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BACTERIAL PLASMID-ASSOCIATED REGIONS DETERMINING NODULATION
IN COMMERCIAL AND PRIMITIVE CULTIVARS
OF PEA (Pisum sativum)

by

© SHENGWU MA, B.Sc., M.Sc.

A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfilment of
the requirements for the degree of
Doctor of Philosophy.

Department of Biology.
Carleton University.
Ottawa, Ontario.
September 28, 1988.

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The undersigned recommend to the Faculty of Graduate Studies
and Research acceptance of the thesis:

"Bacterial Plasmid-associated Regions Determining Nodulation in
Commercial and Primitive Cultivars of Pea (Pisum sativum)"
submitted by Shengwu Ma, B.Sc., M.Sc.
in partial fulfilment of the degree of
Doctor of Philosophy.

Chairman, Department of Biology.

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September 28, 1988.

ABSTRACT

Rhizobium leguminosarum biovar viciae is distinguished from other biovars of this species on the basis of the legume species on which it forms root nodules. For example, the biovar trifolii nodulates clover and the biovar viciae nodulates pea and vetch. The molecular basis of this host specificity is not well-understood although it is known that its genetic determinants are plasmid-borne. Different isolates of a single biovar can also display differences in their ability to nodulate different cultivars of the same legume species. Thus, a particular rare strain of biovar viciae called TOM, isolated from soil in Turkey, can form nodules on the primitive pea cultivar (cv) Afghanistan, as well as cultivars that are now used commercially (e.g. cv. Novella). On the other hand, most other strains of this biovar are unable to nodulate cv. Afghanistan (e.g. strain 248). In both of these different strains of the same biovar (viciae), determinants of host and cultivar specificity were known to be located on one of their plasmids called respectively pRL5JI and pRL1JI.

The structural and functional organization of a set of nod (nodulation) genes in pRL5JI and pRL1JI have been compared using transposon mutagenesis, subcloning and genetic complementation. It was concluded that the overall organization of the genes in the two plasmids is similar and that most genes are exchangeable functionally. A major difference is that pRL5JI but not pRL1JI contains a region of about 2 kb (kilobase pairs), the presence and expression of which is correlated with the ability to nodulate cv. Afghanistan. In addition, there may be subtle differences in their respective nodD and nodIJ genes. A single polypeptide could be ascribed to this 2 kb cultivar extension region on

the basis of polypeptides detected in an E coli in vivo coupled transcription-translation system. On the basis of the size of this polypeptide and the nucleotide sequence of the 2 kb region that was also determined, an initiation and termination codon in this sequence could be preferentially ascribed to this function. This putative polypeptide is inferred to be highly hydrophobic and therefore likely to be associated with the bacterial membrane.

EcoRI-digested total DNA of 41 different field isolates of R. leguminosarum biovar viciae was examined by DNA hybridization (Southern blotting) against radioactive probes containing different nod genes of the characterized pRL5JI. A fragment that hybridized to the 2 kb cultivar extension region of pRL5JI was found in a few cases and these strains also nodulated both pea cultivars. Isolates were also identified that did not show hybridization to this cultivar extension region and yet nodulated cv. Afghanistan. One isolate that displayed hybridization did not nodulate cv. Afghanistan. From these studies and the geographical origin of these isolates, it was concluded that:

- 1) isolates with the ability to nodulate the primitive cv. Afghanistan are rare but not limited to the Middle East;

- 2) such isolates may represent more than one clonal class with possibly different mechanisms of cultivar extension, and;

- 3) isolates from the same geographical location tend to have less polymorphism in restriction fragment length across the nod region than isolates from separated geographical regions.

DEDICATION

This dissertation is dedicated to my parents and to Ziqin Yin.

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First, and most important, I would like to express my sincere gratitude to my Supervisor, Dr. V.N. Iyer. His generous help, advice, patience, encouragement and support is greatly appreciated.

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Thanks to Dr. A.W.B. Johnston for providing Rhizobium pRL1JI::Tn5 nod mutants used in this study.

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LIST OF ABBREVIATIONS

Ap	ampicillin
dATP	deoxyadenosine triphosphate
bp	base pairs
Cm	chloramphenicol
dCTP	deoxycytosine triphosphate
cv.	cultivar
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetra-acetic acid
EPS	exopolysaccharide
EtBr	ethidium bromide
<u>exo</u>	genes for synthesis of exopolysaccharide
<u>fix</u>	nitrogen fixation gene
h	hour(s)
Hac	root hair curling
hsn	host specificity of nodulation
Hup	hydrogen uptake
Inc	incompatibility group
Inf	infection thread formation
kb	kilobase pairs
kd	kilodaltons
Km	kanamycin
lac	lactose utilization
mg	milligram
mL	millilitre
md	megadalton
Met	methionine
min	minute(s)
m.o.i.	multiplicity of infection
Nal	nalidixic acid
Nm	neomycin
Nod	nodulation (phenotype)
<u>nod</u>	nodulation (genotype)
ORF	open reading frame
<u>oriV</u>	origin of vegetative replication
PEG	polyethylene glycol
<u>phoA</u>	alkaline phosphatase structural gene
pol	DNA polymerase
rif	rifampicin
r	resistance
s	sensitivity
Sm	streptomycin
Sp	spectinomycin
Sym	symbiotic
Tn	transposon
<u>Tn5</u>	Transposon 5
Tc	tetracycline
Tra	conjugal transfer
μ Ci	microcurie
μ g	microgram
μ l	microlitre
UV	ultraviolet radiation

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INTRODUCTION

Rhizobium leguminosarum is a species of Gram-negative soil bacteria that can grow as free-living cells but which can also enter into a symbiotic relationship with specific groups of leguminous plants. In the roots of these plants, it forms nodules which are organized and developed structures within which morphologically-altered forms of the bacteria called bacteroids fix atmospheric nitrogen. There are three biovars of R. leguminosarum named on the bases of host-nodulation specificity. These are R. leguminosarum biovar viciae which nodulates pea (Pisum sativum), R. leguminosarum biovar trifolii which nodulates field clover (Trifolium repens) and R. leguminosarum biovar phaseoli which nodulates bean (Phaseolus vulgaris).

Although most cultivars of Pisum sativum (pea) are nodulated by R. leguminosarum bv. viciae, it has been found that certain rare cultivars (cv.) are not (Kneen and LaRue, 1984; Young et al., 1982). One such cultivar, which is of primitive origin, is cv. Afghanistan. Holl (1975) found that this resistance to nodulation by cv. Afghanistan is conditioned by a recessive gene, sym-2. Other genes determining low nodule number and temperature-sensitive nodulation, have also been found in cv. Afghanistan (T. LaRue, personal communication). In 1978, Lie isolated an unusual strain of R. leguminosarum bv. viciae that nodulated cv. Afghanistan in addition to the common pea cultivars, and called this strain, which was isolated from soil in Tomek, Turkey, as strain TOM. The cv. Afghanistan-TOM relationship thus provides one interesting and attractive model system with which to explore the basis of specific symbiotic interactions between a bacterium and its plant host.

This study is concerned with the bacterium, one of its principal objectives being to contribute to the understanding of the

molecular basis of the extended specificity of strain TOM. This seemed to be a reasonable undertaking as there was already evidence in the literature suggesting that a molecular basis of the process would be tractable. Thus, it had been established that the determinants of this cultivar extension was on a self-transmissible plasmid (Brewin, 1980a) and that this plasmid had a region of about 2 kilobase pairs (kb) that was absent in strains of R. leguminosarum bv. viciae with a nodulation range that did not extend to cv. Afghanistan (Hombrecher et al., 1984; Gotz et al 1985). However, neither nodulation region of this plasmid or part of it that conferred cultivar extension had been well characterized. A major goal of this study was such a characterization (Chapter 1) and as part of this the nucleotide sequence of the cultivar extension region was determined (Chapter 2). While this was in progress, Davis et al., (1988) published a sequence of the same region. The sequence I determined (in collaboration with J.H.E. Nash) is in agreement with that of Davis et al., (1988). I disagree with the interpretation of the sequence for reasons that are also discussed in Chapter 2.

For the above analysis of the nodulation region of strain TOM, a series of subclones of the cultivar extension region had been generated. In the experiments described in Chapter 3, I have exploited these subclones to survey the DNA of a collection of field isolates of R. leguminosarum from various geographical origins for the presence of the cultivar extension region, and fragment length polymorphisms in the respective nod regions. This survey included several recently isolated field strains that nodulate cv. Afghanistan.

Each chapter concludes with a separate discussion and the thesis ends with a list of conclusions.

LITERATURE REVIEW.

The Literature review in this section was completed in July of 1988.

The Rhizobiaceae.

Classification and nomenclature.

In the published literature, the nomenclature used for species and genera within the family Rhizobiaceae is confusing. The nomenclature advocated by Bergey's Manual of Determinative Bacteriology (9th Edition, 1984) is authoritative and is therefore used in this thesis.

The family Rhizobiaceae comprises three genera: Agrobacterium, Rhizobium and Bradyrhizobium. The criteria used for classifying genera are shown in Table 0.1. The main feature that differentiates Agrobacterium from Rhizobium and Bradyrhizobium is that Agrobacterium has the capacity to induce plant diseases (crown gall and hairy root disease) on many dicotyledonous plants, but Rhizobium and Bradyrhizobium do not. The two latter genera are characterized by their ability to successfully induce the formation of nodules on roots of members of the plant family Leguminosae.

The differentiation of Rhizobium from Bradyrhizobium is based on their growth rate in yeast-extract-mannitol-mineral salts medium, flagellar arrangement, DNA base composition and the genera of host plants nodulated. The genus Rhizobium contains all fast-growers with a mean generation time of about 2 hours while all Bradyrhizobium species are slow-growers with a mean generation time of about 6-8 hours. There are many biochemical and physiological differences between the two genera. For instance, Rhizobium species can use a wide range

Table 0.1: Key to the genera of the family Rhizobiaceae.

Family: Rhizobiaceae

Genus:	<u>Rhizobium</u>	<u>Bradyrhizobium</u>	<u>Agrobacterium</u>
Differential characteristics of the three genera:	Cause nodule production on roots of leguminous plants that are predominantly of the temperate zone.	Cause nodule production on roots of tropical and some temperate zone plants.	Do not cause root nodule production but do produce other types of heterotrophies (crown gall) on many plants.
	Fast growth on yeast-extract-mannitol agar; production of acid in mineral salts-mannitol medium.	Slow growth on yeast-extract-mannitol agar; production of alkali in mineral salts-mannitol medium.	Do not fix nitrogen.
	Mol% G+C of total DNA is 59 - 64 (T_m).	Mol% G+C of total DNA is 61 - 65 (T_m).	Mol% G+C of the total DNA is 57 - 63 (T_m).

(Adopted from Jordan, D.C., (1984).

(T_m) - DNA melting temperature.

of carbohydrates and organic acids for growth whereas the Bradyrhizobium grow only on a smaller range of substrates (Jordan, 1982). The two genera also differ in several symbiotic properties. For example, Rhizobium species fix nitrogen symbiotically, whereas at least some or possibly all Bradyrhizobium strains can be induced to fix nitrogen in free-living culture (Kurz *et al.*, 1975; McComb *et al.*, 1975). Rhizobium species infect only a few, closely-related legumes, whereas Bradyrhizobium strains, especially the so called "cowpea miscellany", can infect a broad range of diverse legume hosts (Elkan, 1981). Rhizobium species almost invariably infect their hosts via the root hairs whereas Bradyrhizobium species may infect one host, such as pigeonpea, through root hairs and another, such as groundnut, by direct intercellular penetration between the epidermal cells, the so called "crack entry" process (Chandler, 1978; Dart, 1977).

The genus Bradyrhizobium represents a heterogeneous group of nodulating bacteria within which the taxonomic relationships are not well understood. According to Bergey's manual, only a single species, B. japonicum, is designated within this genus. DNA/DNA hybridization studies by Hollis *et al* (1981) suggest that strains of B. japonicum can be separated into at least three DNA homology groups. Other Bradyrhizobium strains have not yet been classified into species or biovars. Bradyrhizobium species cause nodule production on certain species of Lotus (L. uliginosus and L. pedunculatus), as well as on Vigna and species of Lupinus, Ornithopus, Cicer, Sesbania, Leucaena, Mimosa, Lablab, and Acacia.

Very recently a 'new' genus, Azorhizobium, has been proposed to accommodate bacteria that induce nodules on the stems of Sesbania and which, though fast-growing, are more similar to the Bradyrhizobium than to Rhizobium (Pawlowski *et al.*, 1987).

The genus Rhizobium.

Most Rhizobium show a high degree of selectivity towards their host. In fact, the taxonomy of Rhizobium is largely based on the specificity displayed towards different legumes. Three species of Rhizobium have been recorded in Bergey's Manual (see Table 0.2). The three biovars viciae, trifolii and phaseoli indicated for R. leguminosarum are based largely on host-plant specificity. These three biovars are very closely related and nodulation host-specificity can be readily altered in the laboratory (and is presumably altered in nature) by substituting a plasmid from one biovar with that of another. The retention of R. meliloti as a separate species is based on extensive evidence employing numerical taxonomy (Graham, 1964), DNA hybridization (Jarvis et al., 1980), two-dimensional polyacrylamide gel electrophoresis of cellular proteins (Robert et al., 1980) and findings involving the transfer of infectivity via plasmids (Higashi, 1967; Johnston et al., 1978a). R. loti was proposed by Vincent (1974) on the basis of a variety of evidence. It contains those fast-growing polar and nonpolar-flagellated strains which nodulate a wide range of leguminous plants. Criteria separating R. loti from R. leguminosarum and R. meliloti are presented by Jarvis et al. (1982).

From soybean root nodules, fast-growing root-nodulating bacteria have been obtained which are physiologically distinct from members of the genus Bradyrhizobium (Keyser et al., 1982). These bacteria form effective root nodules on wild soybean (Glycine soja). Taxonomically, these bacteria have

Table 0.2: Nodulation characteristics of Rhizobium species

Bacterial sp.:	<u>R. leguminosarum</u>	<u>R. meliloti</u>	<u>R. loti</u>
	biovar <u>viciae trifolii phaseoli</u>		
Plant sp.:			
<u>Pisum sativum</u> ,	+	±	-
<u>Vicia hirsuta</u> ,			
<u>V. sativa</u>			
<u>Phaseolus</u>	(±)	(±)	+
<u>vulgaris</u>			
<u>Trifolii</u>	±	± ^b	(±)
<u>repens</u>			
<u>Lotus</u>	-	-	-
<u>corniculatus</u>			
<u>Medicago</u>	-	-	-
<u>sativa</u>			
<u>Macroptilium</u>	-	-	±
<u>atropurpureum</u>			

Symbols:

+: generally nodulates; ±: sometimes nodulates, nodules commonly effective in nitrogen fixation; (±): rarely nodulates, nodules commonly ineffective; -: does not nodulate.

