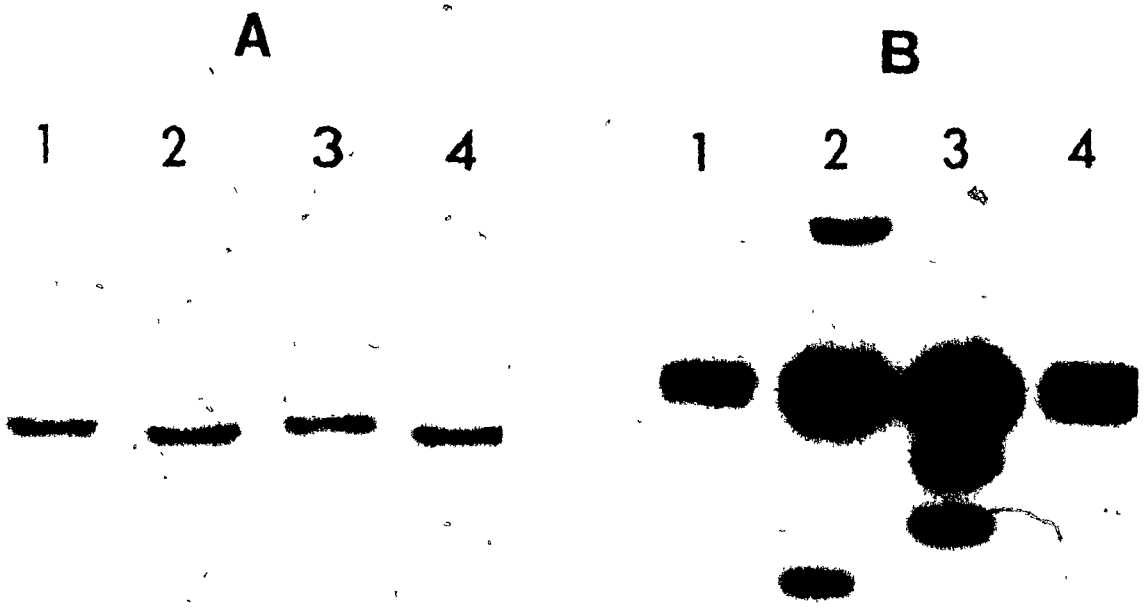
III.2.3 Physical analysis of Tn5 insertions in His mutants and revertants

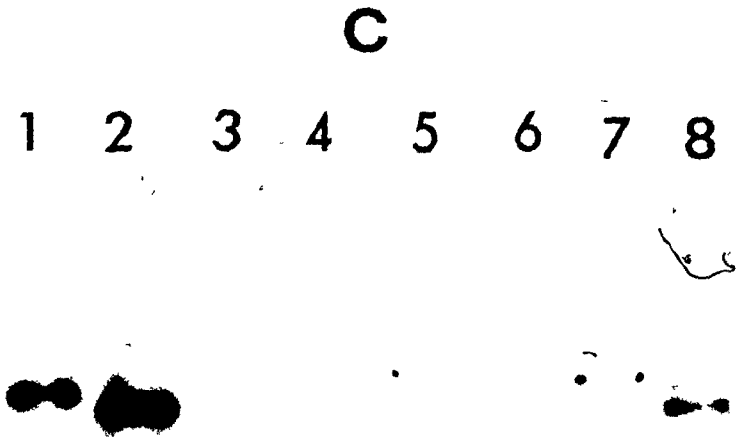
Southern blots of EcoRI and XhoI digests of genomic DNAs from the four histidine mutants were hybridized to radioactively labelled lambda::Tn5. The results shown in Fig 8A indicate that each mutant has a single Tn5 insertion. The results shown in Fig. 8B show that the His2 and His3 mutants have border fragments of different sizes indicating independent sites of Tn5 insertion, but for His1 and His4, some of the border fragments appear to be of a similar size to the internal Tn5 fragments. Hybridizations with R.meliloti nod (pKSK5) and B.japonicum nifKD and nifH probes indicated that Tn5 had not inserted into any of these sequences. Hybridization of genomic DNA from two members of each nodule-isolated phenotype class showed that all kan^s isolates (class 2 and 3) had lost Tn5, whereas kan^r isolates (class 1) had retained Tn5 (Fig. 8C).

III.2.4 Cloning of Tn5-containing EcoRI fragments and isolation of wild-type sequences

The Tn5-containing EcoRI fragments from mutants

Figure 7 : Hybridization analysis of genomic DNA from the histidine-requiring mutants of B. japonicum USDA 122. (A) EcoRI-digested and (B) XhoI-digested genomic DNA from B. japonicum USDA 122 mutants His1 (lane 1), His2 (lane 2), His3 (lane 3), His4 (lane 4) hybridized with ^{32}P-labelled lambda::Tn5 DNA, showing single independent Tn5 insertions. (C) EcoRI digested genomic DNA from independent nodule isolates belonging to the phenotypic revertant class 1 (lanes 1,2), class 2 (lanes 3,4), class 3 (lanes 5,6), mutant His1 (lane 7) and mutant His3 (lane 8).


A
B
1 2 3 4
1 2 3 4

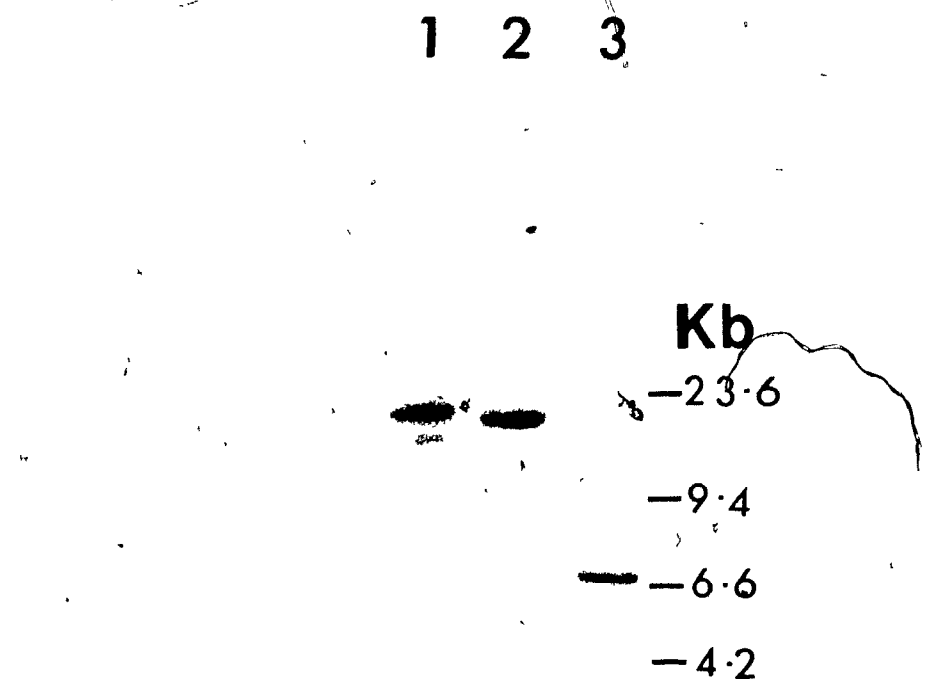

C
1 2 3 4 5 6 7 8

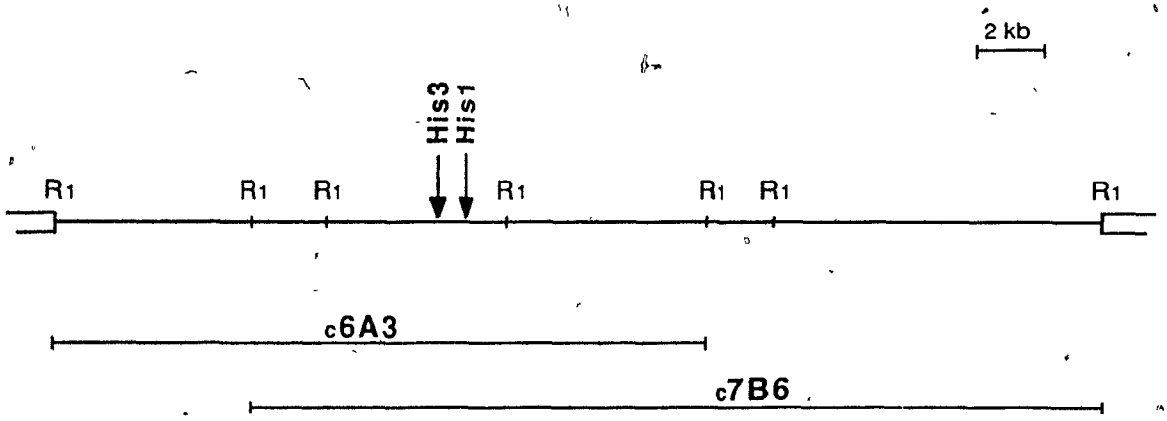
His1 and His3 were cloned in the plasmid vector pBR322. The recombinant plasmids, pH1 and pH3 (for Tn5-containing fragments from mutants His1 and His3 respectively) were hybridized to Southern blots of EcoRI-digested genomic DNA from His1, His3 and wild-type USDA 122 to check the authenticity of the recombinant clones. Fig. 8A shows that even though both mutants appear to have different sized Tn5-containing EcoRI fragments, they lack the same wild-type 6.6 kb EcoRI fragment suggesting that the insertions were in the same fragment. Another intriguing result is that His1 shows two hybridizing bands when the insert from pH1 is used as a probe. The second band is clearly not due to a double insertion as a single band is seen with a Tn5 probe (Fig. 7A). A comparison of the restriction maps of clones pH1 and pH3 confirmed that the Tn5 insertions were in the same fragment, with the insertion sites about 500 bp apart. Therefore, His1 and His3 are presumably mutated in different regions of the same gene that is required for histidine biosynthesis and indirectly, nodule formation.