^a: some nodulation-negative strains have been reported with V. hirsuta and P. sativum.

^b: some nodulation-negative strains have been reported with isolates from T. ambipum and African clovers.

(Adopted from Jordan, D.C., 1984)

been recently considered as a new species within the genus Rhizobium, named R. fredii.

Patterns of infection and of nodule development.

Leguminous root nodules are highly specialized structures which arise as the result of interactions between the host plant and the invading Rhizobium. Many of the biochemical and morphological aspects of nodule development have been studied a great deal, especially in the fast-growing Rhizobia and in Bradyrhizobium japonicum. Several reviews of this subject are available (Libbenga and Bogers, 1974; Dart, 1974, 1977; Goodchild, 1978; Robertson and Farnden, 1980; Meijer, 1981; Newcomb, 1981; Bauer, 1981; Verma and Long, 1983; Sutton, 1983; Halverson and Stacey, 1986). The following review section will deal with these aspects only briefly. Also, steps prior to the attachment of Rhizobium to the root surface (e.g. chemotaxis) are not considered.

Invasion of the root hair cells.

This event is crucial to the establishment and development of the legume-Rhizobium symbiosis and involves the penetration of Rhizobium into the plant cell. Numerous factors control and influence this infection process including temperature, pH, mineral nutrition, number of Rhizobium, competition and specificity of Rhizobium strains, and time of inoculation. These include:

a) Root Hair Curling. The invasion of the plant by Rhizobium is largely by two methods: either via a host root hair or through openings which arise due to lateral root emergence as in the peanut (Arachis hypogea) and in lupine species (reviewed by Sutton, 1983). R. leguminosarum and R.

meliloti infect their host by the first method. Root hair invasion is characterized by curling or deformation of the root hairs (Dart, 1974). In a sample of 20,000 root hairs in Pisum sativum, only 2.8% were observed to be infected (Newcomb, 1981). Infected root hairs develop a bright spot on the root hair cell wall as observed by light microscopy. This bright spot appears to be the region where the infection thread is originated (Dart, 1977; Newcomb, 1981).

b) Infection Thread Formation. Infection thread formation is initiated with localized degradation of the hair cell wall with concomitant deposition of new cell wall-like material to form a tubular structure (called an infection thread) all the way to the base of the same hair cell (Nutman, 1956). In their study of clover infections, Callahan and Torrey (1981) observed that this new layer of wall material is deposited inside the original hair cell wall at the site of penetration.

The mechanism of localized degradation of hair cell wall is not yet understood. Involvement of both bacterial hydrolytic enzymes (Hubbell et al., 1978; Martinez-Molina, 1979) and host hydrolytic enzymes (Fahraeus and Liunggren, 1967; Munns, 1968; Ljunggren, 1969) have been suggested.

Infection of cortical cells.

a) Release of Bacteria from the Infection Thread. As described in the pea by Libbenga and Harkes (1973), Newcomb (1976) and Sutton (1983), while the infection thread penetrates the outer layer of the root cortex, the cells of the inner cortex begin to divide in advance of the arrival of the infection thread. The infection thread advances towards these dividing cells and the Rhizobia inside the thread continue to divide filling the thread as it elongates. When

the infection thread reaches the dividing cortical cells, bacteria are released from its unwallled tip, each becoming enclosed individually in a cell membrane of plant origin, the peribacteroid membrane.

b) Bacteroid Differentiation. During development of the nodule, the peribacteroid membranes become altered in protein, lipid and enzyme composition, probably to facilitate the interchange of material between the inside and the outside of the membrane envelope. Inside the envelope, the bacteria differentiate into bacteroids, this process involves alterations in the structure of the bacterial cell wall and the surface membrane also becomes highly invaginated. The major effect of all of these changes is that the bacteroids become capable of nitrogen fixation.

c) Nodule Shape. The size and shape of effective nodules are controlled by the plant host rather than by the Rhizobium strain (Kidby and Goodchild, 1966). There are two major patterns of nodule development resulting in 'indeterminate nodules' and 'determinate nodules'. Indeterminate nodules formed in peas and clovers are characterized by continuing infection of new formed cells through the spread of infection threads. The nodules formed in this way are generally elongated. The determinate nodules specified by Glycine max and Arachis hypogea have no persistent meristem and the spread of infection is achieved mainly by the division of preinfected cells. The nodules formed in this way are spherical in shape and are limited in size. For detailed morphogenesis of these nodules reviews by Dart (1977) and Newcomb (1981) are recommended.

Symbiotic plasmids of R. leguminosarum.

Plasmids containing genes that are involved in the establishment and/or maintenance of a successful nitrogen-fixing symbiosis are known as symbiotic-plasmids (Sym-plasmids).

Evidence for Sym plasmids in R. leguminosarum.

Although studies on the genetic basis of the Rhizobium-legume interaction had begun many years earlier, it was the finding of Higashi (1967) that indicated that an extrachromosomal element might be involved in the control of nodulation and host-specificity of R. leguminosarum biovar trifolii strains. However, it was not until the studies of Zurkowski and Lorkiewicz (1976), Nuti et al. (1977), Casse et al. (1979a) and Prakash et al. (1980) that plasmids of various sizes were definitely shown to exist in some Rhizobium strains. Zurkowski and Lorkiewicz (1978) subsequently found a method which was suitable for heat-curing many R. leguminosarum biovar trifolii strains of their nodulation capacity. The mutant non-nodulating phenotype was always correlated with the loss of a large plasmid. The role of plasmid-encoded gene in symbiosis was confirmed when it was shown that the transfer of one of these large plasmids could restore the nodulation capacity to a plasmid-cured strain (Zurkowski, 1980).

A more precise role of a particular Rhizobium plasmid with the encoding of both nodulation and nitrogen-fixation functions followed two developments. The first of these was the use of cloned nitrogenase genes (nifH, nifD) from Klebsiella pneumoniae to show the conservation of such DNA sequences in a variety of nitrogen-fixing bacterial strains (Ruvkun and Ausubel, 1980). Definitive evidence that a single

large plasmid of a fast-growing Rhizobium strain encoded nitrogenase genes came from the hybridization of the cloned Klebsiella nitrogenase genes to plasmid DNA resolved by gel electrophoresis. In addition, non-nodulating Rhizobium strains cured of one of their large plasmids and strains with large deletions in one of their plasmid species lacked the DNA sequence which hybridized to Klebsiella nifH, Δ probes (Ruvkun and Ausubel, 1980). The second development was that of transposon mutagenesis using the transposon Tn5 to label different Rhizobium plasmids (Beringer et al., 1978b; Johnston et al., 1982). This construction initially helped the genetic analysis of such plasmids by demonstrating that some Sym plasmids encoded symbiotic genes and were self-transmissible by bacterial mating (Johnston et al., 1978a; Hooykaas et al., 1981). More recently, the use of Tn5-labelled Sym plasmids has permitted the construction of transmissible plasmids which code for both symbiotic functions by cointegration of the Sym plasmids with other small mobilizable plasmids (Kondorosi et al., 1982; Rolfe et al., 1984; Banfalvi et al., 1983; Morrison et al., 1983).

Biological functions determined by Sym plasmids in the biovars of R. leguminosarum.

Using appropriate approaches, Sym plasmids have been examined from various strains of the three biovars of R. leguminosarum. Transposon mutagenesis was then used to study the functions located on these Rhizobium Sym plasmids. Functions ascribed to Rhizobium Sym plasmids now include the following aspects:

a) Production of medium-sized bacteriocins and bacteriocin repression. Plasmids isolated from R. leguminosarum biovar viciae which contain the genes for production of medium-sized bacteriocins, can be introduced

into a strain of R. leguminosarum biovar viciae that normally produces small molecular weight bacteriocins. Not only does the transformed bacterium produce medium-sized bacteriocins, but it no longer produces small bacteriocins (Wijffelman et al., 1983).

b) Plasmid transfer. The bacteriocinogenic plasmids are transferred between strains of R. leguminosarum biovar viciae at high frequencies. It is thought that the transfer factor is located on the plasmid itself, though there may be some contribution from the chromosome.

c) Nodulation. At present it is quite clear that in fast-growing Rhizobium species determinants for the attachment of Rhizobium cells to root hairs, root hair curling, infection thread formation and nodule development are all plasmid-borne.

d) Host specificity. Different species of Rhizobium infect different legumes. Transfer of plasmids from R. leguminosarum biovar viciae which can infect peas, to R. leguminosarum biovar trifolii or R. leguminosarum biovar phaseoli, which cannot, enabled the recipient strain to nodulate peas (Beynon et al., 1980).

e) Nodule function. Mutation of pRL1JI (the plasmid that confers the Nod⁺ phenotype on pea host) resulted in the production of Fix⁻ (failure to fix nitrogen) nodules in peas infected by R. leguminosarum carrying the mutant plasmid (Buchanan-Wollaston et al., 1980). The nif genes (structural genes for nitrogenase) are also located on this plasmid.

f) Pigment production. Old cultures of R. leguminosarum biovar phaseoli produce a melanin-type dark pigment. The synthesis of this pigment from tyrosine may be encoded on the same plasmid as the one containing the nod genes (Lamb et al., 1982).

g) Incompatibility. Certain R. leguminosarum plasmids cannot coexist within the same cell. They are therefore believed to carry an incompatibility function, as yet unidentified (Beringer et al., 1980).

h) Hydrogen-uptake (Hup⁺) system. Hydrogen evolution from root nodules makes nitrogen fixation by some legume-Rhizobium systems inefficient. However, a limited number of Rhizobium strains have the potential to recycle hydrogen by means of an H₂ uptake system and oxidize it into H₂O. The re-utilization of the nitrogenase-generated H₂ in these nodules could provide ATP for nitrogen fixation and protect nitrogenase against O₂. The determinants of a Hup⁺ system in strain 128C53 of R. leguminosarum biovar viciae have been shown to be located on Sym plasmid pRL6JI (Brewin et al., 1980b).

i) Competitive blocking of host nodulation. R. leguminosarum biovar viciae strain TOM nodulates the resistant form of primitive pea cv. Afghanistan effectively. However, some Afghanistan-non-nodulating strains can block nodulation of this cultivar by TOM. In one strain (F2), the determinants for competitive nodulation blocking of TOM on cv. Afghanistan has been shown to be linked to the Sym plasmid of pF2 (Dowling et al., 1987).

The genes carried on Rhizobium leguminosarum Sym plasmids have been studied in some detail during the past few years and are still the subject of intensive research. Among the Sym plasmid genes involved in symbiotic nitrogen fixation are those designated as nod (nodulation), hsn (host specificity for nodulation), fix (nitrogen fixation which includes nif).

The following section will selectively review both nod and hcn genes and their possible regulation.

The more intensively studied Sym plasmids of R. leguminosarum.

Since the discovery of symbiotic determinants being plasmid-borne in some Rhizobium species, many Sym plasmids have been examined in different strains and different species of Rhizobium. In this section, those plasmids which have been studied intensively and which have a bearing on the pea, clover and Phaseolus bean-R. leguminosarum symbiosis will be reviewed.

pRL1JI is the most extensively studied Sym plasmid of R. leguminosarum biovar viciae.

R. leguminosarum 248 is a field isolate, which harbours three megaplasms with molecular weights of about 90×10^6 , 130×10^6 , and 160×10^6 respectively (Johnston et al., 1978a). Hirsch et al. (1980) were the first to provide evidence that one of the plasmids in this strain was a Sym plasmid and designated it as pRL1JI.

pRL1JI was self-transmissible by inter-bacterial mating. When pRL1JI was transferred to non-nodulating strains of R. leguminosarum biovar viciae or to R. leguminosarum biovar trifolii or R. leguminosarum biovar phaseoli which normally nodulate clover or phaseoli beans but not peas, the transconjugants nodulated peas and also their original hosts (Johnston et al., 1978a).

Downie et al. (1983) isolated two cosmid clones from a genomic library of R. leguminosarum pRL1JI and designated

them pIJ1085 and pIJ1089. These two plasmids had a 10 kb DNA region in common. The transfer of either pIJ1085 or pIJ1089 to R. leguminosarum biovar phaseoli strain cured of its indigenous Sym plasmid (strain 8401) could confer to the recipient the ability to nodulate peas instead of Phaseolus beans (the normal host), indicating that the nodulation (nod) and host-specificity (hsn) determinants were present within this 10 kb common nod region.

Structure and function of root hair curling (hac) genes in pRL1J1.

The 10 kb region of overlap between the recombinant plasmid pIJ1085 and pIJ1089 comprises two adjacent EcoR1 fragments of 6.6 kb and 3.2 kb (Figure 0.1). To characterize the nod genes of this region, the two EcoR1 fragments were separately cloned into a broad-host cloning vector and introduced into a Rhizobium strain cured of its Sym plasmid. The strain containing the cloned 6.6 kb fragment (pIJ1216) was capable of inducing root hair curling on peas but no nodules were formed. In contrast, the cloned 3.2 kb fragment induced no root hair curling on peas. The finding that the 6.6 kb EcoR1 fragment contained hac genes was consistent with other studies that Tn5 mutations which abolished root hair curling and nodulation ability were mapped within this 6.6 kb region (Downie et al., 1983; 1985. See Figure 0.1). Further analysis was by subcloning a 3.2 kb Bgl11-EcoR1 fragment from pIJ1216 (to give plasmid pIJ1389) and to test this for root hair curling. A root hair curling response was observed with pIJ1389, suggesting that very few genes were involved in this initial step of the interaction of Rhizobium with its host plant. Rossen et al. (1984) sequenced the region of DNA on pIJ1389 and identified three putative genes called nodA, nodB and nodC respectively. Similar work has been conducted with R. leguminosarum biovar trifolii (Schofield and Watson, 1986)

Legend to Figure 0.1: Nodulation region contained in different plasmids and their derivatives.