The gel-purified insert from pH1 was radioactively labelled and used to screen a genomic cosmid library of wild-type B.japonicum USDA 122, constructed in the broad host range cosmid vector pLAFR1 (Friedman et al.,1982). Two strongly hybridizing cosmid clones 6A3 and 7B6 were identified. Restriction analysis of the two clones showed that both clones contained the wild-type 6.6 kb EcoRI fragment. The overlapping

Figure 8A : Hybridization of Southern blots of EcoRI-digested genomic DNA from B. japonicum USDA 122 mutant His1 (lane 1), mutant His3 (lane 2) and wild-type (lane 3) hybridized with the insert from plasmid pH1.

Figure 8B. Restriction map of the DNA region contained in two overlapping cosmids that hybridize to the radioactively labelled insert from pH3.


1 2 3
Kb
23·6
9·4
6·6
4·2


2 kb
His3
His1
R1
R1
R1
R1
R1
R1
R1
c6A3
c7B6

clones contained about 34 kb of wild-type B.japonicum USDA 122 DNA in this region (Fig. 8B).

To test for complementation of the His mutation with the wild-type DNA, cosmid clone 6A3 was conjugated into mutant His3 using the triparental mating system (Ditta et al., 1980). Colonies appearing on tetracycline (tet), kan and str (150 ug/ml each) were tested for auxotrophy and symbiosis. Mutant His3 containing clone 6A3 resembled the wild-type with respect to auxotrophy and symbiosis. The cosmid clone 6A3 therefore contains the authentic wild-type region of the mutation in His3. While complementation of His1 was not tested, it is presumed that 6A3 would complement that mutant as well.

III.3 Isolation and characterization of cell surface mutants of Bradyrhizobium japonicum 61A76

Stacey et al. (1982) have reported a correlation between bacteriophage resistance and defective symbiosis in B.japonicum 61A76. However, the mutation conferring phage resistance was not characterized in detail and molecular cloning of the genes involved was also not attempted. The Tn5-induced mutants isolated in the present study were screened for bacteriophage resistance, as a prerequisite to the identification and characterization of the genes involved.

III.3.1 Isolation of bacteriophage resistant mutants

4000 Tn5-induced mutants of B.japonicum 61A76 were

transferred from microtiter wells onto selective YM medium plates which had previously been spread with 10^8 pfu of B.japonicum 61A76-specific bacteriophage. The plates were incubated for 7 days and 28 vigorously growing colonies were identified. These colonies were streaked from the microtiter wells and retested for bacteriophage resistance by streaking across a previously streaked phage suspension. About 20 colonies were found to be phage resistant on retesting, giving a frequency of 0.5%. The frequency of spontaneous mutation to phage resistance was 10^{-4}.

III.3.2 The phage resistance mutation results in an altered cell surface

As the mutants were initially isolated by screening for bacteriophage resistance, it seemed likely that some would be altered in their cell surface structure. It was reasoned that such surface alterations could result in the attachment of fewer phage particles to the mutant cells and this possibility was tested. Bacteriophage adsorption studies were done as follows. About 10^5 pfu/ml of phage particles were allowed to infect log phase cells of the individual mutants for 30 minutes, after which the cells were centrifuged and free phage particles estimated in the supernatant by plating on a lawn of phage-sensitive wild-type cells. The data indicate that less than 20% of the added phage bound to mutant cells whereas >95% bound to wild-type cells.

Figure 9 : Demonstration of alterations in the cell surface of B. japonicum 61A76 bacteriophage-resistant mutants. 20 ug of total proteins from mutant 23H11 (lane 1), mutant 24F6 (lane 2) and wild-type (lane 3) were separated by SDS-PAGE, transferred to nitrocellulose, and (A) stained with 0.1% amido black and (B) immunoblotted with an antiserum raised against whole wild-type B. japonicum 61A76 cells.

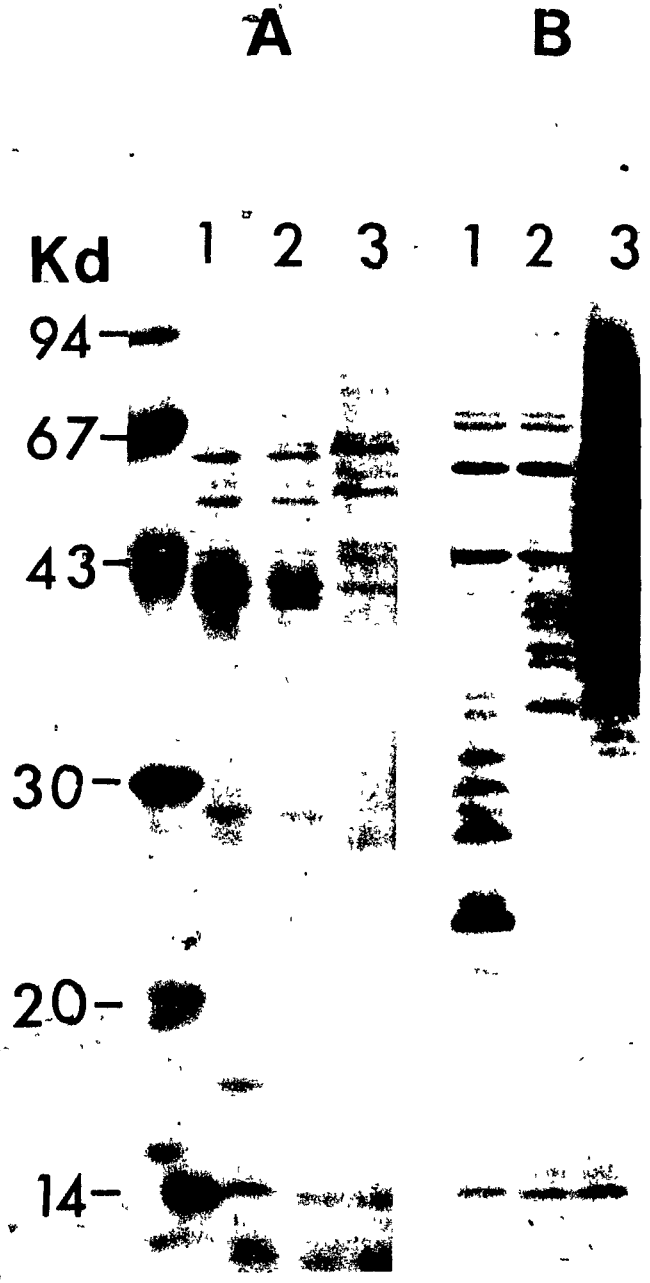
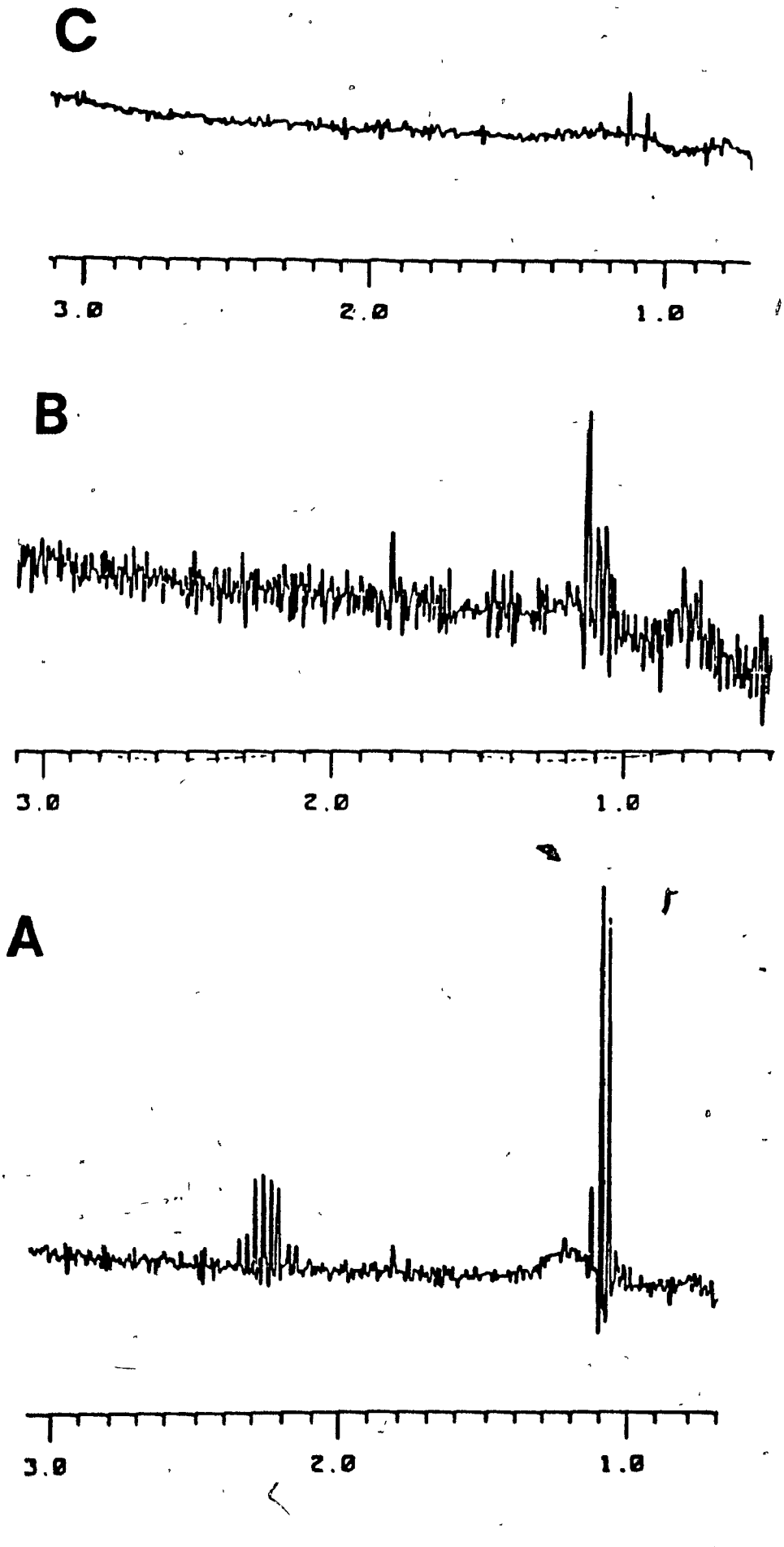

A
B
Kd
1 2 3 1 2 3
94
67
43
30
20
14

Figure 10 : Proton NMR spectroscopy of crude EPS extracted from B. japonicum 61A76 (A) wild-type, (B) bacteriophage-resistant mutant 23H11, and (C) bacteriophage-resistant mutant 24F6. The analysis was performed as described by Hollingsworth et al., (1984).