Plasmids pIJ1085 and pIJ1089 were described by Downie et al. (1983) and each contains about 30 kb of DNA from pRL1JI the native R. leguminosarum biovar: viciae plasmid. The nodulation genes are contained within the 10 kb region of DNA common to both plasmids. The restriction enzyme sites EcoR1 and Bgl11 used to subclone the region of DNA in plasmids pIJ1216 and pIJ1389 are indicated. The extent and location of nodAB and C genes are indicated by arrows.

Restriction sites: B - BamH1, E - EcoR1

and R. meliloti (Torok et al., 1984; Egelhoff et al., 1985b). A comparison of these sequences indicates that they are highly conserved in these different species or different biovars. They have therefore been considered to be "common" nodulation genes. Further study confirmed that they are also functionally conserved (Djordjevic et al., 1985b; Fisher et al., 1985).

The common nodABC genes of R. leguminosarum biovar viciae appear to be organized into a single transcriptional unit since they all read in the same direction and the intergenic region is only about 20 to 30 bp (Rossen et al., 1984). The deduced molecular weights of the polypeptide products of these three genes are 18 kd, 23 kd and 46 kd respectively. In an in vitro transcription-translation system, the nodC gene directed the synthesis of a polypeptide with a molecular weight of 46 kd (Downie et al., 1985), in good agreement with the value inferred from the DNA sequence. Analysis of the predicted polypeptide sequences of nodABC suggested that NodA and B are cytosol proteins but NodC is an inner-membrane protein which has a very high hydrophobic carboxy terminus (summarized in Table 0.3).

nodD gene.

Upstream from nodA, Downie et al. (1985) mapped a region in which several Tn5 insertions abolished nodulation and caused a delay in root hair curling. The DNA sequence of this region showed one putative gene. It was designated nodD (Shearman et al., 1986). The nodD gene was found to be transcribed in a direction opposite to that of nodABC. It was reasoned that the space between nodD and nodA must contain the promoters for both groups of genes. The deduced molecular weight of the nodD product is 33 kd, in good agreement with the results of in vitro synthesis (Downie et al., 1985. See Figure 0.2 and Table 0.3).

Table 0.3: Characteristics of defined Rhizobium nod genes.

gene	Comments on properties of gene product	Phenotype of mutant strains in <u>R. leguminosarum</u> biovar	
		<u>viciae</u>	<u>trifolii</u>
<u>nodA</u>	located in cytoplasm	Hac ⁻	Hac ⁻
<u>nodB</u>	"	Hac ⁻	Hac ⁻
<u>nodC</u>	located in membrane	Hac ⁻	Hac ⁻
<u>nodD</u>	regulatory	Hac ⁻	Hac ⁻
<u>nodE</u>	unknown	delayed (Nod ⁺ on pea)	delayed (Nod ⁺ on pea)
<u>nodF</u>	similar to acyl carrier protein	?	delayed (Nod ⁺ on pea)
<u>nodI</u>	homologous to membrane bound transport proteins	delayed	NI
<u>nodJ</u>	probably membrane bound protein	delayed	NI
<u>nodL</u>	unknown	Reduction in nodulation of peas, <u>Lens</u> and <u>Lathyrus</u> but not of <u>Vicia</u>	NI
<u>nodM</u>	some homology with amidophosphoribosyl transferase	?	NI
<u>nodN</u>	unknown	?	NI

Hac⁻: fails to curl root hairs and to nodulate.

Nod⁻: fails to nodulate but still deforms root hairs.

delayed: a reduction in the number of nodules and a delay in their time of onset.

NI: phenotype not identified; ?: no mutants available.

REGULATION OF nod GENE EXPRESSION

As with the nodABC genes, the function of nodD also appears to be conserved, in that the cloned nodD gene from one Rhizobium species could complement the same mutants in other species without changing selectivity for a plant host (Djordjevic et al., 1985b; Fisher et al., 1985). Therefore, nodD is referred to as a "common" nod gene as well. A region of deduced nodD gene product resembles the proposed DNA-binding sites of the products of the E. coli araC and lysR genes.

nodE and F genes.

To the left of nodD (as shown in Figure 0.2) had been located a nod::Tn5 insertion which caused a delay in nodulation and a reduction in nodule number. This mutation did not appear to inhibit root hair curling (Downie et al., 1985). The DNA sequence of a region covering this Tn5 insertion has been determined and the gene in which the Tn5 was inserted was termed nodE. This gene specifies a polypeptide of 48 kd (Shearman et al., 1986). The proposed translational start of nodE is immediately preceded by a stop codon that represents presumably the translational stop codon of another ORF (open reading frame) which is upstream from nodE and would be transcribed in the same orientation as nodE. No mutations in this ORF have been isolated. It represents a putative gene designated as nodF and specifies a potential polypeptide of 14 kd. The predicted amino acid sequence of the putative nodF gene was compared to that of other previously identified proteins. One acyl-carrier protein isolated from E. coli and barley, showed significant homology to the NodF protein (Shearman et al., 1986). Acyl-carrier proteins are involved in fatty acid biosynthesis. If the observed homology of amino acid sequences reflects functional

conservation, the NodF protein may act as an acyl-carrier protein, involving acetylation, synthesis of fatty acids or lipopolysaccharides (eg. of sugars or fatty acids).

There is an extensive amino acid sequence homology between nodFE gene products of pRL1JI and the same gene products of both R. leguminosarum biovar trifolii and R. meliloti (Schofield and Watson, 1986; Horvath et al., 1987). However, it appears that they are not functionally homologous on the basis of genetic complementation tests.

nodI and nodJ genes.

Mutations in these two genes cause only a slight delay (one or two days) in the onset of nodulation and they do not appear to affect root hair curling and nodule number (Downie et al., 1985). The two genes lie immediately downstream of the nodC gene and are both transcribed in the same direction as nodABC genes (see Figure 0.2). DNA sequence analysis suggested that nodI codes for a protein with molecular weight of 34 kd whereas nodJ codes for a 27 kd protein. The predicted amino acid sequence of the nodI gene showed considerable homology to the hisP and malK gene products from Salmonella typhimurium and E. coli respectively (Evans and Downie, 1986). The hisP-coded protein is involved in the active transport of histidine and the E. coli malK gene is involved in the high-affinity system for transport of maltose. Therefore, it seems reasonable to propose that the NodI protein is involved in active transport of a low molecular weight molecule. The predicted product of the nodJ is very hydrophobic, suggesting that it may be an integral membrane protein (summarized in Table 0.3). nodJ is also likely to be

involved in active transport because it bears some similarity to the consensus sequence of malF, malG, hisQ, hisM, pstC, pstA and oppC genes which are all involved in the active transport of small molecules (Evans and Downie, 1986). The relatively short distance (130 nucleotides) between nodC and nodI suggested that nodI and nodJ are probably in the same transcriptional unit as nodABC.

Genes similar to nodI and nodJ have been identified in R. trifolii (Djordjevic et al., 1986) and in R. meliloti (Torok et al., 1984; Egelhoff and Long, 1985a; Horvath et al., 1987).

nodI and J mutants of R. leguminosarum biovar viciae could also be complemented by the corresponding genes of the other biovars or R. meliloti. These genes are therefore designated as common nod genes as well (Spaink et al., 1987).

nodL, nodM and nodN genes.

As mentioned previously, the 10 kb region of overlap between two cosmid clones (pIJ1085 and pIJ1089) contained essential nodulation genes. However, it was noted that pIJ1089 conferred better nodulation of peas and V. hirsuta than did pIJ1085, suggesting that additional nodulation genes were present on pIJ1089 but absent from pIJ1085. Surin and Downie (1988) sequenced a 4.2 kb region of DNA (on pIJ1089) to the left of pIJ1085. Translation of this DNA sequence showed that there were three long open reading frames, all reading in the same direction as nodF (Figure 0.2). The putative genes corresponding to these open reading frames were named nodL, nodM and nodN and the molecular weights of their predicted gene products are 20, 65.7 and 18 kd respectively.

Mutation of nodL strongly inhibited nodulation in peas, Lens and Lathyrus; but mutations within nodM or nodN only had a slight effect on the number of nodules formed on the same plants. A comparison of amino acid sequences of nodL, nodM and nodN with other protein sequences indicated that nodM is homologous to two amidophosphoribosyl transferases (purF gene products), i.e. one from E. coli and one from Bacillus subtilis. The amidophosphoribosyl transferase catalyses the first step in purine biosynthesis using phosphoribosyl pyrophosphate as a substrate.

Nod gene regulation in pRL1JI.

The regulation of nod genes in pRL1JI of R. leguminosarum biovar viciae has been studied by an approach of gene fusions. These fusions were between the E. coli lacZ gene (specifies β -galactosidase which can be easily monitored) lacking its promoter (transcriptional fusions) or lacking its ribosomal binding site (translational fusions) and the individual nod genes (Shearman et al., 1986; Spaink et al., 1987; Surin et al., 1988). The following conclusions were drawn from these gene fusions:

a) The 11 nod genes of pRL1JI are organized into 4 transcriptional units: nodABCIJ, nodD, nodFEL and nodMN.

b) nodD is transcribed constitutively both in normal growth media and in media supplemented with exudate from pea roots. In normal media without exudate, nodFE, nodABCIJ and nodGH are transcribed at very low levels, if at all.

c) nodD has at least two regulatory functions; in media which either contains or lacks pea exudate it represses its

own transcription (autoregulation) and it activates high-level expression (70 fold increase over background) of the nodFE, nodACBIIJ and genes when cells were exposed to root exudate from peas. A schematic illustration of nod gene regulation in pRL1JI is shown in Figure 0.3.

d) DNA sequence analysis revealed a strongly conserved region, the nod box, which is located about 200 bp upstream of the start codon of the first gene. The complete inducibility of a 114 bp clone of the nodA promoter, a 330 bp fragment of the nodF promoter, and a 630 bp fragment of the nodM promoter of R. leguminosarum, strongly suggest that these nod boxes are important elements of nod promoters. This same (or very closely related) sequence is also found in R. leguminosarum biovar trifolii (Schofield and Watson, 1986) and R. meliloti (Torok et al., 1984; Rostas et al., 1986). A consensus sequence for nod boxes of different Rhizobium species is given in Figure 0.4.

Organization, structure and function of nod genes in R. leguminosarum biovar trifolii.

Strain ANU843 is the most studied bacteria in this biovar. Six genes called nodABCDEF have been defined by DNA sequencing and mutational analysis. They are organized into three operons nodABC, nodD and nodFE (Djordjevic et al., 1985a; Innes et al., 1985; Schofield and Watson, 1986). In addition, nodulation genes are also found in ANU843 on the symbiotic plasmid in regions currently designated as II, IV and V (Djordjevic et al., 1986). The nodFE (region III) genes and genes in regions IV and V confer host specificity (Djordjevic et al., 1985b, 1986) while region II genes appear

Legend to Figure 0.3: Locations and mode of regulation of the 11 *nod* genes of the Sym plasmid pRL1JI in *R. leguminosarum* biovar *viciae*.

The directions of transcription of the *nod* genes are shown as solid arrows. The boxed triangle indicates the position of the conserved "*nod* box". Note that *nodD* is autoregulatory and that its product activates transcription of the other *nod* genes in the presence of plant inducer.

REGULATION OF nod GENE EXPRESSION

Legend to Figure 0.4: Conserved sequences (nod box) of Rhizobium species.

Sequences of nod box lie upstream of different nodD-activated transcriptional units in different Rhizobium species. It is believed that the nod box acts as a regulatory region. Squares indicate the complete conserved sequences in the given Rhizobium species.

R.leguminosarum
biovar viciae nodA
nod F
nodM
biovar trifolii
nodA
nod F
R.meliloti
nodA

ATCCA	TTCCATA	GATG	AT	TGCC	ATCCA	AA	CAATCA	ATTTT	TAC	CAAT	CTTTC
ATCCA	TAGTGTG	GATG	CT	TTTG	ATCCA	CACA	ATCA	ATTTT	TAC	CAAT	GATGC
ATCCA	TATCGTG	GATG	ATA	GCT	ATCCA	CA	CAATCA	ATTTT	TAC	TAAT	CTGTT
ATCCA	CGCTGTA	GATG	AT	TGCG	ATCCA	AA	CAATCA	ATTTT	TAC	CAAT	CTTTC
ATCCA	TACTGCG	GATG	CT	TGCG	ATCCA	AT	CAATCA	ATTTT	TAC	CAAT	CCTTC
ATCCA	TATCGCA	GATG	AT	CGTT	ATCCA	AA	CAATCA	ATTTT	TAC	CAAT	CTTGC

to be functionally homologous to the nodIJ genes of R. leguminosarum biovar viciae (Evans et al., 1986, see Figure 0.2).

Characteristics of nodFE as host-specificity genes.

Like many other strains of R. leguminosarum biovar trifolii, strain ANU843 naturally nodulates Trifolium (subterranean clover and white clover), but not Pisum (pea) or Vicia (vetch). Djordjevic et al. (1985a) mapped several Tn5-insertion mutants to region III (see Figure 0.2) which showed altered host-range specificity, i.e. nodulate subterranean clover and peas but are unable to nodulate white clover. Analysis of the nucleotide sequence of region III by Schofield and Watson (1986) revealed open reading frames corresponding to the nodFE genes of R. leguminosarum biovar viciae. This implied that nodFE act as host specificity genes. Since the nucleotide sequences of these two genes are highly conserved between biovars trifolii and viciae (Schofield and Watson, 1986), it suggests that host range may be determined not by widely-differing genes products, but by differences in the active sites of these nod gene products.

Region IV nod genes.

Region IV nod genes (see Figure 0.2) were recognized by the nodulation defect of Tn5 mutants, which are consistently delayed in their ability to induce nodules on clover (Innes et al., 1985). Region IV mutants were not complemented by the R. meliloti recombinant plasmid pRmSL26 which carries common nod genes including nod A, B, C and D (Egelhoff and Long. 1985a),

implying their host-specific properties (Djordjevic et al., 1986).

The possible function of region IV nod genes is to enhance the phenotypic expression of the common nod genes, since the introduction of a plasmid carrying region IV to strain ANU845 (cured of its Sym plasmid) containing pRmSL26 stimulated the phenotypic expression of root hair curling ability on clover plants but not alfalfa plants. ANU845 (pRmSL26) does not induce marked root hair curling or nodules on clover plants.

Region V nod genes.

The phenotypic analysis of specific subcloned DNA fragments from the nod region resulted in the identification of a fifth region (Region V) involved in determining host range ability. In R. leguminosarum biovar trifolii strain ANU843, nodulation and host-range specific determinants are localized on a 14 kb DNA fragment from the Sym plasmid (see Figure 0.2). The introduction of an 11.4 kb HindIII-XhoI fragment (region V is absent) to its ANU845, a derivative of ANU843 cured of its Sym plasmid, restored root hair curling and infection thread formation on clovers; however, only a small number of nodules were induced on T. subterraneum plants and none appeared on T. repens (Djordjevic et al., 1986). This indicated that genes located on region V were important for conferring nodulation proficiency on all clover species.

Since the introduction either to R. leguminosarum biovar viciae or to ANU845 (pRmSL26) of DNA fragments carrying region III (nodFE), IV and V conferred nodulation of clover plants to these recipients, these three distinct loci (regions)

appeared to determine the host range characteristics of this strain.

The expression of R. leguminosarum biovar trifolii nod genes is regulated by the plant factor(s).

Innes et al. (1985) isolated lac operon transcriptional fusions to several genes within the 14 kb nod region of ANU 843 Sym plasmid using the mini-Mu-lac bacteriophage transposon MudI1734 (Castihlo et al., 1984) for study of gene regulation in this biovar. They found that expression and regulation of nod genes in this biovar are analogous to those described in biovar viciae, that is, nodD is constitutively expressed while nodABCIJ, nodFE and region IV genes are activated by nodD product and root exudates.

Identification of inducer and anti-inducer molecules.

As mentioned in the previous section, the nod genes are induced by root exudates in the presence of nodD product. The inducing molecules in the root exudate of various host plants are flavonoids. The major inducer of R. meliloti nodA gene was isolated from alfalfa seed extract and was characterized as the flavone luteolin (Peters et al., 1986). The R. leguminosarum biovar trifolii nodA gene inducers present in white clover seedlings are 7,4'-dihydroxyflavone, geraldone and 4'-hydroxy-7-methoxyflavone, in order of decreasing activity (Redmond et al., 1986). The major inducer of R. leguminosarum biovar viciae nodA gene in Vicia sativa root exudate co-purifies with the flavanone fraction and can be replaced by naringenin and a number of other flavonoids (Zaat et al., 1987). Seed extracts of pea are reported to contain

two major inducers of R. leguminosarum biovar viciae nodA gene, one of which was tentatively identified as apigenin-7-O-glucoside (Firmin et al., 1986). In addition to these naturally occurring inducers, a number of other flavones and flavanones with appropriate hydroxylation and methoxylation patterns can induce nod genes.

Other molecules made by plants antagonize induction of nod gene transcription by flavones and flavanones (Firmin et al., 1986). Isoflavanoids, one flavanol and some acetophenones at concentrations as low as 10 uM inhibited activation. Some of the acetophenones had been shown to induce the virulence genes of Agrobacterium tumefaciens and are made in increased amounts in wounded tissue (Stachel et al., 1985). Isoflavanoids are common in legumes but rare in other plant families. The function of these anti-inducer molecules needs to be further investigated.

Structure and function of nodulation genes in R. leguminosarum biovar phaseoli.

Unlike the other two biovars, the genetic studies of nod genes in R. leguminosarum biovar phaseoli are less advanced. The individual nod genes have not been characterized in detail, except that Lamb et al. (1985) demonstrated that the cloned R. leguminosarum biovar phaseoli nodulation region shared extensive DNA homology with the nod genes of R. leguminosarum biovar viciae.

MATERIALS AND METHODS.

In this chapter, procedures that are fully described in other publications are cited and not described in detail. New procedures or modifications of published procedures are described in detail.

Bacterial strains, plasmids and bacteriophages.

The strains of Rhizobium and E. coli used in this study are listed in Table 0.4 and Table 0.5, respectively. The derivatives of strains constructed in this study (i.e. resistant mutants, transposon insertions, and recombinant plasmids) are given in Appendix 1. The plasmids constructed or used in this study are listed in Table F.

Four transposon Tn5 insertion derivatives of the plasmid pRL1JI with insertions in nodA, B, C, D and J respectively were provided by Dr. A.W.B. Johnston. Bacteriophage lambda b₂₂₁ cI₈₅₇ P_{am} O_{am} rex::TnphoA (Hoffman et al., 1985), abbreviated as lambda::TnphoA, was obtained from Dr. C. Manoil.

Media, chemicals and antibiotics.

Strains of Rhizobium were grown at 30°C on TY medium of Beringer (1974). The CaCl₂ in this medium was added to the rest of the medium that was sterilized by autoclaving separately. For solid media, agar (Difco) was used at a concentration of 1.5%. Strains were stored on this medium at 5°C and also in 40% glycerol at -20°C.

c) Conjugation. The most detailed chromosomal mapping has arisen from studies of conjugal gene transfer. R-plasmids belonging to the P-1 incompatibility group such as RP4, were shown to be transmissible between a wide range of Gram-negative bacteria, including R. leguminosarum (Datta et al., 1971; Beringer, 1974; Dixon et al., 1976) and R. meliloti (Kondorosi and Johnston, 1981). These plasmids are stably maintained in these two Rhizobium species. The mobilization of the chromosome of R. leguminosarum (also R. meliloti) was promoted by P-1-type plasmids, but sometimes it was inefficient (Beringer et al., 1978b). The plasmid R68.45, a variant of R68 with enhanced chromosome-mobilizing ability in many Gram-negative bacteria (Haas and Holloway, 1976) promoted high-frequency chromosomal transfer in R. leguminosarum (Beringer et al., 1978b), in R. meliloti Rm41 (Kondorosi et al., 1977) and in R. meliloti RmGR4 (Casadesus and Olivares, 1979).

Chromosomal mapping.

Based on linkage analysis of auxotrophic and antibiotic resistance markers, genetic maps of chromosomes of R. leguminosarum biovar viciae strain 300 (Beringer et al., 1978b), a R. leguminosarum biovar trifolii strain M555 (Megias et al., 1982) and three R. meliloti strains 2011, 41 and GR4 (Meade and Signer, 1977; Kondorosi et al., 1977; Casadesus and Olivares, 1979) have been obtained. Comparison of these maps showed remarkable similarities (Hooykaas et al., 1982). Recombination between chromosomal DNA of different R. leguminosarum strains was found to occur at high frequencies in crosses between these strains. In crosses between a R. leguminosarum strain and a strain of R. meliloti virtually no

chromosomal recombination was detected (Johnston *et al.*, 1978b). This provides a genetic basis for placing *R. leguminosarum* and *R. meliloti* in two different species. In contrast, R68.45-mediated crosses between the biovars of *R. leguminosarum* yielded haploid recombinants for many alleles and at frequencies similar to those found in crosses between different strains of a single *R. leguminosarum* biovar *viciae*. This finding provides a basis for the close taxonomic relatedness of these three biovars.

Mapping of symbiotic mutations on *Rhizobium* chromosome.

The chromosomal linkage maps of *Rhizobium* provide a framework for the localization of any chromosomal mutations. Using a P-1 group plasmid-mediated recombination, a non-selectable mutation linked to a selectable marker on the linkage map can be identified. In this way, some chromosomal regions involving symbiotic functions are localized in both *R. leguminosarum* and *R. meliloti*.

Recently, more chromosomal mutants which affect either the formation of nodules or differentiation of bacteroid or nodule functions have been isolated from *R. meliloti* (Forrai *et al.*, 1983; Selvaraj *et al.*, 1986). It is likely that homologous chromosomal regions occur in *R. leguminosarum* but they remain to be uncovered. From this review of chromosome genetics, it can be seen that studies on genes and functions determined by the *Rhizobium* chromosome are in early stages. It appears that following the discovery of Sym plasmids and the observation that most functions related to symbiosis are determined by regions on these plasmids, there has been a justifiable concentration of effort on these plasmids and the genes they contain. Figure 0.5 provides chromosome maps of

Legend to Figure G: Rhizobium linkage maps.

A represents R. leguminosarum biovar viciae strain 300 (Beringer et al., 1978; Johnston et al., 1978).

B represents R. meliloti (Kondorosi et al., 1977a, 1980; Svab et al., 1978; Kiss et al., 1979; Vincze et al., 1981).

The two linkage maps were constructed mainly by conjugation using IncP incompatibility group plasmids. It is assumed that the linkage maps for R. leguminosarum biovar trifolii and biovar phaseoli were very similar to that for biovar viciae, because in crosses between these three biovars the frequency of recombination were the same as in crosses between the different strains of the same biovar.

Mapping of symbiotic mutations on the chromosome of R. meliloti was described by Forrai et al. (1983). The inside broken line indicates a region on the chromosome where symbiotic mutants were isolated. Arrows represent the Tn5 insertions.

B

A

R. leguminosarum and of R. meliloti, the only two species of Rhizobium on which such information exists. The map of R. leguminosarum includes all of its three biovars, viciae, trifolii and phaseoli.

Other plasmids in the strain of Rhizobium.

In most strains of Rhizobium, more than one large plasmid was present. For instance, most R. leguminosarum biovar viciae strains were found to carry at least three plasmids of more than 90×10^5 molecular weight (Casse et al., 1979a,b; Hirsch et al., 1980). In R. meliloti, the presence of two megaplasms was the general feature (Hynes et al., 1986). As mentioned in the previous section, in fast-growing Rhizobium, all of the nodulation genes that have so far been well characterized were ascribed to one megaplasmid (Sym plasmid). Mutations in these nod genes on the Sym plasmid either do not evoke root hair curling and fail to induce nodule formation (i.e. inactivation in common nod genes) or are affected in host-range determination (i.e. inactivation in hcn genes). During the past few years, a different class of ineffective mutants has been identified, which is exopolysaccharide-deficient (EPS⁻) and induces Fix⁻ nodules (Chakravorty et al., 1982; Leigh et al., 1985; Vandebosch et al., 1985; Borthakur et al., 1986; Gardiol et al., 1987; Djordjevic et al., 1987). In R. meliloti, the genes inactivated in these mutants (designated exo) have been located on the non-symbiotic megaplasmid, the megaplasmid 2 (some of these mutants have been mapped to the chromosome, see Finan et al., 1985, 1986; Hynes et al., 1986). Alfalfa seedlings inoculated with these exo mutants formed ineffective nodules that contained few if any bacteria and none of the

morphologically differentiated forms termed bacteroids (Leigh et al., 1985).

Examination of nodules elicited by an exoB mutant revealed that no infection threads are formed following inoculation with this mutant. Plant cells in the interior of nodules elicited by this strain are empty and do not contain bacteria or bacteroids (Finan et al., 1985). Alfalfa seedlings inoculated with exoH mutants exhibit delayed root hair curling compared with plants inoculated with wild-type bacteria. They form infection threads that abort at an early stage and produce nodules unoccupied by bacteria ("empty" nodules, see Leigh et al., 1987). Whether these exo mutants are also located on the non-symbiotic plasmids, in other Rhizobium species, needs to be further investigated.

MATERIALS AND METHODS.

In this chapter, procedures that are fully described in other publications are cited and not described in detail. New procedures or modifications of published procedures are described in detail.

Bacterial strains, plasmids and bacteriophages.

The strains of Rhizobium and E. coli used in this study are listed in Table 0.4 and Table 0.5, respectively. The derivatives of strains constructed in this study (i.e. resistant mutants, transposon insertions, and recombinant plasmids) are given in Appendix 1. The plasmids constructed or used in this study are listed in Table F.

Four transposon Tn5 insertion derivatives of the plasmid pRL1JI with insertions in nodA, B, C, D and J respectively were provided by Dr. A.W.B. Johnston. Bacteriophage lambda b₂₂₁ cI₈₅₇ P_{am} O_{am} rex::Tnp_{hoA} (Hoffman et al., 1985), abbreviated as lambda::Tnp_{hoA}, was obtained from Dr. C. Manoil.

Media, chemicals and antibiotics.

Strains of Rhizobium were grown at 30°C on TY medium of Beringer (1974). The CaCl₂ in this medium was added to the rest of the medium that was sterilized by autoclaving separately. For solid media, agar (Difco) was used at a concentration of 1.5%. Strains were stored on this medium at 5°C and also in 40% glycerol at -20°C.

Table 0.4: Characterization of natural Rhizobium strains studied.

Strain:	Source:	Origin:	Relevant reference or information or method of isolation:	Root nodulation on Nov. ¹ Afg. ¹
<u>R. leguminosarum</u>				
biovar				
<u>viciae</u>				
TOM	T.A.Lie	soil [Tomek, Turkey]	Lie, 1978.	+
A1	K.Engvild	nodules of Afghanistan	Jensen <u>et al.</u> 1986 An attempt was made to use Afghanistan pea as a non-nodulating reference crop. Unexpectedly, it was found that Afghanistan nodulated early and abundantly in one particular field.	+
A6	"	"	"	+ ^d
A7	"	"	"	+
A8	"	"	"	+
A10	"	"	"	+

(continued)

Table 0.4 (continued).

Strain:	Source:	Origin:	Relevant reference or information or method of isolation:	Root nodulation on Nov. ¹ Afg. ¹
A-3	S.A. Chetkova	soil, USSR	Chetkova <u>et al.</u> , 1986. Of 40 strains tested, 3 were found to be able to nodulate Afghanistan pea.	+ +
D38B	Y.P.Zhang	nodules of [<u>Vicia villosa</u>], Xin-Jiang, China	Personal communication. Three randomly chosen strains isolated from nodules.	+ +
D52	"	nodules of [<u>Vicia faba</u>], Xin-Jiang, China	"	+ +
T14	Y.P.Zhang	nodules of [<u>Vicia craca</u>], Xin-Jiang, China.	"	+ +
2386	US Dept.of Agriculture	nodules of [<u>Pisum sativum</u>] Yugoslavia		+ +

(continued ...)

Table 0.4 (continued).