C
3.0
2.0
1.0
B
3.0
2.0
1.0
A
3.0
2.0
1.0

To test for altered cell surface components more directly, Western blots of total proteins from mutant and wild-type cells were probed with an antiserum raised against wild-type whole cells. The results shown in Fig. 9 reveal marked differences in protein profiles between the mutant and the wild-type.

Crude preparations of total EPS from the mutants were compared with that of wild-type by ^{1}H NMR spectroscopy. The results shown in Figure 10 suggest that the mutants are deficient in a methylene group modification of 3-hydroxybutanoic acid, which has been shown to be a component of the acidic EPS of R. trifolii (Hollingsworth et al., 1984). In addition to this defeciency, mutant 24F6 also appeared to lack a methyl group modification of 3-hydroxybutanoic acid.

A comparison of crude extracts from one of the mutants to that of the wild-type indicated that the mutant 23H11 accumulated an oligosaccharide component which the wild-type did not (see Figure 20). A comparison of the electrophoretic mobility of this component with that reported by Puvanesarajah et al. (1987) suggests that it is a component of LPS.

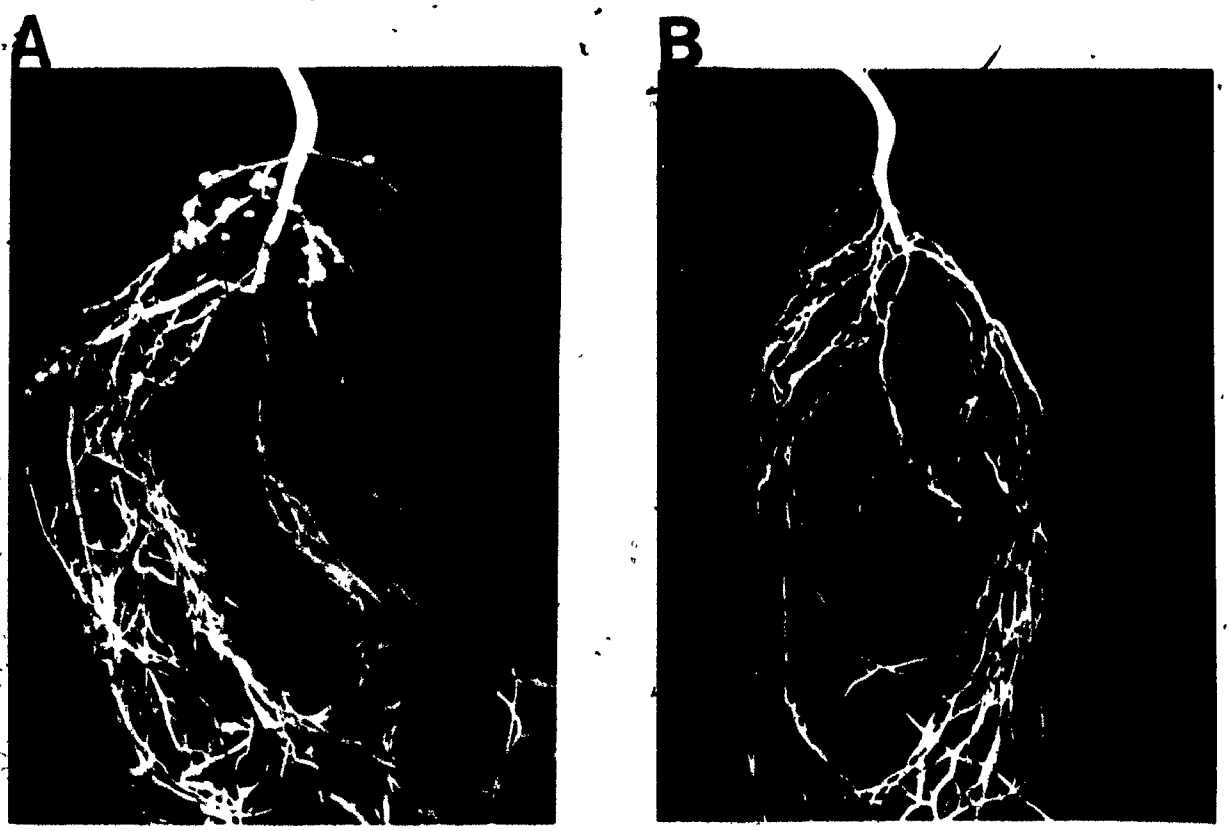
These results taken together clearly suggest that the phage-resistant mutants are altered in the cell surface in such a manner that few phage particles can associate with the cell surface.

III.3.3 Symbiotic phenotype of bacteriophage-resistant mutants

The 20 bacteriophage resistant mutants were inoculated onto Glycine soja and plants were examined after 3 weeks for their symbiotic phenotype. Two mutants, 23H11 and 24F6 were found to be defective in nodulation. A similar defect in nodulation was seen on the North American soybean cultivar G. max. While the wild-type induced normal looking, pink nodules, these mutants induced numerous, very small, white "bumps" or root deformations, on soybean roots (see Fig. 11). The mutant-inoculated plants were yellow and appeared to be starved for nitrogen as compared to the wild-type-inoculated plants, and nitrogen fixation capacity as measured by acetylene reduction was completely absent. The two nodulation-defective mutants were further characterized. The other bacteriophage-resistant mutants induced normal-looking nodules on soybean plants and acetylene reducing activity was comparable to the wild-type.

Studies on root hair curling (Hac) inducibility by the mutants on G. soja showed that mutants 23H11 and 24F6 were capable of initiating the Hac response by soybean root hairs, suggesting that the block in symbiosis was beyond this step (see Fig 12). Light microscopic studies of mutant-induced nodule-like structures showed that both mutants induced cortical cell division in soybean roots. However, nodule development appeared to be arrested at that stage even after 21 days post-inoculation. The wild-type-elicited nodules, on the other hand, showed invaded cells and differentiated

Figure 11 : Nodules and root deformations on Glycine max after inoculation with B. japonicum 61A76 (A) wild-type (B) bacteriophage-resistant mutant 23H11 (C) A higher magnification of (B). Note small, numerous, irregular-shaped nodule-like structures.


A
B


C

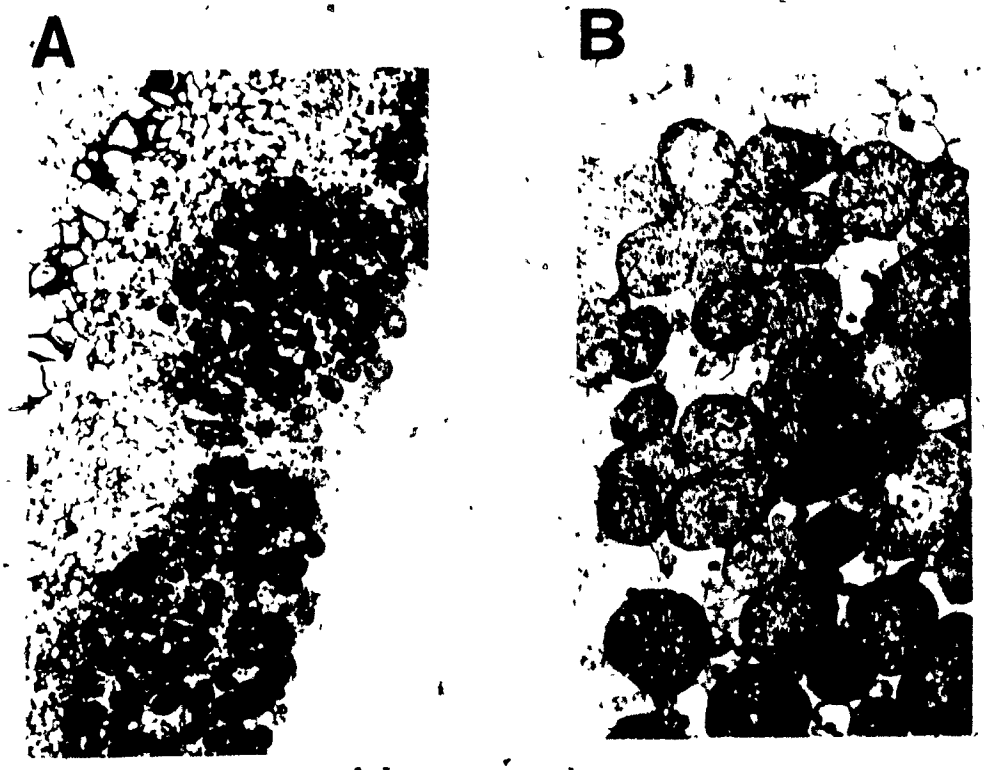
Figure 12 : Root hair curling of Glycine soja inoculated with B. japonicum 61A76 (A) uninoculated control (B) wild-type (C) bacteriophage-resistant mutant 23H11. The microscope slide assemblies used in this assay were described by Fahreus (1957) and photomicrographs of G. soja root hairs shown here were taken 3 days after inoculation with the relevent bacteria.