Strain:	Source:	Origin:	Relevant reference or information or method of isolation:	Root nodulation on Nov. ¹ Afg. ¹
I9	P.T.C.Nambiar	soil, India	Personal communication No history of cultivation of cv. Afghanistan or any other pea where strain I9 was isolated.	+ +
I2	"	"	"	+ -
I7	"	"	"	+ -
I8	"	"	"	+ -
JISP18	J.P.W.Young	nodules on [<u>Pisum sativum</u>] Haiford, England.	Young, 1985. Two test pea plants (J11194, a commercial pea type) and Afghanistan pea (J1103). Peas J11194 were nodulated well, but J1103 did not form any nodules in the field where JISP18 was isolated.	+ -

(continued ...)

Table 0.4 (continued).

Strain:	Source:	Origin:	Relevant reference or information or method of isolation:	Root nodulation on Nov. ¹ Afg. ¹
JISP24	"	"	Isolated from same peas (J11194) in the same field (Haiford).	+ -
JISP36	"	"	"	+ -
JISP48	"	"	"	+ -
JISP75	"	"	"	+ -
JIM1104	"	nodules of [<u>Pisum sativum</u>], Morley, England.	Young, 1987 Nodules were collected from a field pea (<u>P. sativum</u> cv. Maro, a commercial pea type).	+ -
JIM1121	"	"	"	+ -
JIM1122	"	"	"	+ -
JIM1201	"	"	"	+ -
JIM1325	"	"	"	+ -
JIM1331	"	"	"	+ -

(continued ...)

Table 0.4 (continued).

Strain:	Source:	Origin:	Relevant reference or information or method of isolation:	Root nodulation on Nov. ¹ Afg. ¹
JIM1418	"	"	"	+ -
JIM1726	"	"	"	+ -
JIM1934	"	"	"	+ -
2397	US Dept. of Agriculture	nodules of [<u>Pisum sativum</u>] Florida, USA		+ -
2429	"	nodules of [<u>Lens culinaris</u>]		+ -
2449	"	nodules of [<u>P. sativum</u>] Brazil		+ -
2478	"	nodules of [<u>Vicia sativum</u>] Morocco		+ +
SMY8501	this study	nodules from <u>P. sativum</u>	seeds (125839*) from Zebak, Afghanistan ^a	+ -

(continued ...)

Table 0.4 (continued).

Strain:	Source:	Origin:	Relevant reference or information or method of isolation:	Root nodulation on Nov. 1 Afg. 1
SMY8502	"	"	seeds (125840*) from Laghman, Afghanistan ^a	+ -
SMY8510	"	nodules from <u>P. sativum</u>	seeds (126341*) from Nagil, Afghanistan ^a	+ -
SMY8513	"	"	seeds (1770669*) from Kocaeli, Turkey ^a	+ -
SMY8514	"	"	seeds (169600*) from Kocaeli, Turkey ^a	+ -
SMY8516	"	"	seeds (135846*) from Aoi Khurak, Afghanistan ^a	+ -
SMY8517	"	"	(as above)	+ -
SMY8519	"	"	seeds (212112*) from Faisbad, Afghanistan ^a	+ -
SMY8520	"	"	"	+ -

(continued ...)

Table 0.4 (continued).

Strain:	Source:	Origin:	Relevant reference or information or method of isolation:	Root nodulation on Nov. ¹ Afg. ¹
SMY8522	"	"	seeds (223527*) from Kunar, Afghanistan ^a	+ -
SMY8524	"	"	seeds (167363*) from Torus, Turkey ^a	+ -
SMY8525	"	"	"	+ -
SMY8527	"	"	seeds (169600*) from Izmir, Turkey ^a	+ -
SMY8529	"	"	seeds (171812*) from Niskar, Turkey ^a	+ -
SMY9001	"	soil	soil from Northern, Ontario ^b (west of Sudbury), Canada	+ -
SMY9002	"	"	"	+ -
SMY9003	"	"	"	+ -
SMY9004	"	"	"	+ -

continued

Table 0.4 (continued).

Strain:	Source:	Origin:	Relevant reference or information or method of isolation:	Root nodulation on Nov. ¹ Afg. ¹
SMY9006	"	soil	soil from Northern, Ontario ^b (Sudbury), Canada	+ -
SMY9008	"	"	soil from Ontario (north of Sudbury), ^b Canada	+ -
SMY9009	"	"	"	+ -
SMY9011	"	"	"	+ -
SMY9012	"	"	"	+ -
248	N.J. Brewin	nodule from <u>P. sativum</u>	Brewin <u>et al.</u> , 1980a.	+ -
300	"	"	Johnston and Beringer, 1975.	+ -
128C53	"	"	Brewin <u>et al.</u> , 1980c.	+ -

(continued ..)

Table 0.4 (continued).

Strain:	Source:	Origin:	Relevant reference or information or method of isolation:	Root nodulation on Nov. ¹ Afg. ¹
<u>R. meliloti</u>				
1027	US Dept of Agriculture	nodule from <u>Medicago sativa</u> , Turkey.		-
1030	"	"		-
1066	"	nodule from <u>M. polymorpha</u> , Turkey.		-
1096	"	nodule from <u>M. sp.</u>		-
1098	"	nodule from <u>M. orbicularis</u>		-
1171	"	Nodule from <u>M. arborea</u>		-

(continued . .)

Table 0.4 (continued).

Strain:	Source:	Origin:	Relevant reference or information or method of isolation:	Root nodulation on Nov. 1 Afg. ¹
<u>R. leguminosarum</u>				
biovar				
<u>trifolii</u>				
ANU843	N.J.Brewin	nodules from clover.	Rolfe <u>et al.</u> , 1980.	- .
<u>Bradyrhizobium</u>				
IC3554	P.T.C. Nambiar	nodules		- .
<u>A. tumefaciens</u>				
315	A.L.Hamel			- .

(continued ...)

Table 0.4 (continued).

Abbreviations: Nov. and Afg. refer to the two pea cultivars "Novella" and "Afghanistan". P. sativum cv. Novella is a dwarf, anthrocyaninless, commercial pea with green, wrinkled seed. It was purchased from Stokes Seeds inc., Buffalo, New York, USA. P. sativum cv. Afghanistan is a tall anthrocyanin, yellow mottle, smooth seeded wild pea. Its seeds were obtained from Prairie Regional Laboratory, National Research Council, Saskatoon, Canada.

- *: Seeds from different sources were surface sterilised by immersion in 15% v/v Javex for 20 minutes, followed by 5 minutes in 70% (v/v) ethanol. After this, the seeds were washed five or six times with sterile distilled water and then carefully sown in a plot at the Central Experimental Farm, Ottawa. Procedures used for isolation of rhizobia from nodules were described in Materials and Methods.
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(c. ntinued ..)

Table 0.4 (continued).

^b: Each soil sample was well mixed, shaken with sterile water (120mg/ml), and used to inoculate pea plants (both cv."Novella" and cv."Afghanistan") grown on vermiculites in individual flasks. Each plant received 5-7 ml of soil suspension. No nodules formed on uninoculated control plants. Isolation and characterization of bacteria from nodules were carried out as described in Materials and Methods.

^d= delayed nodulation on cv. Afghanistan. For details, see Chapter 3 section 3.2.

*= Pisum sativum plant introductions from NE Regional Plant Introduction Station, Geneva, New York, USA.

Table 0.5: Escherichia coli K-12 strains used in this study.

<u>E. coli</u>	Relevant characteristics:	Reference/source:
CC118	<u>recA</u> , <u>phoA</u> , <u>galK</u> , <u>rpsL</u>	Manoil <u>et al.</u> , 1984.
LE392	<u>supE</u> , <u>supF</u> , <u>hsdR</u> , <u>met</u> <u>trpR</u> , <u>lac</u>	Enquist <u>et al.</u> , 1979.
HB101	<u>hsdS20</u> , <u>recA13</u> , <u>ara14</u> , <u>polA2</u> , <u>lacY1</u> , <u>rps20</u> , <u>xyl5</u> , <u>mtl-1</u>	Boyer and Roulland-Dussoix, 1969.
JM103	$\Delta(\text{lac pro})$, <u>*hi</u> , <u>strA</u> , <u>supE</u> , <u>endA</u> , <u>sbcB</u> , <u>hsdR</u> , /F' <u>traD36</u> , <u>proAB</u> , <u>lacI9</u> , 2 M15	Messing <u>et al.</u> , 1981.
CM1070	<u>metE70</u> , <u>thyA36</u> , <u>deoC2</u> , <u>polA1</u>	Kelley and Grindley, 1976a.
CSR603	<u>recA1</u> , <u>uvrA6</u> , <u>lacY1</u> , <u>rpsL31</u> , <u>thr-1</u> , <u>supE44</u>	Sancar and Rupert, 1978.

For the growth of E. coli strains LB (Miller, 1972) was used. The minimal medium M9 (Miller, 1972) with appropriate supplements was used to test or grow mutant strains. The growth temperature for all strains was 37°C unless otherwise stated.

For the growth of E. coli strain JM101 containing M13mp18 or M13mp19 recombinant phage, 2XYT broth was used (16 g tryptone, 10 g yeast extract, 5 g NaCl per litre).

The concentration of antibiotics or other inhibitors used in solid media was as follows ($\mu\text{g/ml}$): ampicillin (Ap), 40; carbenicillin (Cb), 200; chloramphenicol (Cm), 75; gentamycin (Gm), 25; kanamycin (Km), 80; nalidixic acid (Nal), 100; spectinomycin (Sp), 100; streptomycin (Sm), 100 for E. coli, 200 for Rhizobium; rifampicin (Rif), 50 for E. coli 150 for Rhizobium; tetracycline (Tc), 10 for E. coli, 20 for Rhizobium. In liquid media, these antibiotics were used at half the above concentrations, unless otherwise stated. The antibiotics were from Bristol-Myers (Canada) Ltd., and from Sigma Chemical Co (USA).

Bacterial matings.

Donor and recipient cells were grown in liquid culture to exponential phase, mixed in Eppendorf tubes (at a ratio of 1:1 in E. coli matings and 1:5 in E. coli/Rhizobium matings) and centrifuged together for 30 seconds. The supernatant was then decanted, the cells were resuspended in 50 μl of the medium and spread onto sterilized filters (porosity 0.45 μm ; Millipore Corp.) on LA or TY (when the recipient was Rhizobium) plates. Mating mixtures on the filters were incubated for 4 to 15 h (depending on the recipient), and

afterwards were resuspended and diluted in physiological saline (when *E. coli* was the recipient) or distilled water (when *Rhizobium* or *Agrobacterium* were recipients). Aliquots of the suspension and serial dilutions of it were then plated on selective plates.

When the donor strain carried the plasmids with the pLAFR1, pKT210, pKT230 or pKT240 based recombinant plasmids, the matings were carried out triparentally with the helper plasmid pRK2013 (Ditta *et al.*, 1980) or pBRK4013 (Hamel, 1987). Other unpublished studies in this laboratory have shown that these two artificial constructs carrying the P *tra* system can efficiently mobilize and transfer the four vectors listed above (N.B. Ramsey, personal communication). Merodiploid *Rhizobium* strains for complementation tests were constructed by transferring individual subclones (carrying different *nod* regions cloned into one of the above IncQ group plasmid vectors) into *R. leguminosarum* biovar *viciae* 8401 carrying either pIJ1095 *nod::TnphoA*-mutations or pRL1JI *nod::Tn5*-mutations. In triparental matings of the above type, selection was for streptomycin resistance or neomycin resistance specified by T^r along with another vector marker (Cm^r in the case of pKT210 and Cb^r in the case of pKT240). Transconjugants were picked, purified on the same selective medium and tested for their nodulation phenotype.

**Transposon *TnphoA* mutagenesis of *nod* region of pIJ1095.
Preparation of lambda431 (*lambda::TnphoA*) phage stocks.**

Phage *lambda::TnphoA* lysates were prepared by the method of de Bruijn and Lupski (1984).

Mutagenesis of plasmid pIJ1095 with transposon TnphoA.

Introduction of TnphoA into plasmid pIJ1095 was accomplished by two different methods. In one, the plasmid pIJ1095 DNA was transformed into E. coli strain CC118 which is sensitive to phage lambda infection. The presence of the plasmid was physically verified using the rapid plasmid preparation protocol described by Holmes and Quigley (1982). Plasmid-bearing cells were then grown to stationary phase in 5 ml LB supplemented with maltose (final concentration of maltose, 0.2%) at 37°C. The culture was diluted 100 fold and grown at 37°C in 5 ml LB plus maltose to an A_{600} of 0.8 (approx. 10^9 cells/ml). Then 1 ml of cells were mixed with lambda::TnphoA phage at an m.o.i. of 1 to 10 and placed at 30°C for 1.5 h. Infected cells were then plated in 0.2 ml aliquots onto LB plates containing 40 µg/ml kanamycin and 10 µg/ml tetracycline (the latter was for selection of the plasmid). The plates were incubated at 37°C for 48 h. Km^r Tc^r colonies were pooled and conjugated with the E. coli polA mutant strain C2107 by a triparental mating system as described above using pBRK4013 as the helper. In this mutant recipient strain, pBRK4013 (which contains the ColE1 replicon) could not be maintained. Transconjugants were obtained by selection of Km^r and Tc^r . Individual colonies were picked and purified. Plasmid DNA was isolated from each of them and the position of the TnphoA insert mapped by restriction enzyme analysis. Those plasmids into which TnphoA was mapped within the cloned nod region were mobilized into R. leguminosarum biovar viciae strain B151 or biovar phaseoli strain 8401 (the strains cured of their respective Sym plasmid). The resulting strain was purified and used for nodulation and host range tests on appropriate pea cultivars.

A second method that was used to introduce TnphoA into pIJ1095 was by using generalized suicide plasmid mutagenesis of R. leguminosarum biovar viciae. An adaptation of the suicide plasmid techniques described by Selvaraj and Iyer (1984) to mutagenize R. meliloti was used. A derivative of the plasmid pCU101 (Thatte and Iyer, 1983) was constructed to carry TnphoA in a non-essential region of the plasmid. This plasmid, called pMA100 is shown in Figure 0.6. As the plasmid pMA100 does not replicate when transferred to R. leguminosarum biovar viciae, kanamycin-resistant transconjugants should contain the TnphoA transposon inserted at sites in the Rhizobium genome.

E. coli strain 803 containing pMA100 was mated with R. leguminosarum biovar viciae strain B151 carrying pIJ1095; and Km^rSm^r transconjugants were selected and then tested for sensitivity to chloramphenicol. On average, 90% of the kanamycin resistant colonies were chloramphenicol sensitive and therefore candidates for strains carrying TnphoA transpositions to the R. leguminosarum biovar viciae replicons. For selection of TnphoA transpositions into pIJ1095, all Km^rSm^r transconjugants were pooled and mated with R. leguminosarum biovar viciae strain WA801 (same as B151 but also Sp^r). Transconjugants were selected for the $Sp^rKm^rTc^r$ phenotype. Individual $Sp^rKm^rTc^r$ transconjugants were then inoculated onto plants for the nodulation tests. The position of the TnphoA insertions on the plasmid were also mapped.

Legend to Figure 0.6: Construction of plasmid pMA100.

pMA100 was constructed by inserting TnphoA (a derivative of Tn5 in which a 2.3 kb DNA fragment containing an E. coli alkaline phosphatase gene was cloned into IS50L of Tn5) into plasmid pCU101. pCU101 was described by Selvaraj and Iyer (1984) as a plasmid that could not be stably maintained in Rhizobium hosts and which could therefore be used as a suicide vehicle for transposon mutagenesis in this genus. The position of TnphoA on pCU101 was determined by restriction mapping analysis. The size of pMA100 is 32.8 kb.

Cm: chloramphenicol; Km: kanamycin; Ori: origin of replication from the p15A replicon pACYC185; tra: conjugal transfer region from the IncN group plasmid pCU101.

Determination of nodulation and host range of R. leguminosarum biovar viciae strains containing TnphoA insertions.

The primitive pea cultivar (cv. Afghanistan) and the commercial pea cultivar (cv. Novella) were used in all tests for nodulation and host-range. In some experiments two other commercial peas cv. Alaska and cv. Trapper were also used.

a) Growth of seedlings. The seeds were surface sterilized by immersion in a 20% commercial household bleach (hypochlorite) solution, stirring the contents for 30 min. followed by a wash with 70% (v:v) ethanol for approximately 1 min. and three times with sterile distilled water. The seeds were germinated on petri plates containing 1% agar in water. After two days (3 to 4 days for Novella seeds) incubation, any seeds in contact with bacterial or fungal growth were discarded. Germinated seeds free of contamination were transferred, using sterile forceps, to 1 litre glass bottles containing sterile vermiculite moistened with sterile Jensen's nitrogen-free nutrient solution. Jensen's nitrogen-free nutrient solution contained, in g/L, 0.272 KH_2PO_4 ; 0.349 K_2SO_4 ; 0.249 MgSO_4 ; 0.004 H_3BO_3 ; 1.034 CaSO_4 ; 0.015 Fe-EDTA; and in mg/l, 0.990 $\text{MnCl}_2 \cdot 2\text{H}_2\text{O}$. Chemicals were from Baker, Sigma and Fisher Scientific companies. The pH was 6.5 - 7.0.

b) Inoculation of seedlings with bacterial strains. After the seedlings were transferred to the bottles they were grown for two days before being inoculated with bacteria. 10^9 - 10^{10} cells of the R. leguminosarum biovar viciae strain, washed and resuspended in sterile distilled water, were added to the surface of the roots. The inoculated seedlings were then grown in the growth chamber with a light photoperiod of 18 h light/ 6 h dark. The temperature was between 23° and

25°C. The number of seedlings used (replicates) varied and is mentioned for the specific experiments.

c) Nodulation assays. The inoculated peas were observed periodically during their growth. The timing of nodule initiation and the number of nodules were recorded. In particular, the plants inoculated with those TnphoA mutants which showed a delayed nodulation phenotype were examined at 4-5 day intervals. To ensure that the delayed mutant phenotypes were indeed due to the insertion of TnphoA and not to reversion to Nod⁺, bacteria were isolated from nodules, checked for their genetic markers, and retested for the nodulation characteristics on the relevant cultivars.

Assays for root hair curling and infection thread formation were performed according to the method of Vasse and Truchet, (1984) using methylene blue-stained root segments which were examined at 3 to 4 days interval following infection using light microscopy.

Isolation of bacteria from nodules.

The nodules were excised intact from the roots and placed in sterile distilled water for 2 min., 95% (v:v) ethanol for 30 seconds and sterile distilled water for 2 minutes. Next they were placed in a solution of hydrogen peroxide [33% (v:v)] for 30 seconds and rinsed with sterile distilled water. These 'surface-sterilized' nodules were transferred to a sterile petri dish containing 1 ml sterile distilled water and crushed with a sterile scalpel. To make serial dilutions, another 4 petri dishes were prepared, with each containing 1 ml sterile distilled water. Each plate was mixed with 5 loops of suspension in a sequential manner and then 100 μ l of each

dilution was plated on TYC agar with no antibiotic and incubated at 30°C for 2 days. Single colonies were purified and tested for their ability to grow in the presence of antibiotics.

Determination of the plasmid composition of bacterial strains.

The plasmid composition of Rhizobium strains was determined by a modification of the analytical procedure of Eckhardt (1978) as described by Rosenberg et al. (1982). 0.5 ml of culture was spun for 15 seconds in an Eppendorf microfuge. The cell pellet was resuspended in 1.0 ml TBE buffer (89 mM Tris-Base, 89 mM boric acid, 2.5 mM disodium EDTA at pH 8.2) and the cells were again pelleted as above. This was followed by a wash with 1 ml of 0.2% Sarkosyl in TBE buffer. The cells were pelleted for 5 minutes and resuspended in 150 µl of Loading buffer [10% sucrose, 0.001% xylene cyanol, 0.1 mg/ml lysozyme, 0.01 mg/ml RNase A in TBE] and then vortexed for a few seconds and immediately loaded into the wells of an 0.6% agarose (in TBE buffer) horizontal gel. Horizontal gel electrophoresis was carried out for 45 minutes at 30 V and then for 4 hours at 200 V. Gels were stained in a 0.4 µg/ml solution of ethidium bromide for 10 minutes, destained in distilled water for 30 minutes and then photographed under long-wave UV illumination. Estimation of plasmid molecular weights were made by comparing their migration distances with those plasmids of R. leguminosarum biovar viciae strain TOM (containing three plasmids with molecular weight of 140, 160 and 220 Megadalton (md) respectively, Brewin et al., 1980a) and R. meliloti (containing two plasmids with molecular weight of 135 and 350 md respectively, Adachi et al., 1983).

For the preparation of the horizontal gel, we have used a double comb system with a piece of lucite set directly against the back of the toothed comb as suggested by Dr. L. Barran. The slot was filled in with a solution of 1% agarose, 1% SDS and 0.001% bromophenol blue.

DNA isolation procedure.

Rhizobium genomic DNA was prepared from 5 ml culture by the method of Meade et al. (1982) with minor modifications. Cells grown in TY broth were harvested by centrifugation, washed with 5 ml cold acetone, and suspended in 10 ml of 1.0 M NaCl, shaken for 30 min at 4°C. Next the pelleted cells were suspended in 10 ml of cold TES buffer (Tris, 0.01 M, pH 8.0; 0.025 M EDTA; 0.15 M NaCl), centrifuged and resuspended in 5 ml of cold TE buffer (TES without NaCl). A 0.5 ml lysozyme solution (2 mg of lysozyme per ml of sterile distilled water) was added to the resuspended cells. After 15 minutes of incubation at 37°C, 0.6 ml of a Sarkosyl-pronase (10% Sarkosyl and 5 mg/ml pronase in TE) solution was added, and the mixture was incubated for an additional 1 h at 37°C. The lysed cells were extracted twice with 5 ml of STE-saturated phenol (0.1 M NaCl, 0.01 M Tris (pH 7.5), 0.001 M EDTA) and then with 5 ml of chloroform. The aqueous phase was brought to 0.3 M ammonium acetate, and DNA was precipitated with 0.54 volumes of isopropanol at room temperature. The precipitated DNA was removed with a glass rod and then dissolved in 0.5 - 1 ml TE buffer (Tris, 0.01 M, pH 8.0; EDTA, 0.001 M). This DNA was suitable for endonuclease digestion analysis and DNA cloning.

Preparative isolation of plasmid DNA from Rhizobium followed the method of Hirsch et al. (1980). The plasmid DNA

was further purified by CsCl-ethidium bromide density gradient ultracentrifugation, according to Clewell and Helinski (1969).

Preparative amounts of *E. coli* plasmid DNAs were isolated by a modification of the cleared lysate procedure of Clewell and Helinski (1969) using 1% Triton X-100 instead of Brij-58 deoxycholate. Small amounts of *E. coli* plasmid DNAs prepared by the method of Holmes and Quigley (1981) were used for identification of the right recombinant plant.

Single-stranded M13 phage DNA for DNA sequencing reactions was prepared by the procedure of Messing (1983) with some modifications. A single white plaque was inoculated into 2.5 ml 2XYT broth in a sterile 10 ml tube and incubated with aeration for between 5 and 7 hours. A 1.4 ml sample of the growth was pelleted in an Eppendorf tube. To 1.0 ml of supernatant, 125 μ l of 5 M NaCl and 155 μ l of 40% PEG were added to precipitate the phage DNA and the mixture was left on ice for 1 h (in some cases, overnight). DNA was precipitated and resuspended in 120 μ l TE buffer. The DNA solution was extracted twice with equal amounts of phenol and once with chloroform. 22 μ l of 10 M ammonium acetate and 250 μ l of absolute ethanol were added to the upper aqueous phase to precipitate DNA. The DNA pellet was washed twice with 70% ethanol and then vacuum dried and dissolved in TE buffer.

Plasmid DNA manipulations.

Restriction endonucleases were purchased from Bethesda Research Laboratories, USA (BRL) or Amersham Canada Ltd. Endonuclease digestion of the DNA and DNA ligations were carried out following the manufacturer's instructions. When digestion was necessary with two or more endonucleases

requiring different reaction buffers, the TA buffer (O'Farrell *et al.*, 1980) was used. For the use of endonucleases Sall and Xho1, sodium chloride solution was added to the TA buffer to give the final concentration of 100 mM in order to get the maximum enzymatic activity.

Digests were analyzed in horizontal 0.8% agarose gels prepared in TBE buffer. The sizes of the digestion fragments were calculated using the sizes of bacteriophage lambda DNA digested with Hind111 or EcoR1 plus Hind111 as molecular weight markers.

DNA ligation and transformation.

Ligation of the DNA fragments was preformed according to Maniatis *et al.* (1982), using T4 DNA ligase (BRL laboratories). DNA concentrations were 80 µg/ml for intermolecular ligation and 20 µg/ml for intramolecular ligation. The ratio of vector DNA and DNA containing desired fragment was 1:3.

Transformation was carried out according to the method of Cohen *et al.* (1977).

Plasmid construction.

All plasmids constructed in this study are shown in Table F. Most plasmids were constructed using restriction sites present on the nod DNA region of pRL5JI cloned in pIJ1095 into the same restriction sites of the vectors used.

For the construction of pMA540 and pMA545, an intermediate plasmid was used: the 2.4 kb Sall fragment

Table 0.6: Plasmids secured or constructed and used in this study.

Plasmids:	Relevant characteristics:	Reference/source:
pGSS33	IncQ(rep), Ap ^r , Cm ^r , Sm ^r , Tc ^r , broad host cloning vector, Mob ⁺	Sharpe, 1984.
pLAFR1	IncP-1(rep), Tc ^r , broad host cloning vector, Mob ⁺	Friedman <u>et al.</u> , 1982.
pBR328	ColE1(rep), Ap ^r , Tc ^r , Cm ^r , cloning vector	Soberon <u>et al.</u> , 1980.
pRK2013	ColE1(rep), Km ^r , Mob ⁺ , Tra ⁺ (RP4 <u>tra</u>)	Ditta <u>et al.</u> , 1980.
pBRK4013	ColE1(rep), Cb ^r , Mob ⁺ , Tra ⁺ (RP4 <u>tra</u>)	Hamel, 1987.
M13mp18	Derivative of M13 wild type, cloning vector	Norrandar <u>et al.</u> , 1983.
M13mp19	Derivative of M13 wild type, cloning vector	Norrandar <u>et al.</u> , 1983.
pCU101	p15A(rep), Cm ^r , Tra ⁺ (pCU1)	Thatte and Iyer, 1983.
pGV1106	IncW(rep), Km ^r , Sm ^r , broad host vector	Leemans <u>et al.</u> , (1978).
pKT210	IncQ(rep), Cm ^r , Sm ^r , broad host vector	Bagdasarian <u>et al.</u> , 1981.
pKT230	IncQ(rep), Km ^r , Sm ^r , broad host vector	Bagdasarian <u>et al.</u> , 1981.
pKT240	IncQ(rep), Ap ^r , Km ^r , promoter probing vector	Bagdasarian <u>et al.</u> , 1983.
pUC7	pMB1(rep), Ap ^r , <u>lacZa</u> ⁺ cloning vector	Veira and Messing (1982)

(continued . .)

Table 0.6 (continued).

pMA100	pCU101::Tnp _h oA	This study
pMA215	pBR325 derivative carrying 2.4 kb <u>Sal</u> I cultivar extension region of pRL5JI	This study
pMA500	4.5 kb <u>Eco</u> R1 fragment from pIJ1095 subcloned in pKT230	This study
pMA510	2.7 kb <u>Eco</u> R1+ <u>Sst</u> I fragment* subcloned in pKT210	This study
pMA520	1.8 kb <u>Eco</u> R1+ <u>Sst</u> I fragment* subcloned in pKT230	This study
pMA521	As in pMA520, but the 1.8 kb <u>Eco</u> R1+ <u>Sst</u> I fragment subcloned in pGSS33.	This study
pMA525	6.8 kb <u>Eco</u> R1 fragment cloned cloned into pKT230	This study
pMA526	As in pMA525, but the fragment cloned into pBR328.	This study
pMA530	7 kb <u>Sst</u> I fragment* cloned in pKT210	This study
pMA535	As in pMA530, but the 7 kb fragment in opposite orientation	This study
pMA540	As in pMA215, but the 2.4 kb <u>Sal</u> I fragment cloned in pKT230	This study
pMA545	As in pMA540, but the fragment cloned in the opposite orientation	This study

(continued ...)