A

B

C

Figure 13 : Light microscopy of nodules and nodule-like structures on G. max cv Lee, 21 days after inoculation with B. japonicum 61A76 (A) and (B) wild-type, (C) and (D) bacteriophage-resistant mutant 23H11. Note well differentiated cell types in the wild-type and a lack of differentiation in the mutant-induced structures. Magnifications (A) and (C) 60x, (B) 100x, (D) 80x

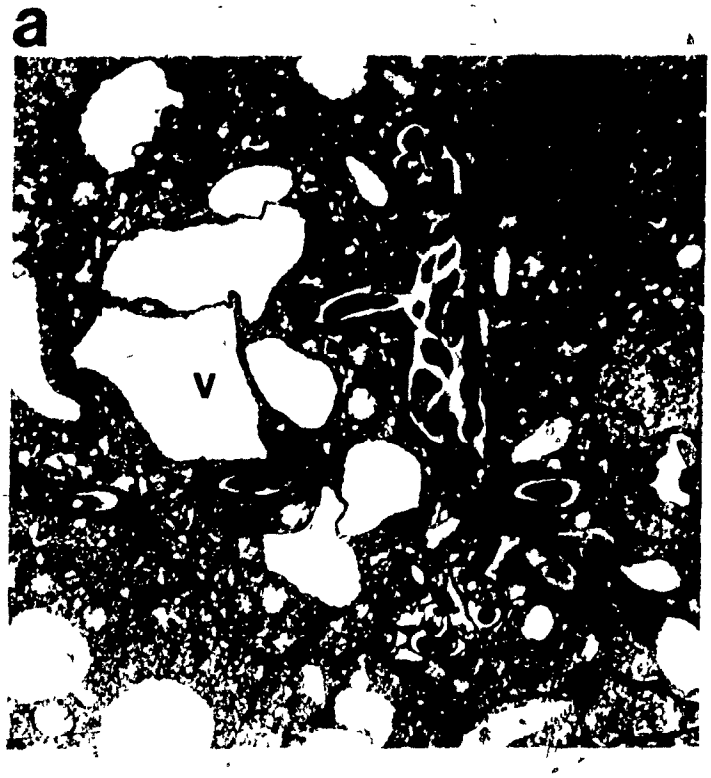

A
B
C
D

infected and uninfected cells. Fig. 13 shows representative light microscopic sections of mutant and wild-type-induced nodule structures on G. max cv Lee.

A noticeable difference between the two mutants was that when the plants were allowed to grow for 5-6 weeks, 23H11 produced a mixture of "bumps" and larger, more differentiated nodule-like structures. These structures, while resembling wild-type-induced nodules, were different in that they were of irregular shape and lacked the ability to fix nitrogen.

Electron microscopic studies of mutant-induced structures showed empty cortical cells for 24F6 whereas 23H11 occasionally appeared to initiate infection threads. A wild-type-induced infection thread is shown in Fig. 14A seven days after inoculation, and a fully differentiated nodule structure (Fig. 14B) thirteen days after inoculation. Fig. 15 shows electron micrographs of 23H11-induced infection threads, taken 21 days after inoculation. Note the disarrayed infection thread boundaries and empty spaces surrounding it. The bacteria are apparently not released from the threads. The host cytoplasm appears normal; however, there is a complete lack of differentiation between infected and uninfected cells in the plant after 3 weeks (Fig. 16A) and numerous amyloplasts can be seen. Even though 23H11 induced infection threads can be seen prior to 21 days, electron micrographs taken at 21 days are shown here to illustrate the arrest of development. Fig. 16B shows the empty cells that are induced by mutant

Figure 14 : Electron micrographs showing B. japonicum 61A76 wild-type induced infection threads on G. max cv Lee (a) 7 days post-inoculation and (b) 13 days post-inoculation. Note continuous infection thread wall (ITW), granular cytoplasm, vacuoles (v) and nucleus (N).
Magnification (a) 5308x, (b) 3010x


a
v

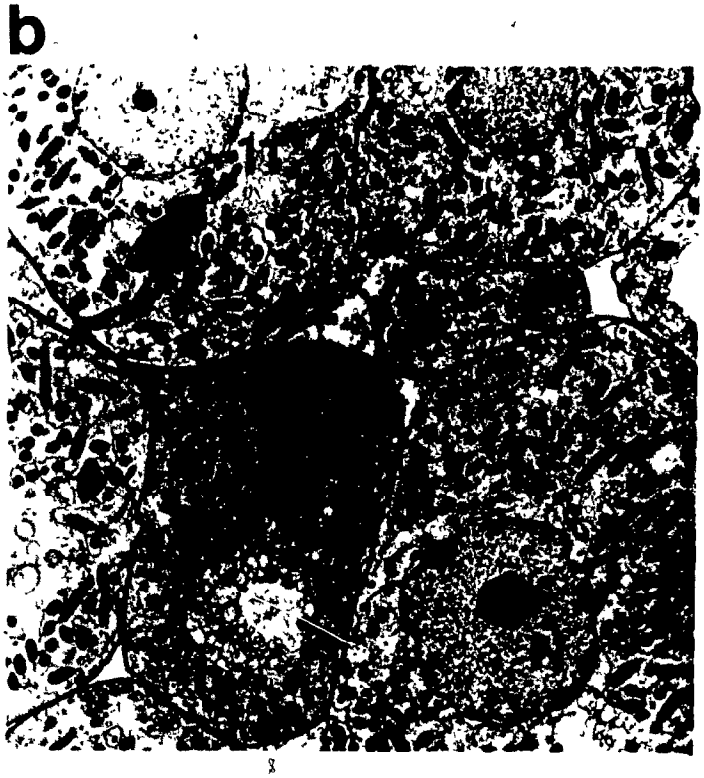
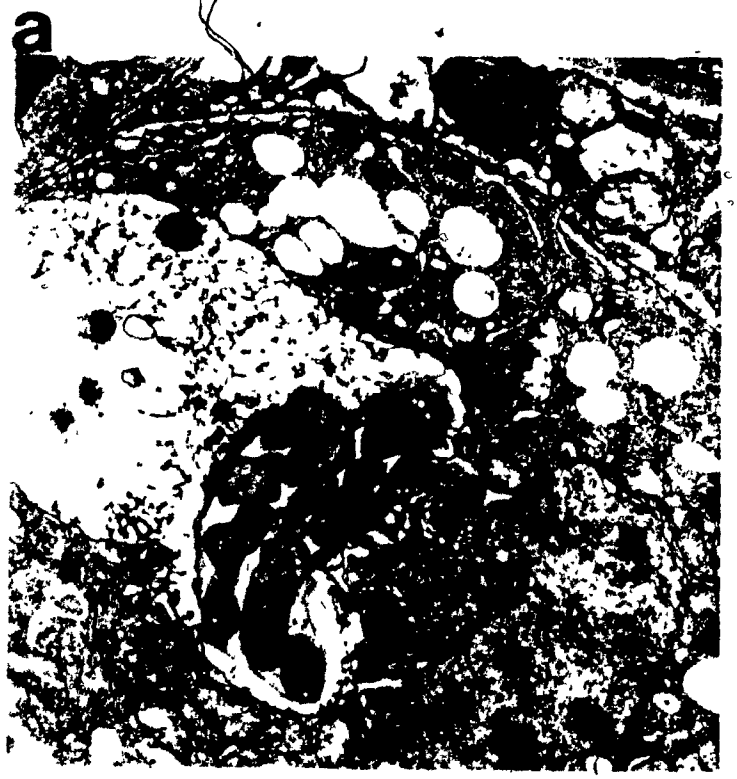

b

Figure 15a,b : Electron micrographs of B. japonicum 61A76 bacteriophage-resistant mutant 23H11 induced structures on G. max, 21 days after inoculation. Note disarrayed infection thread wall (ITW, open triangles) and empty spaces surrounding it. Bacteroids (b) are enclosed and little release into the cytoplasm is seen. Also note lack of differentiation between the two nodule cell types.

Magnification 12250x

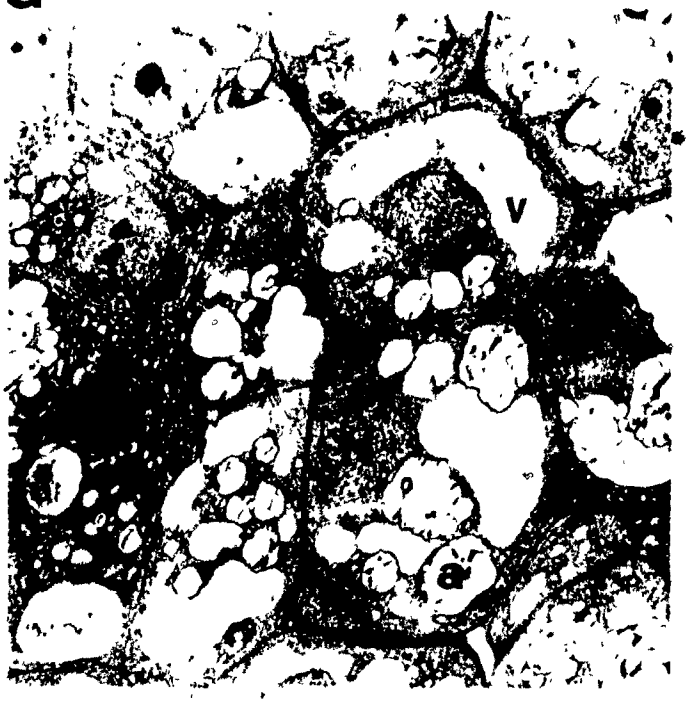

a

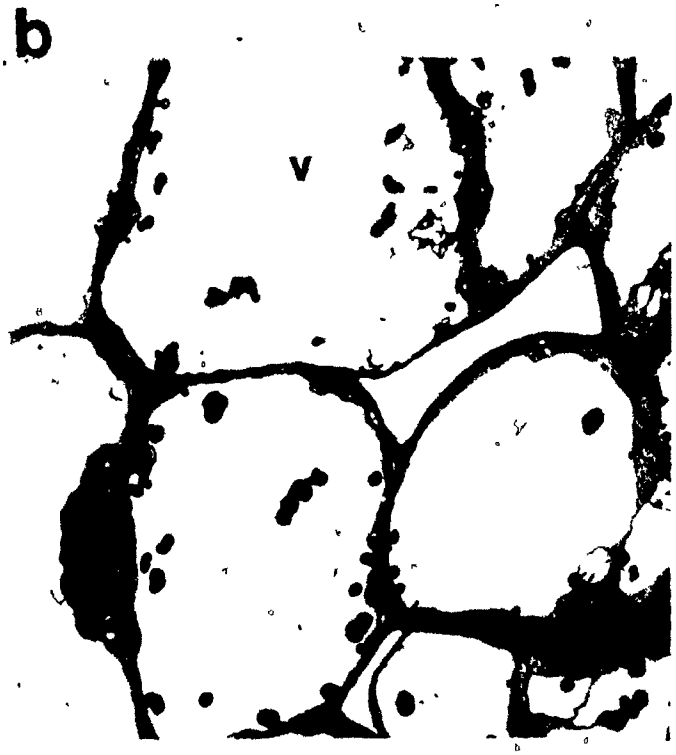

b

Figure 16a : Electron micrograph of 21 day old nodule-like structures from G. max cv Lee inoculated with B. japonicum 61A76 bacteriophage-resistant mutant 23H11, illustrating a lack of differentiation of cell types along with prominent amyloplasts (a), vacuoles (v) and nucleus (N).

Figure 16b : Electron micrograph of 21 day old nodule-like structures from G. max cv Lee inoculated with B. japonicum 61A76 bacteriophage-resistant mutant 24F6. Note large vacuole (v), numerous electron dense particles and lack of differentiated cell types.

Magnification (a) and (b) 3010x


a
v


b
v

24F6.

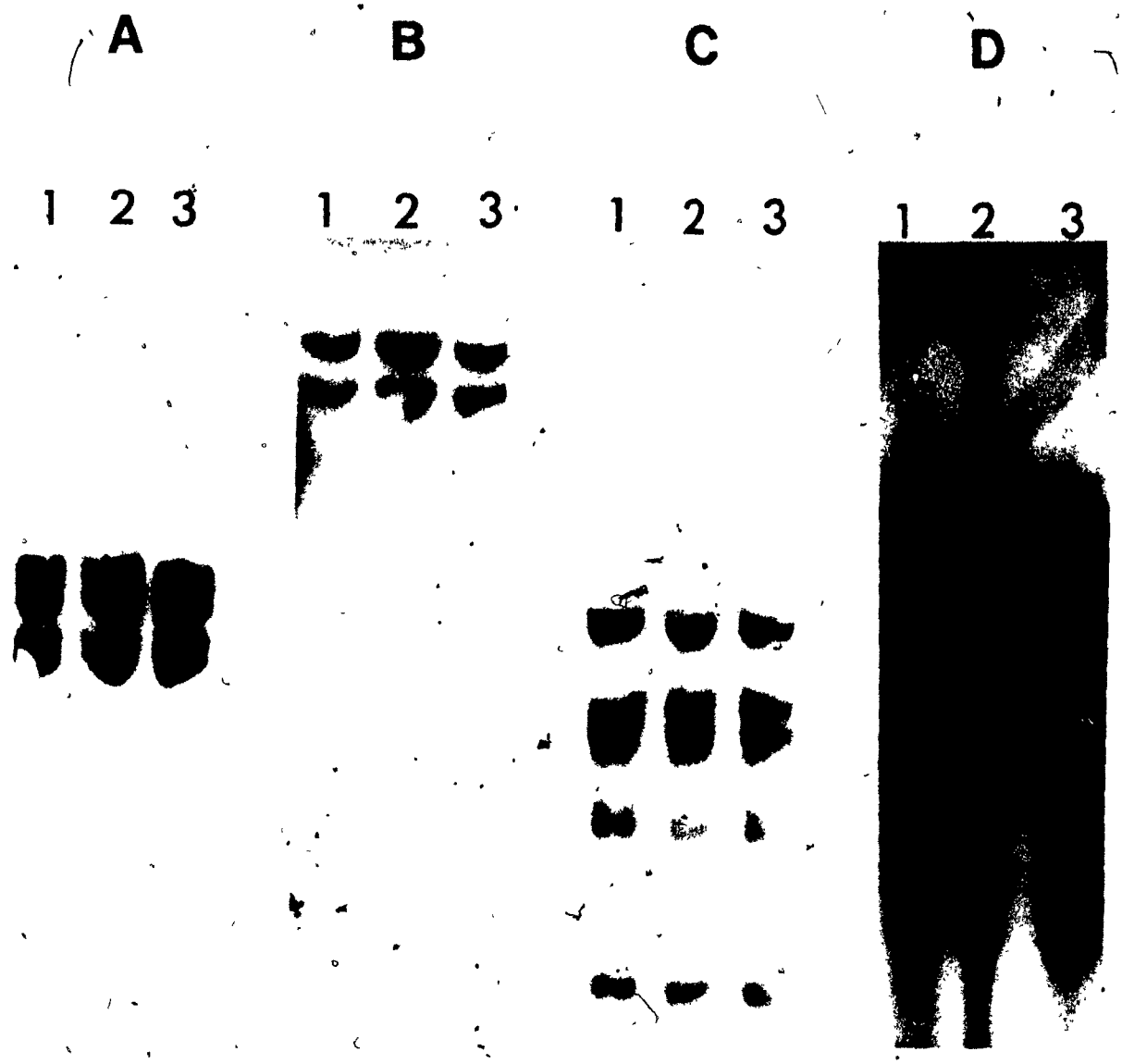
III.3.4 The nif and nod gene regions are unaffected in the nodule development mutants

To test the possibility that Tn5 had inserted into the nif and nod genes, cosmid clones containing these DNA regions were isolated by using pSA30 which contains nifKDH (Canon et al., 1979) and pELa which contains B. japonicum 61A76 nodABC (Lifson and Verma, unpublished) as hybridization probes. The cosmids were then radioactively labelled and hybridized to Southern blots of EcoRI-digested genomic DNA from the mutants and wild-type. The results in Figure 17A,B show that both mutants are indistinguishable from the wild-type in their hybridization pattern suggesting that Tn5 had not inserted into the previously characterized nif or nod regions.

III.3.5 Cloning of Tn5-mutated fragments from 23H11 and 24F6

To isolate the gene mutated by transposon insertion, the Tn5-containing EcoRI fragments from 23H11 and 24F6 were cloned in pBR322 to give pSP23 and pSP24 respectively. The authenticity of the cloned fragments was checked by hybridizing with Southern blots of EcoRI-digested genomic DNA from the mutants and wild-type. A genomic library of B.japonicum 61A76 constructed in the broad host range vector pVK102 (Knauf and Nester, 1982) was screened for wild-type-containing sequences by using gel-purified inserts from clones

Figure 17 : Hybridization of Southern blots of EcoRI-digested genomic DNA from B. japonicum 61A76 bacteriophage-resistant mutant 23H11 (lane 1), 24F6 (lane 2) and wild-type (lane 3) with radioactively-labelled cosmids containing (A) nif DNA region, (B) nodABC DNA region, (C) SP23 DNA region and (D) SP24 DNA region. Note that both mutants are indistinguishable from the wild-type with respect to the hybridization pattern of the four different probes.


A
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1 2 3

pSP23 and pSP24, as hybridization probes. One cosmid was found to hybridize to the SP23 fragment and two cosmids to the SP24 fragment. Restriction analysis showed that none of the 3 cosmids contained complete wild-type fragments. as they did not contain the DNA region flanking the site of Tn5 insertion. Attempts to complement the mutations with these cosmids were unsuccessful. It was therefore concluded that the genomic library in pVK102 did not contain complete DNA sequences corresponding to the wild-type region of the two insertion mutations. A second approach to isolate complete wild-type DNA regions was taken. Mini libraries were constructed in the broad host range plasmid vector pKT210 (Bagdasarian et al., 1979) by size fractionating EcoRI-digested genomic DNA (3 kb for 23H11 and 15 kb for 24F6) from the wild-type strain, and ligating into EcoRI cut pKT210. The recombinant colonies obtained after transforming E.coli HB101 were screened using gel-purified inserts from pSP23 and pSP24 as hybridization probes. The hybridizing colonies were isolated and two clones, designated pWP23 (for wild-type fragment of 23H11) and pWP24 (for wild-type fragment of 24F6) respectively were used in further tests. However, complementation attempted with these plasmids containing complete wild-type sequences was also unsuccessful. A second cosmid library was constructed using the broad host range vector pLAFR3, a derivative of the broad host range cosmid vector pLAFR1 (Friedman et al., 1982). Several cosmids hybridizing to radioactively labelled probes

made from pSP23 and pSP24 were isolated and the complementation test repeated. Again, these mutants could not be complemented, leading to the conclusion that the characterized Tn5 insertion alone was not the primary cause of the nodulation-defective phenotype, but that there was a another mutation elsewhere in the genome, leading to the block in proper nodule development. To eliminate the possibility that the insertion of Tn5 had caused genetic rearrangement in the flanking DNA region, Southern blots of EcoRI-digested genomic DNA from the mutants and wild-type were hybridized to radioactively labelled cosmids that contained wild-type regions corresponding to the fragments into which Tn5 had inserted. Figure 17C,D shows that the mutants are also indistinguishable from the wild-type with respect to these DNA regions.