Table 0.6 (continued).

pMA550	4.3 kb fragment* cloned in pKT230	This study
pMA560	1.7 kb <u>EcoRI</u> + <u>PstI</u> fragment* cloned in pBR328	This study
pMA570	0.8 kb <u>PstI</u> + <u>BglII</u> fragment cloned in pGV1106.	This study
pMA580	4.3 kb <u>EcoRI</u> + <u>SstI</u> fragment* cloned in pKT230	This study
pMA585	As in pMA580, but the fragment cloned in pKT240	This study
pMA600	As in pMA540, but the fragment cloned in M13mp19	This study
pMA605	As in pMA600, but the fragment cloned in opposite orientation	This study

*: For the relative position of these fragment see Figure 1.1.

containing cultivar extension region from pIJ1095 (Figure 1.1) was first inserted in the same Sall site of pUC7 and then the 2.6 kb EcoR1 fragment from this intermediate plasmid (containing the 2.4 kb cultivar extension region) was inserted into the EcoR1 site of pKT230 to generate pMA540. pMA545 contains the same 2.6 kb EcoR1 insert but with opposite orientation.

DNA restriction mapping.

For mapping of plasmid pIJ1095, the DNA was prepared as described in the Materials and Methods. The restriction enzymes used were Bgl11, EcoR1, Hpa1, Pst1, Sall, Sst1 and Xho1. Briefly, the DNA was first digested with single enzyme and then digested with different combinations of two enzymes. All digested fragments were separated on agarose gels and the size of each fragment was estimated as described in the Materials and Methods. An example is given in Appendix 2. The relative position of each individual restriction site was deduced as described by Rodriguez and Tait (1983).

For mapping the location of each TnphoA insert in pIJ1095::TnphoA, plasmid DNA preparations were made from the individual pIJ1095::TnphoA colonies and digested with a restriction enzyme that cuts within TnphoA and within the plasmid pIJ1095 DNA. (The restriction map of TnphoA is shown in Figure 0.7). For instance, 6 EcoR1 fragments were present within the cloned nod DNA of pRL5JI. The TnphoA insertions were first mapped to one of these 6 fragments using EcoR1. The more accurate location of TnphoA within this EcoR1 fragment was then determined by using a second restriction enzyme. The maximum error of the estimated TnphoA locations is less than 300 base pairs.

Legend to Figure 0.7: Restriction map of TnphoA.

TnphoA was constructed by inserting a 2.3 kb fragment containing an E. coli alkaline phosphatase (phoA) gene into IS50L of Tn5 (Manoil and Beckwith, 1985). The two small open boxes that are the uppermost represent the coding regions of the genes determining kanamycin (Km)/neomycin (Nm) and streptomycin (Sm) resistance respectively. The dashed line indicates the uncertainty of the transcription termination site of the gene determining Sm^r . The arrow represents the translational direction of E. coli phoA gene.

Restriction sites are abbreviated as follows:

B - BglII; Bm - BamHI; H - HindIII; Hi - HincII; P - PstI;
Pv - PvuI; S - SalI; Sm - SmaI; X - XhoI. Coordinates in kb
are listed on the line below.

DNA blotting and hybridization.

EcoR1-digested genomic DNA fragments were separated on 0.8% agarose gels and transferred to Hybond N nylon membranes by the bi-directional blot method of Smith and Summers (1980) with some modifications. After the denaturation step, the gels were neutralized with a solution of 1 M ammonium acetate, pH 7.5 (adjusted with 1 N NaOH). To fix the DNA onto the membrane after blotting, the membrane was placed face-down and irradiated with UV light for five minutes. The DNAs used as probes were labelled by nick-translation with (³²)dATP or (³²)dCTP, using DNA polymerase I (Klenow fragment), as described by Maniatis et al. (1982). Prehybridization was done for one hour at 65°C in a solution containing 6X SSC (10X SSC: 1.5 M NaCl; 0.15 M sodium citrate), 5X Denhardt's solution, 0.5% SDS and denatured salmon sperm DNA (500 µg/ml). Hybridization was for 18 - 24h at 65°C in the same solution supplemented with the heat-denatured hybridization probe. After hybridization, the membranes were first washed twice with 2X SSC at 65°C each time for 15 minutes. They were then washed with 2X SSC containing 0.1% SDS at 65°C for 30 minutes and finally washed with 0.1 X SSC at 65°C for 10 minutes. In some cases, a more stringent washing (2X SSC at 65°C for 1 h and then 2X SSC containing 0.1% SDS at 65°C for 1 h) was used to distinguish the specific binding from non-specific binding of the probe to the membrane. The membranes were then dried at room temperature and exposed to Kodak X-Omat AR film at -70°C for 1 or 2 days.

DNA sequence analysis.

A 2.4 kb SalI fragment carrying the host-range extension region was ligated into the same restriction site of M13mp19;

the clone (pMA600) carrying the insert was used to generate a series of sequential overlapping clones using the procedures of Dale et al. (1985) and a synthetic 22-mer oligonucleotide primer (International Biochemical Industries). To obtain the complementary strands of the clone which has different orientation of the same insert for DNA sequencing the cloned DNA was analyzed by the C-test described by Messing (1983). The DNA clones that contained complementary strands of the insert were hybridized to form dimers which migrate slower during electrophoresis through agarose gels than do unhybridized monomers. DNA sequencing was carried out by the dideoxy termination method of Sanger et al. (1977), except that a modified T7 DNA polymerase was used instead of E. coli DNA polymerase (Klenow fragment). The modified T7 DNA polymerase (commercially known as "Sequenase") and a kit containing the reagents for the sequencing reactions were obtained from US Biochemicals, and were used under conditions recommended by the manufacturer. The "universal" primer used in the sequencing reactions was obtained from Amersham (Canada). Both strands of each clone were sequenced, one strand being independently sequenced by Dr. John Nash. The complete nucleotide sequence of the coding strand of the 2 kb DNA carrying cultivar extension region (the remaining 0.4 kb sequence is not shown because this sequence was found not to code for any cultivar extension function) is shown in Figure 2.2. DNA sequence data was compiled and analyzed using the MicroGenie programs of Beckmann Instruments Limited.

TnphoA genome junction sequencing.

Two plasmids carrying TnphoA insertion within the 2.4 kb host-range extension region (nod2::phoA and nod121::TnphoA, see Figure 1.1) were sequenced with a synthetic primer complementary to the end of Tn5. The Tn5 sequencing primer

(3'-TTCATCGCAGGACTTGC-5') was obtained from Allelix Canada. This sequence was selected as it possessed the least homology to other sequences at the ends of Tn5 (Auerswald *et al.*, 1981). DNA fragments extending from HindIII sites within Tnp_{hoA} to Sall sites in the flanking Rhizobium DNA were first cloned into M13mp18 and then the single stranded DNA was prepared for sequencing analysis.

nod gene expression in a maxicell system.

Plasmid pMA215 carrying the 2.4 kb Sall fragment was transformed into the maxicell-producing E. coli strain CSR603 and labelled with [³⁵S]-methionine (Amersham) as described by Sancar and Rupert (1978). The radioactive samples were subjected to electrophoresis on the SDS-polyacrylamide gel described by Laemmli (1970) using ¹⁴C-labelled proteins (Amersham) as molecular weight standards. Gels were fixed in 4% sulphosalicylic acid, washed, dried, and exposed to Kodak X-Omat AR film at -70°C for several days.

CHAPTER 1: STRUCTURAL AND FUNCTIONAL ANALYSIS OF NODULATION REGIONS IN PLASMID pRL5JI OF R. leguminosarum BIOVAR viciae (STRAIN TOM).

Each Rhizobium species recognizes and infects only a few leguminous plants. Even within a single cross-inoculation group, certain restrictions on host-range are present. For instance, most European R. leguminosarum biovar viciae strains are unable to nodulate a primitive pea designated cv. Afghanistan. However, a strain called TOM isolated from soils in Turkey is able to nodulate cv. Afghanistan as well as most of the commercial pea varieties (Lie, 1978). Brewin et al. (1980) experimentally showed that the nodulation and host-range specificity of strain TOM is associated with a single self-transmissible plasmid, designated pRL5JI. Plasmid pRL5JI could be transferred by conjugation to the same or different Rhizobium species at a frequency of 10^{-5} to 10^{-6} per recipient.

Hombrecher et al. (1984) cloned a continuous region of approximately 30 kb of pRL5JI into the broad host-range cosmid vector pLAFR1 (Friedman et al., 1982) to yield the hybrid cosmid pIJ1095. When R. leguminosarum biovar viciae or biovar phaseoli strains are cured of their respective Sym plasmids, and pIJ1095 is introduced into such cured derivatives, the resulting strains nodulate both commercial (cv. Novella) and primitive (cv. Afghanistan) cultivars of pea. This indicates that most genes for nodulation (nod) and also cultivar extension are present in pIJ1095.

Gotz et al. (1985) physically and genetically demonstrated that a region of about 2 kb from pIJ1095 is involved in host-range extension to cv. Afghanistan. These workers also used a 0.8 kb PstI-BglII fragment originating

from the internal part of this 2 kb region of DNA as a hybridization probe to search for nucleotide sequence homology to the most extensively studied Sym plasmid, pRL1JI of R. leguminosarum biovar viciae strain 248. They did not detect any hybridization.

In this chapter, the general organization of those nodulation genes of pRL5JI that are contained in pIJ1095 and their possible regulation will be described. It will be shown that the structure and function of the nod genes of pIJ1095 (and therefore of pRL5JI) and of pRL1JI share a significant degree of similarity with the notable exception that only the former carries the 2.0 kb region that was referred earlier. Evidence will also be presented to indicate that some of the genes are similar and functionally exchangeable but not identical.

1.1 Physical mapping of the cloned nodulation (nod) region of pRL5JI.

Plasmid pIJ1095, which contains the nod region of pRL5JI, was transferred to Rhizobium strain B151 (a derivative of R. leguminosarum biovar viciae in which the native Sym plasmid had been cured) by triparental mating with the help of pRK2013. The resultant transconjugants were tested for nodulation on cv. Afghanistan and on cv. Novella. Small white nodules were observed on the roots of both plants, supporting the previous observation (Hombrecher et al., 1984). The DNA of pIJ1095 was isolated and a restriction map was constructed using seven different restriction endonucleases BglII, EcoRI, HpaI, PstI, SalI, SstI and XhoI). This map (Figure 1.1) is consistent with that of Gotz et al., (1985) for a region of about 15.6 kb.

Legend to Figure 1.1: Physical and genetic map of the R. leguminosarum biovar viciae strain TOM nodulation region.

The large horizontal bar represents the 15.5 kb nod region from the Sym plasmid pRL5JI. The vertical lines above the large bar represent TnphoA insertions into the recombinant plasmid pIJ1095. Below the large bar are small horizontal bars representing different subclones derived from pIJ1095, oriented with respect to the relevant restriction sites of the Nod region of pRL5JI. Insertions into the subclone pMA530 are also designated by vertical lines. The numerical designation of the mutation is indicated at the top of the line. Insertions which have no effect on the nodulation phenotype of their plasmids are marked with an asterisk. Mutants which are affected in nodulation of cv. 'Afghanistan' peas, but not modern pea cultivars, and which contain insertions mapping within the cultivar-extension region have boxed designations. The phenotypes of the mutations in the areas designated I, II, III, IV, and V are shown in Table 1.

B - Bgl11; E - EcoR1; H - Hind111; P - Pst1; Sa - Sal1; S - Sst1; X - Xho1. pMA450 and pMA545 represent insertions of the same fragment into the vector in alternative orientations.

(PMA500)

(PMA520)

(PMA540, PMA545)

(PMA510)

(PMA550)

(PMA580, PMA585)

1.2. Distribution of transposon insertions and their phenotypic effects.

Following transposon insertion by the procedures described in the Materials and Methods, a total of 98 *TnphoA* insertions were mapped with reference to the restriction map. Several of these insertions mapped within vector sequences. The position of 29 insertions that mapped at positions within the cloned *Rhizobium* DNA is shown in Figure 1.1 (top). Each such cosmid derivative into which the *TnphoA* was localized was then transferred by conjugative mobilization (for details, see Materials and Methods) to the *R. leguminosarum* biovar *viciae* or biovar *phaseoli* strain that had been cured of its respective indigenous Sym plasmid (strain B151 or 8401). Transconjugants were purified and tested for their nodulation phenotype on both cv. Novella and cv. Afghanistan peas. Five phenotypic classes were distinguished. The classes are described in Table 1.1 and the positions of each insertion giving rise to the respective class are shown in Figure 1.1. Class I mutants (see top of Figure 1.1) were all totally defective in nodulation on both pea cultivars and mapped to the *EcoRI*-*SstI* region in the 4.5 kb *EcoRI* fragment (between coordinate 6.0 and 8.8 kb). When strains carrying these mutants were examined for their ability to induce curling or deformation of root hairs, root hair curling or deformation was absent and these mutants could not be distinguished from strain 8401 or B151.

Class II mutants mapped to the left of Class I mutants on the same 4.5 kb *EcoRI* fragment but between coordinates 4.3 and 6 kb; Figure 1.1). Strains containing these mutants gave a normal level of nodulation on cv. Afghanistan, but substantially fewer nodules on cv. Novella (approximately 4-7 nodules per plant, compared to 25-30 nodules per plant for the strain carrying pIJ1095).

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Table 1.1: Phenotypic characterization of Tn_{phoA} induced Nod mutants in plasmid pIJ1095.

Class of mutant:	Mutant designation:	Phenotype affected:				Map position (kb co-ordinates, see Fig 1.1)
		Hac	Inf	Nov	Afg	
I	18, 37, 42, 63, 66, 78, 81, 87, 94	-	-	-	-	6.1 - 8.8
II	44, 51, 57, 58	+	+	±	+	4.3 - 6.1
III	99, 100, 101, 105, 110, 115, 117	+	+	*	+	1.5 - 3.5
IV	24, 27, 28, 69	+	+	-	-	8.8 - 10.8
V	2	+	+	+	-	11.2 - 12.4

Approximately 27-33 nodules per plant were formed on Novella pea when a R. leguminosarum strain containing plasmid pIJ1095 was tested during a control experiment. Strains carrying a Class II mutation induced 6-8 nodules per plant. For each test, six replicates of each pea cultivar were used.

Hac: root hair curling; Inf: infection thread formation; Nov: nodule formation on cv. 'Novella' (modern) peas; Afg: nodule formation on cv. 'Afghanistan' peas.

+: indicates phenotype is expressed.

-: indicates phenotype is not expressed.

±: indicates a reduction (to about 30%) in the number of nodules formed on Novella peas.