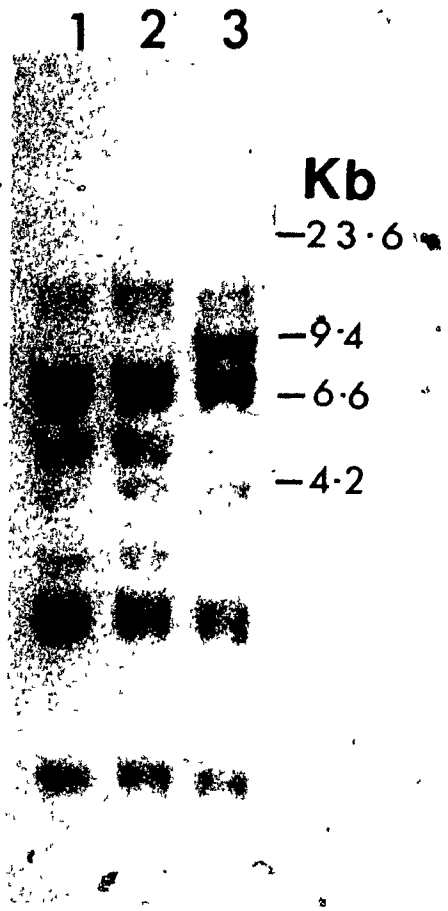
III.3.5 Complementation by sub-pools of a genomic cosmid library

In an alternative approach to isolate the DNA region encompassing the mutations in 23H11 and 24F6, the pLAFR3 cosmid library was pooled into groups of 48 clones each. The sub-pools were conjugated into both mutants separately and tet^r colonies (presumably containing cosmid clones of the genomic library) were inoculated onto soybean plants. Several normal-looking nodules were found on plants inoculated with 23H11 containing three different sub-pools of the genomic

library. A curious observation was that 24F6 conjugated with the same pools did not induce any nodules. The bacteria isolated from the wild-type-like nodules elicited by the presumably complemented mutant, designated 23H11/C were found to be tet^r and bacteriophage-sensitive, suggesting the presence of a complementing cosmid. Cosmid DNA was prepared from 23H11/C and transformed into E.coli cells. Cosmid preparations from E.coli cells showed the presence of a cosmid, designated pPS23A, which contained about 27 kb of insert DNA. To check the authenticity of the cosmid clone, it was nick-translated and hybridized to Southern blots of EcoRI-digested genomic DNA from mutants 23H11, 24F6 and the wild-type. Fig. 18 shows that both mutants lack a specific EcoRI fragment (approximately 8 Kb) that is present in the wild-type.

As distinct cell surface changes were detected in the phage-resistant nodulation-defective mutants, it was important to test whether the complementation by cosmid pPS23A would also restore these alterations to wild-type patterns. Total proteins were isolated from 23H11, 23H11/C and wild-type and analysed by Western blots as before. Figure 19 shows that complementation of the mutation in 23H11 also restores some surface components to the wild-type pattern. An analysis of crude extracts specifically stained for oligosaccharide components also reveals that the wild-type pattern is restored in the complemented mutant 23H11/C (see Figure 20).

Figure 18 : Hybridization of Southern blots of EcoRI digested genomic DNA from B. japonicum 61A76 bacteriophage-resistant mutant 23H11 (lane 1), 24F6 (lane 2) and wild-type (lane 3), with radioactively labelled cosmid pPS23A. Note both mutants are lacking a specific EcoRI fragment (approximately 8 Kb) that is present in the wild-type.
A restriction map of the cosmid pPS23A showing the relative position of the EcoRI fragment unique to B. japonicum 61A76 wild-type. R_1-EcoRI, P-PstI, X-XhoI.



pPS23A

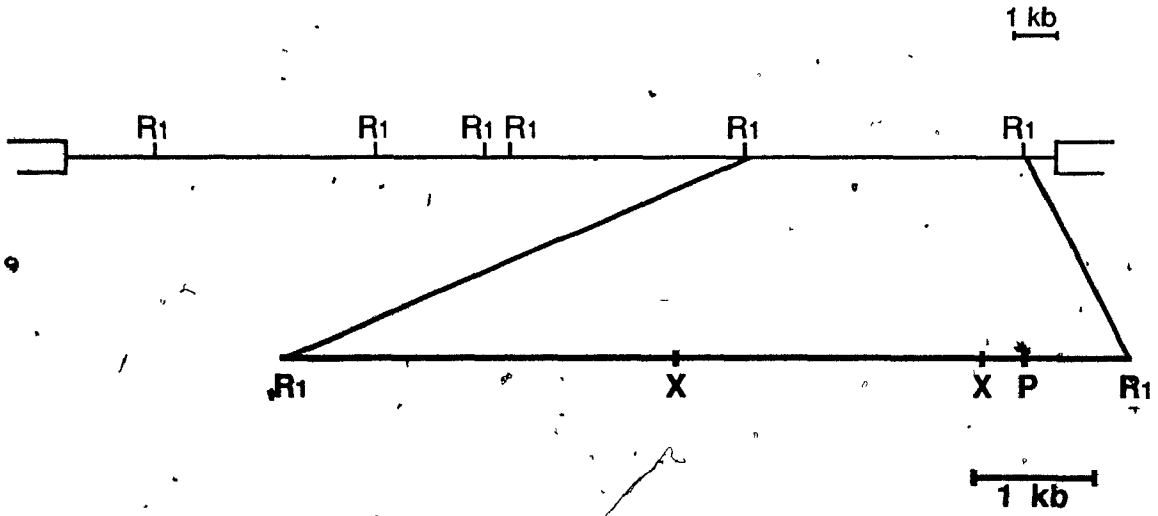


Figure 19 : Demonstration of partial restoration of surface protein components in B. japonicum 61A76 bacteriophage-resistant mutant 23H11 by the presence of the complementing cosmid pPS23A. 5 ug of total proteins from 23H11 (lane 1), 23H11/C (lane 2) and the wild-type (lane 3) were separated by SDS-PAGE, and (A) transferred to nitrocellulose and immunoblotted with an antiserum raised against whole wild-type cells, (B) stained with silver stain.

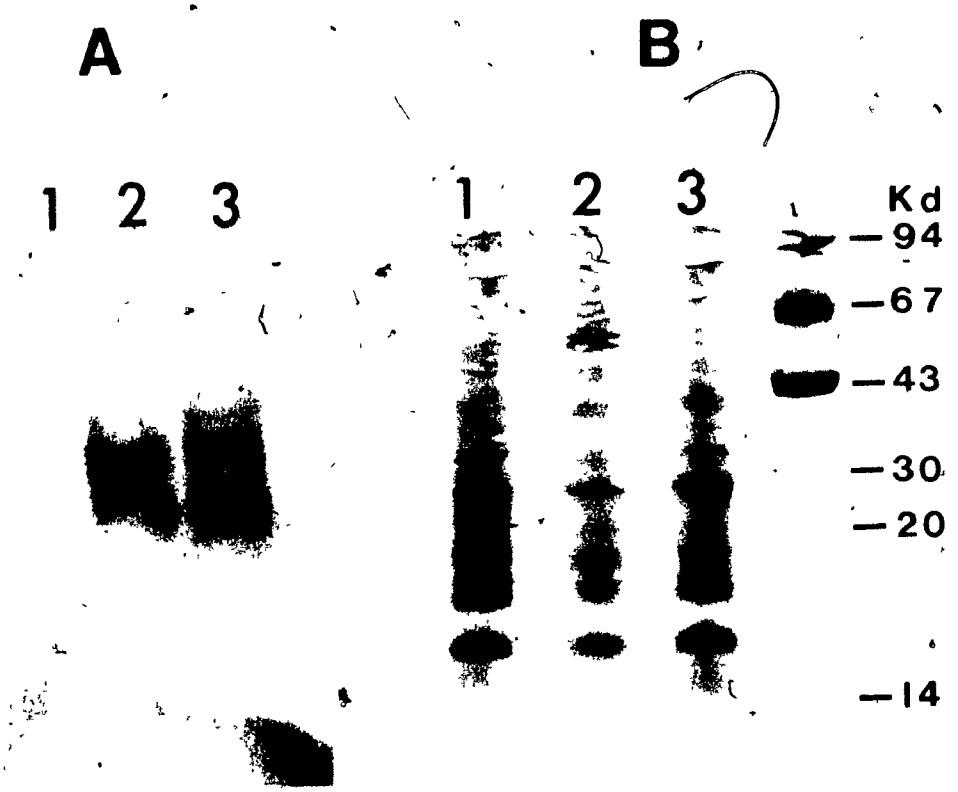

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Figure 20 : Demonstration of restoration of wild-type pattern of surface polysaccharides by complementing cosmid pPS23A. Crude polysaccharide extracts from B. japonicum 61A76 bacteriophage-resistant mutant 23H11 (lane 1), 23H11/C (lane 2) and wild-type (lane 3) were separated by SDS-PAGE and (A) gel stained before periodic treatment (B) gel stained by the periodic acid method of Dubray and Bezard (1982) to visualize polysaccharides. Only the relevant areas of the gel are shown here.


A
B
1
2
3
1
2
3

III.3.6 Possibility of a double mutation in 24F6

As it was not possible to complement mutant 24F6 with pPS23A, it was probable that another mutation existed elsewhere in the genome. The possibility of Tn5 causing the other mutation was re-examined. RNA extracted from 24F6 and the wild-type was subjected to Northern blot analysis. Fig 21 shows a SP24-probed Northern blot of RNA from free-living cultures of 24F6 and the wild-type. The mutant clearly shows a transcript smaller than the wild-type, possibly caused by the insertion of Tn5. It is therefore probable that in addition to the deletion/rearrangement, mutant 24F6 has lost some other function due to the insertion of Tn5 and that this lost function is also required for proper nodule development.

Figure 21 : Northern blot analysis of RNA extracted from the bacteriophage-resistant mutant 24F6 (lane 1) and the wild-type (lane 2) probed with the radioactively labelled Tn5-containing EcoRI fragment from pSP24. The Tn5-encoded transcripts are not shown.

1 2

Chapter IV/Discussion

The most successful approach used in the study of Bradyrhizobium japonicum symbiotic genes has been to look for genes which share structural or functional homology with previously identified Rhizobium symbiotic genes. Thus, the nitrogenase genes, the common nodulation genes, and homologues of other Rhizobium genes required for nitrogen fixation, have been identified in Bradyrhizobium (Henecke, 1981; Fuhrman et al., 1985; Marvel et al., 1985; Russel et al., 1985). However, as this approach is unlikely to identify genes that are unique to Bradyrhizobium, such as those responsible for the differences in host range specificity and other aspects of symbiosis, it is important to have a random mutagenesis procedure to identify previously uncharacterized symbiotic genes. This work represents a step in this direction.

IV.1 Transposon mutagenesis

The suicide vector, pJB4JI, was found to be unsuitable for transposon mutagenesis in Bradyrhizobium japonicum. Even though it has been successfully used to generate genuine Tn5 insertion mutations in several species, frequent problems associated with its use include cotransposition of Mu sequences, transposition of endogenous insertion sequences into symbiotic genes and the induction of complex DNA rearrangements and deletions (Meade et al., 1982; Ruvkun et

al., 1982). Moreover, pJB4JI is believed to behave differently in various species of Rhizobium as well as in different strains of the same species (Meade et al., 1982). Therefore, the failure of pJB4JI to yield large numbers of genuine transposon insertion mutants in Bradyrhizobium japonicum is not surprising.

The use of an alternative suicide vector, pGS9 (Selvaraj and Iyer, 1983), allowed the establishment of single, random Tn5 insertion mutations in Bradyrhizobium japonicum 61A76 (Rostas et al., 1984). The transposition frequency of Tn5 (10^{-6} per cell) was comparable to that reported for other Rhizobium species such as R. meliloti (Meade et al., 1982) and permitted the isolation of a large number of transposon Tn5 mutants (Rostas et al., 1984). Putnoky et al. (1983) and Selvaraj and Iyer (1984) have reported that Tn5 carries a str^r gene which is not expressed in E. coli but is expressed in several Rhizobium species. Therefore, the isolation of Tn5 mutants in this work was facilitated by the selection for str^r along with Tn5-encoded kan^r. This step not only counter selected the donor E. coli strain but also prevented the isolation of spontaneous kan^r mutants. Consistent with an earlier report (Meade et al., 1982), physical analysis of genomic DNA from several mutants revealed a lack of site-specificity for transposition (Fig. 4). Hybridization analysis as well as microbiological tests confirmed that the incidence of vector cointegration was extremely low.

extremely low.

The incidence of auxotrophy confirmed that Tn5 insertion is not restricted to a specific region of the genome. However, the frequency of auxotrophs observed in this study (0.05%) was much lower than that reported by Meade et al. (1982) for R. meliloti (0.3%). Parke and Ornston (1984) conducted an auxanographic nutritional survey of aromatic and hydroaromatic compounds for several representative strains of the members of Rhizobiaceae. They concluded that members of the slow-growing Bradyrhizobium group showed the greatest nutritional diversity in terms of their capability to utilize aromatic compounds, whereas the fast-growing Rhizobium species were more fastidious in their nutritional requirements. These observations suggest the occurrence of alternative pathways for the biosynthesis of growth compounds in Bradyrhizobium which probably explains the lower frequencies of auxotrophy within this group.

Tn5 mutants defective in symbiosis were detected by plant tests. All of these mutants were capable of forming nodules but were defective in nitrogen fixation capability. Characterization of the Tn5 insertion by Southern blot analysis showed that all mutants had a single Tn5 insertion and that Tn5 had not inserted in the nitrogenase structural genes nifKD (Fig. 5). Morrison and Verma (1987) further characterized one of these mutants (T8-1) which is blocked in the endocytosis of bacteria from infection threads and showed

affected the expression of some peribacteroid membrane nodulins.

IV.2 Histidine auxotrophy and symbiosis

An association between auxotrophy and symbiotic defects has been recognised for several years. Adenine auxotrophs of R. leguminosarum were unable to nodulate pea plants (Pain, 1979; Schwinghamer, 1969; Pankhurst and Schwinghamer, 1974) whereas adenine auxotrophs of R. meliloti formed ineffective nodules on alfalfa (Scherrer and Denarie, 1971). Similarly leucine (Denarie et al., 1976) and histidine auxotrophs (Malek and Kowalski, 1977) of R. meliloti and a riboflavin requiring mutant of R. trifolii (Schwinghamer, 1970) elicited ineffective nodules on their respective hosts.

A study of auxotrophs of B. japonicum USDA 122 showed that two mutants which were auxotrophic for histidine were also defective in nodulation (Sadowsky et al., 1986). Mutants His1 and His3 are representative of this class of auxotrophs. However, in addition to these two nod$^-$ mutants, two other histidine auxotrophs designated His2 and His4 were found to be symbiotically competent. A similar linkage between histidine auxotrophy and nodulation has also been reported in B. japonicum USDA 110 (So et al., 1987).

Biochemical studies using intermediates of the histidine biosynthetic pathway showed that all four histidine auxotrophic mutants grew in the presence of L-histidinol but

failed to grow in the presence of L-histidinol phosphate. While the exact nature of the his$^-$ phenotype is unknown, presumptive evidence based on a comparison to the histidine biosynthetic pathways in E. coli and Salmonella spp indicate that the step mediated by the enzyme L-histidinol phosphate phosphatase may be affected. However, it is not clear whether the lack of growth on L-histidinol phosphate is due to the mutants inability to transport this compound or to the presence of a different histidine biosynthetic pathway in B japonicum.

The histidine auxotrophs examined in this study behaved similarly to R. meliloti histidine auxotrophs (Malek and Kowalski, 1977) in that they were rendered symbiotically competent when inoculated onto plants supplemented with histidine. While the His1 and His3 bacteroids recovered from histidine-supplemented plants were all kan^r auxotrophs (mutant type), the bacteroids from the occasional nodules produced on non-histidine-supplemented plants had a variety of phenotypes. Some of the nodule isolates of non-supplemented plants inoculated with mutant His1 were kan^r auxotrophs. When these isolates were reinoculated onto plants, they were capable of nodulating soybeans. Other nodule-isolated bacteria from mutant His1 were kan^s prototrophs or true revertants. However, the bacteria isolated from nodules of non-histidine-supplemented plants inoculated with mutant His3 gave three different phenotypes. While only one of five plants had

nodules, the nodules contained either kan^r auxotrophs, kan^s prototrophs, or kan^s auxotrophs. All of these nodule isolates effectively nodulated soybeans when reinoculated onto plants. Hybridization analysis of DNA from the kan^s isolates showed that Tn5 was absent (Fig. 7C). A precise excision of Tn5 could lead to a kan^s prototroph while an imprecise excision could lead to a kan^s auxotroph. The kan^r auxotrophic nodule isolates from mutants His1 and His3 may have arisen by a second mutation or a slightly different mechanism. For example, it is possible that in these revertants Tn5 has transposed to a different site creating mutations analogous to His2 and His4.

The nod^- phenotype of mutants His1 and His3 appear closely related to auxotrophy, since supplementation of the plant growth medium with histidine or reversion to prototrophy restores symbiotic competence. In addition, neither mutant has Tn5 inserted in any of the known nif and "common-nod" sequences. However, the exact relationship between auxotrophy and symbiotic performance remains unclear since two of the His^- mutants, His2 and His4, are symbiotically competent. These results are similar to those found by Federov and Zaretskaya (1978) in which EMS-induced methionine auxotrophs of R. meliloti could either be effective or ineffective with respect to nitrogen fixation. It is quite possible that the two His^- symbiotic classes have different mutations and that the Nod^- mutants are unable to utilize plant-secreted histidine or its derivatives. While there appears to be

sufficient histidine in root-extracts to support growth of mutants His1 and His3 in petri-dish assays, it may be insufficient to support growth *in planta*.

Hybridization of the cloned Tn5-containing EcoRI fragments from the nod$^-$ mutants His1 and His3 to Southern blots of genomic DNA from His1, His3 and the wild-type showed that both mutants lacked the same EcoRI fragment (Fig. 8A), indicating that Tn5 had been inserted into the same EcoRI fragment in these mutants. Restriction mapping analysis of the cloned Tn5-containing EcoRI fragments from His1 and His3 (plasmids pH1 and pH3 respectively) further supports this hypothesis. Unexpectedly, however, the inserts from pH1 and pH3 were of different sizes and two His1 genomic fragments hybridized to the insert from pH1. The most plausible explanation is that the Tn5 insertion in nodulation mutant His1 also caused some kind of genome rearrangement in the neighbouring DNA region, such that restriction site polymorphisms were created in that region of the genome.

IV.3 Cell-surface mutations

In this study, four thousand Tn5 mutants were screened for cell surface alterations by selecting for bacteriophage resistance. The frequency of mutation for the phage resistance phenotype amongst the Tn5 mutants was remarkably high (0.5%). The frequency of spontaneous bacteriophage resistance was determined to be 10^{-4}. As the frequency of Tn5-induced

determined to be 10^{-4}. As the frequency of Tn5-induced mutations was much higher than that of spontaneous mutations, it is probable that Tn5 was the cause of most mutations, either directly by insertion or indirectly by other means. The high frequency of mutation suggests that there is either a hot spot for the phage resistance locus, or that there are a large number of genes involved in phage binding. However, the data at hand do not allow the verification of either possibility.

Two of the phage-resistant mutants 23H11 and 24F6, were further shown to be defective in nodulation capability. Characterization of these mutants revealed that the mutations were not directly linked to the Tn5 insertions. Hybridization analysis suggested that there were no alterations in the DNA region flanking the Tn5 insertions nor were the nif and nod regions perturbed in any way (Fig. 17). These results taken together strongly implied that the mutations were in loci different from the Tn5 insertions, nif and nod regions. En masse complementation with a genomic cosmid library led to the isolation of a cosmid, pPS23A, that could correct the symbiotic deficiency of one bacteriophage-resistant mutant, 23H11. Southern blot hybridization analysis with EcoRI-digested genomic DNA from the mutants and wild-type indicated that in both mutants a specific EcoRI fragment (approximately 8 Kb) had either been deleted or rearranged to give smaller-sized fragments (Fig. 18). While the conclusion that Tn5 had caused the observed rearrangement cannot be substantiated with

the probable cause of the observed rearrangement.

Kaluza et al. (1985) have reported that several copies of two types of repeated sequences (RS) were present in the B. japonicum 110 genome. Their analysis of these sequences suggested that they possessed features characteristic of bacterial insertion elements. Even though they found several copies of the RS elements in the DNA region flanking the nif structural genes, they found no evidence that these RS elements were functionally involved in symbiotic nitrogen fixation. However, an analysis of similar endogenous insertion elements in R. meliloti has revealed that the element designated ISRm1 preferentially inserts into the nif genes, giving rise to a high frequency of fix$^-$ mutations (Ruvkun et al., 1982).

Two recent reports describe the use of transposon Tn5 in the generation of symbiotic mutants in B. japonicum USDA 110 (Regensburger et al., 1986; So et al., 1987). Both studies have observed Tn5-induced deletions leading to a defective symbiotic phenotype. Their observations are consistent with the results reported in this study that Tn5-induced genome rearrangements do occur and can lead to a defective symbiosis. Moreover, So et al. (1987) also reported an instability of Tn5 insertion mutants and a significant level of vector cointegration. Due to the secondary genetic changes observed they advocate a cautious approach in the use of Tn5 mutagenesis of B. japonicum strains. While the mechanism of

mutagenesis of B. japonicum strains. While the mechanism of Tn5-induced genetic changes is not fully understood at present, it is tempting to speculate that the transposase activity introduced by Tn5 may lead to a high instability of the endogenous insertion-element-like repeated sequences in the B. japonicum genome. The high frequency of phage resistance in Tn5-induced mutants could be explained if one imagines that several RS sequences are present in the vicinity of genes encoding functions required for phage adsorption and proper nodule development. By the introduction of transposase activity, these RS elements could be activated to insert into the flanking region, in a manner analogous to the ISRm1 insertion into the nif genes of R. meliloti. A detailed genetic characterization of the DNA region contained in cosmid pPS23A should allow a test of this hypothesis.

The phenotypes exhibited by the two nodulation defective mutants are complex and resemble that of pleiotropic mutants. Studies quantitating the adsorption of phage to mutant cells clearly implicated an alteration in the cell surface. Immunoblot analysis of total proteins probed with antiserum raised against whole wild-type cells showed distinct differences in surface protein profiles (Fig. 9). It is hard to concieve of a single mutation leading to such drastic alterations of cellular components, unless the mutation was in a key regulatory gene or in some general metabolic or processing function, which has far reaching consequences.

However, the isolation of a complementing DNA region will permit further work to explore the gene(s) and its function(s). The lack of complementation of mutant 24F6 by cosmid pPS23A in addition to the lack of alterations in the nif and nod suggests the possibility of a second mutation elsewhere in the genome. Even though complementation experiments failed with cosmids containing wild-type DNA region encompassing the Tn5 insertion site, it was possible that Tn5 had inserted into a gene required for symbiosis. The detection of a truncated transcript gives credence to this hypothesis. However, it is not known with certainty if the Tn5 insertion has resulted in the loss of any symbiotic function.

The LPS of the bacterial surface has been implicated in the symbiotic process. Non-nodulating mutants of B. japonicum 61A76 have been shown to lack a surface antigen that is associated with the O-antigen of the LPS (Maier and Brill, 1978). Other studies have also implicated an involvement of LPS in symbiosis (Stacey et al., 1982; 1984). Recently, Puvanesarajah et al. (1987) have demonstrated specific differences in the LPS structure of a phage-resistant non-nodulating mutant of B. japonicum. Preliminary analysis of crude extracts from one mutant 23H11, showed an abnormal accumulation of a polysaccharide component which regains the wild-type pattern when complemented with cosmid pPS23A. While the chemical identity of the polysaccharide component is not known at present, a comparison of the electrophoretic mobility

of the polysaccharide with that reported by Puvanesarajah et al. (1987) suggests that it could be a LPS component. Comparison of EPS preparations from the nodulation development mutants with the wild-type EPS by proton NMR spectroscopy showed that both the mutants lacked a methylene modification of the 3-hydroxybutanoic acid component of the acidic EPS (Dr. Frank Dazzo personal communication). The observation of a genetically diverse set of exopolysaccharide deficient (exo) mutants of R. meliloti that are also defective in nitrogen fixation (Finan et al., 1985; Leigh et al., 1985) strongly implies a role for EPS in symbiotic nitrogen fixation. Convincing evidence for such a role comes from the fact that the exogenous addition of purified EPS to fix$^-$ exo mutants of Rhizobium species NGR234 and R. trifolii, restores symbiotic fixation capability (Djordjevic et al., 1987b). Defects in several genetic loci give rise to the exo phenotype suggesting that some comprise genes encoding proteins involved in the biosynthesis of acidic polysaccharides. Other mutations may cause more general alterations in the cell membrane or cell surface that interfere indirectly with EPS production. The R. meliloti exoC mutants which are resistant to nine phages are possibly of this type (Leigh et al., 1985).

The B. japonicum 61A76 mutants described in this study exhibit gross cell surface alterations at the protein level as well as in certain components of the EPS and possibly LPS. The mutants appear to have defects in general cell surface

structures/functions that take on added importance in the symbiotic state. They may be analogous to the pleiotropic exoC mutants of R. meliloti.

The symbiotic phenotype of the nodulation-defective mutants resembles that of the exo (Finan et al., 1985; Leigh et al., 1985) and ndv (Dylan et al., 1986) mutants of R. meliloti. An examination of root hairs of G. soja inoculated with the mutants showed that the mutants were able to evoke a visible reaction by the root hairs (Fig. 12). While the characteristic shepherds crook was not seen, the root hairs were bent and distorted similarly to those of wild-type-inoculated plants. Uninoculated control plants as well as plants inoculated with R. meliloti showed no visible reaction. The mutants can therefore be called Hac^+ according to the classification of Vincent (1980). The nodule like bumps elicited by the phage-resistant nodule-development mutants were varied in size and shape, and were numerous. Light microscopy showed that these structures comprised a mass of meristematic cells initiated from the root cortex (Fig. 13). The tissue was characterized by a lack of Bradyrhizobium-infected cells as well as an absence of differentiation into infected and uninfected cell types. Mutant 23H11 infrequently produced infection threads containing bacteria which were rarely released into the host cytoplasm (Fig. 15). According to the terminology proposed by Vincent (1980), these mutants are Hac^+ (capable of curling root hairs) and Inf^- (incapable

of initiating infection threads), though 23H11 is strictly Inf^+. However, these are also Noi^+ (for nodule initiation) even though this stage is considered to be later than infection. A similar paradox has been reported by Finan et al. (1985) in R. meliloti. Therefore the phenotypic descriptions should not be taken to imply any particular linear sequence of events. To avoid confusion with this nomenclature, the term nodule development (ndv) is more appropriate to describe the mutational block in these mutants. To speculate on the nature of the defect that leads to altered symbiosis, it is possible that recognition between the host and symbiont has been altered due to cell surface changes in the bacteria. Several studies in different Rhizobium and Bradyrhizobium species support the idea that cell wall integrity plays an important role in proper nodule development and function. Perhaps the deficient surface component serves as a signal for the induction of a particular step in the development process. Alternatively, the component may be a part of the infection thread matrix (Leigh et al., 1985) or may serve as a substrate for some plant enzyme, the product of which is required for the continuation of the development process (Finan et al., 1985). Another possible consequence of altered cell surface is that it might cause the rejection of what is normally a compatible symbiont by the host plant (Dylan et al., 1986). A breakdown in signal exchanges between the symbiotic partners due to any of these reasons could lead ultimately to improper

nodule development and defective symbiosis.

In conclusion, random Tn5 mutagenesis of B. japonicum 61A76 has led to the isolation of several mutants defective in symbiosis. A characterization of the DNA region that has been mutagenized by the Tn5 insertion will allow the identification of genes that are important for symbiosis in B. japonicum. A step in this direction is the work of Morrison and Verma (1987) who reported that in B. japonicum mutant T8-1, even though the Tn5 insertion caused a block in the release of bacteria from infection threads, the expression of pbm nodulins in the host plant were unaffected. A selection for cell surface alterations by screening for bacteriophage resistance has led to the identification of two mutants that are also defective in nodulation. An analysis of surface proteins and polysaccharides showed that there were gross alterations in these cell components. While the Tn5 insertion was not the primary cause of the mutations, complementation tests led to the isolation of a cosmid that overcame the symbiotic defect in one of the two mutants. An analysis of the surface proteins and polysaccharide showed a partial restoration of these surface components to wild-type patterns. A detailed characterization of the DNA contained in the complementing cosmid, pPS23A, will show if the mutation is in a regulatory gene or in a gene coding for a key biosynthetic/processing step that affects several functions. Tn5 mutagenesis of the cosmid followed by marker exchange with

wild-type region will demarcate the genes that are essential for proper cell surface integrity and nodule development.

The isolation of Tn5-induced histidine auxotrophs of B. japonicum highlights the importance of histidine in nodulation. The isolation of a cosmid that complements the biochemical defect for histidine as well as nodulation further confirms the relationship. Further characterization of the cosmid clone might help elucidate the exact role of histidine in symbiosis. Sequencing of the cloned gene and comparison to published DNA sequence of the E. coli hisB gene that encodes histidinol phosphate phosphatase, would also show if the histidine biosynthetic pathway in B. japonicum is analogous to that in E. coli.

Chapter V References

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