*: indicates a delay of about 2 - 3 days in the onset of nodulation on Novella, and a slight reduction (to about 80%) in the number of nodules formed on both plant hosts.

Class III mutants mapped to the left as represented in Figure 1.1 (between coordinates 1.5 and 3.2 kb) and were characterized by a delay of about three days in the onset of nodulation as compared to the strain containing pIJ1095 and a reduction (to about 50%, i.e. approximately 10-15 nodules per plant) in the number of nodules formed on cv. Novella peas. There was no delay in the onset of nodulation on cv. Afghanistan pea with Class III mutants and the number of nodules per cv. Afghanistan was not significantly different.

Class IV mutants mapped to the right of Class I mutants (between coordinates 8.8 and 10.8 kb). These mutants failed to nodulate either cv. Afghanistan or cv. Novella. Examination, by microscope, of the roots of both cultivars inoculated with mutants nod24::TnphoA and nod28::TnphoA (see Figure 1.1) indicated the presence of both root hair curling and of infection threads.

Class V mutants mapped to the right of Class IV mutants (between coordinates 11.2 and 12.5). They were able to nodulate cv. Novella but not cv. Afghanistan. The appearance and number of nodules on cv. Novella was similar to that caused by the strain carrying pIJ1095.

Six insertions (nod 13,14,64,82,83 and nod95) which were all mapped to the right of the Class V mutants, did not affect nodulation on either cultivar suggesting that this region is not essential for nodulation.

No insertions mapping to the left of the class III mutants were obtained. Since TnphoA 99, the class III mutant mapping farthest to the left, delayed the nodulation of cv. Novella, it is possible that the DNA region to the left of class III mutants is also concerned with nodulation and/or

host-range specificity (i.e. extending to the terminal EcoRI site or even beyond into a region not cloned in pIJ1095).

1.3. Microscopic study of class V mutants.

Class V mutants (Figure 1.1) were interesting because they affected the nodulation of only one host, the primitive cultivar. To determine the stage of nodulation on cv. Afghanistan that was affected, the mutant carrying the nod2::TnphoA insertion was inoculated onto cv. Afghanistan and tested microscopically for root hair curling and infection thread formation. Figure 1.2 shows the development of an infection thread on a cv. Afghanistan root inoculated with this mutant. Thus, mutations in this region seemed to affect a late nodulation function i.e. after the initiation and development of the infection thread.

1.4 Phenotypic correction of the five classes of nod mutants by the introduction of the corresponding wild-type nod region of pRL5JI.

To determine whether the TnphoA insertion mutations were dominant or recessive, it was useful to introduce the corresponding wild-type nod region into strains carrying each mutation. To do this, a 4.5 kb EcoRI fragment corresponding to the region defined by the class I and class II (Figure 1.1 coordinate 4.3 to 8.8 kb) mutants was subcloned from plasmid pIJ1095 into the EcoRI site of the broad-host range plasmid pKT210 to form plasmid pMA500. Similarly, the 6.8 kb EcoRI fragment (coordinate 8.8 to 15.6 kb) in which class IV and class V mutants were located was subcloned into pKT230 to form pMA525; the 4.3 kb EcoRI fragment corresponding to class III

Legend to Figure 1.2: Light micrograph of a class V mutant on cv. 'Afghanistan' pea root hairs.

A class V mutant (nod-2) was found to be defective in nodulating cv. 'Afghanistan' pea plants. To determine if the mutation was affecting root hair curling or infection thread formation, strain 8401 carrying the mutation was inoculated onto cv. 'Afghanistan' pea plants. Root hairs were examined 7 to 12 days after inoculation. As shown in the figure, root hair curling and infection thread formation had occurred suggesting that later stages in the nodulation process are blocked by the mutation.

mutants (coordinate 0 to 4.3 kb) was subcloned into pKT230 to form pMA550 (see Figure 1.1 for the limits of the cloned regions in these constructs). When pMA500 was transferred to Rhizobium strain 8401 containing class I or class II but not when transferred to the remaining three classes of mutant, the transconjugants could nodulate cv. Afghanistan but showed fewer nodules than the control strain carrying pIJ1095 (see Table 1.2a). The transfer of pMA525 to strains carrying class IV or class V mutants restored their nodulation ability to a level comparable to that of the strain carrying pIJ1095 (see Table 1.2b). Similarly, nodulation became normal with the introduction of pMA550 into the strain associated with class III mutants.

1.5 Isolation of additional transposon insertions on a clone containing the cultivar-extension region.

As demonstrated in the following section, 1.7, plasmid pMA530 was able to complement the class V mutant. Therefore this plasmid must contain the cultivar-extension region which can act in trans to correct the defect in the class V mutant. Attempts to isolate more TnphoA insertions in this region using plasmid pMA530 as a target were made. As shown in Figure 1.1, a total of 8 TnphoA insertions could be isolated. When pMA530 containing individual TnphoA insertions were transferred to the Rhizobium strain 5535 (strain 8401 containing Tn1831-marked pRL1JI (Lamb, 1982) and tested for cv. Afghanistan, the recipient carrying insertion mutants 109, 121, 122 and 124 did not nodulate Afghanistan pea, but insertions 13 and 14 extended the host-range of the recipient to Afghanistan pea. Restriction mapping analysis of these insertions showed that mutants 122, 123 and 124 had TnphoA inserted outside to the left of the cultivar-extension region;

TnphoA in mutants 109, 120 and 121 was located to the PstI and BglII fragment of cultivar-extension region and mutants 13 and 14 had TnphoA mapped the right-end of the cultivar-extension region. Since mutants 122 and 124 also failed to extend host-range to the recipient, it appeared that these three mutants exerted a strong polar effect on the downstream cultivar-extension region.

Mutations 13 and 14 were capable of extending the host-range to the recipient, implying that the cultivar-extension gene did not read through the right 500 bp DNA sequence of the cultivar-extension region.

1.6 Genetic analysis of nod genes of pIJ1095 by using subclones of its region to complement defined R. leguminosarum pRL1JI::nod mutants.

A total of 11 nod genes of the Sym plasmid pRL1JI have been identified and characterized in detail (see Literature Review). So far, this has been the most intensively studied Sym plasmid of R. leguminosarum biovar viciae. As indicated in Table 1.3, some Tn5 nod mutants of pRL1JI were made available. Attempts could therefore be made to correlate the nod region of pRL5JI with that of the individual nod genes of pRL1JI by using the subclones of pRL5JI to complement pRL1JI nod mutants.

Table 1.2a: Complementation analysis of the five classes of pIJ1095::TnphoA mutants by introduction of different cloned wild type Nod regions of pRL5JI.

R. leguminosarum 8401 containing <u>nod</u> mutants										
Plasmid: _____										
	Class I		Class II		Class III		Class IV		Class V	
	Nov	Afg	Nov	Afg	Nov	Afg	Nov	Afg	Nov	Afg
None	0	0	0	0	0	0	0	0	0	0
pMA500	+	+	+	+	-	-	-	-	-	-
pMA510	+	+	-	-	-	-	-	-	-	-
pMA520	-	-	+	+	-	-	-	-	-	-
pMA560	-	-	-	-	+	+	-	-	-	-
pMA525	-	-	-	-	-	-	+	+	+	+

Approximately 27-33 nodules per plant were formed on Novella pea when 8401 containing pIJ1095 was tested during a control experiment.

Nov = nodulation of cv. 'Novella' (modern) peas. Afg = nodulation of cv. 'Afghanistan' peas.

+: indicates complementation. Note: Class I mutants containing either pMA500 or pMA510 only produce very few nodules (1 to 3) per plant.

-: indicates no complementation.

0: indicates no nodules.

Table 1.2b: Complementation analysis of mutants carrying insertion mutations in different nod genes of pRL1JI by subclones of the wild type nod regions of pRL5JI.

Introduced plasmid	<u>R. leguminosarum</u> 8401 containing <u>nod</u> mutants							
	<u>nodB::Tn5</u>		<u>nodC::Tn5</u>		<u>nodD::Tn5</u>		<u>nodJ::Tn5</u>	
	Nov	Afg	Nov	Afg	Nov	Afg	Nov	Afg
none	-	-	-	-	-	-	*	-
pMA500	+	-	+	-	+	-	-	-
pMA510	+	-	+	-	-	-	-	-
pMA520	-	-	-	-	+	-	-	-
pMA540	-	-	-	-	-	-	-	-
pMA580	-	-	-	-	-	-	+	**

Approximately 28-35 nodules per plant were formed on Novella pea when 8401 containing a Sym plasmid was tested during a control experiment. For each test, seven duplicates of each pea cultivar were used.

Nov = nodulation of cv. 'Novella' (modern) peas; Afg = nodulation of cv. 'Afghanistan' peas.

+: indicates complementation. Note: There were fewer nodules formed (3 to 5 per plant) than found on the control.

-: indicates no complementation.

*: indicates delayed nodulation.

** : nodules were formed during this experiment when pMA580 was introduced into the pRL1JI nodJ mutant strain.

Table 1.3: Defined nod mutants of R. leguminosarum biovar viciae Sym plasmid pRL1J1.

Phenotypes were of strain 8401 carrying these plasmids.

<u>nod</u> mutant	observed phenotype*:
<u>nodB</u> ::Tn5	root hair curling is absent, nodules are not formed
<u>nodC</u> ::Tn5	same as above
<u>nodD</u> ::Tn5	root hair curling occurs, nodules are not formed
<u>nodJ</u> ::Tn5	root hair curling occurred, infection thread is formed in a normal way but a delay of about 3 days in the onset of nodulation, the number of nodules formed was similar to that with the wild-type strain

*: cv. Novella was used as the host plant. For each nod mutant at least seven plants were inoculated in each of two separate experiments.

1.6.1 A cloned 4.5 kb EcoRI fragment contains genes analogous to "common" nod genes.

Those nod genes which are functionally exchangeable between different Rhizobium species are usually called "common" nod genes. When plasmid pMA500 [already inferred to carry the region complementing Class I and II mutants (section 1.5 and Figure 1.1)] was transferred into strain 8401 carrying different pRL1JI nod mutants, nodB, nodC and nodD (nodA was not available), the mutant phenotypes were corrected although they did not reach a wild-type pRL1JI level in their efficiency of nodulation (number of nodules) on cv. Novella (Table 1.2b). These results taken with those in section 1.5 suggested that the cloned 4.5 kb region of pRL5JI codes for at least three nod genes equivalent to nodB, C and D. A second line of evidence for this conclusion came from experiments in which the Rhizobium strain carrying pMA500 alone without pRL1JI was inoculated on pea seedlings. The strain carrying pMA500 alone was able to induce root hair curling on both cv. Afghanistan and cv. Novella but without the formation of nodules on either host. Since it has now been well established that the common nod genes nodABC are involved in root hair curling, this supports the conclusion that pMA500 contains the genes with an analogous function.

1.6.2 The nodD gene is located on the 1.7 kb EcoRI and SstI region.

Two smaller plasmids, pMA510 and pMA520 (Figure 1.1), were constructed (Table 0.6) to determine the location of nodD. pMA510 contained a 2.8 kb EcoRI-SstI fragment (coordinates 6.0 to 8.8 kb) cloned into pKT210 while a smaller 1.7 kb fragment (coordinate 4.3 to 6.0) was cloned into pKT230

to form pMA520. When tested on plants, only pMA520 could complement the pRL1JI nodD::Tn5 mutant suggesting that a gene homologous to nodD of pRL1JI resided on pMA520. Although pMA510 failed to complement the nodD mutant, it was capable of complementing the nodB and nodC mutants, implying that the nodB and C genes were on pMA510.

1.6.3 nodJ gene sequence of pRL5JI is located on the 1.8 kb region to the right of the common nod(A)BC genes.

The class IV mutants that mapped to the right of the common nod(A)BC genes (Figure 1.1) completely abolished nodulation on both hosts. To compare the gene of this region with that of the pRL1JI nod genes, we constructed two subclones of pRL5JI, pMA540 and pMA580, for complementation tests. pMA540 carries the 2.4 kb SalI insert (coordinates 10.8 to 13.2 kb), whereas pMA580 contains 4.3 kb EcoRI-SstI insert (coordinates 8.8 to 13.4 kb), which overlaps the 2.4 kb SalI fragment of pMA540 (see Figure 1.1). As summarized in Table 1.2b, pMA580 was able to complement the nodJ mutant of pRL1JI, but pMA540 was not, indicating that the 1.8 kb region of pMA580 corresponding to class IV mutant region, contains a gene analogous to nodJ.

1.7 Regulation of the nod genes in pRL5JI.

When tested in a Rhizobium carrying pRL1JI, pMA540, having only the cultivar-extension region, was able to extend the host range of the recipient to cv. Afghanistan. However, it appeared that the expression of this cultivar extension gene is dependent on a vector promoter, because the same insert but in the opposite orientation (plasmid pMA545) could not extend

the host range to the recipient. From this evidence and the evidence in the literature (Literature Review) on the organization of nod genes in R. leguminosarum biovar viciae, it is reasonable to infer that the cultivar extension region of pRL5JI is co-transcribed with upstream nodulation genes and regulated by a common regulatory sequence (i.e. a nod box). To further test this inference, we compared pMA580 and pMA585 for their cultivar extension ability. The only difference between pMA580 and pMA585 is that pMA580 has the vector pKT230 whereas pMA585 has the vector pKT240. The introduction of pMA580 into a Rhizobium carrying pRL1JI extended the host-range of the strain to cv. Afghanistan but pMA585 did not do so. Unlike pKT230, no vector promoter is available near the EcoRI site of pKT240. This is consistent with the above observation and the inference that the expression of the cultivar extension gene(s) in pMA580 is through a vector promoter.

To search for a complete transcriptional unit in which the expression of cultivar-extension gene is independent of vector promoter, a clone containing the 7 kb SstI insert corresponding to class I, IV and V mutant regions (coordinates 6.1 to 13.1 kb, Figure 1.1) was constructed (vector pKT210 was used) and designated pMA530 (Figure 1.1). In both orientations (the clone with opposite orientation and which is not shown in the figure is designated as pMA535), i.e. pMA530 and pMA535 could extend the host range when transferred to a R. leguminosarum biovar viciae strain carrying pRL1JI. This indicates that in the native state, the cultivar-extension region belongs to the same operon as that of nodA, B and C. Thus, the overall genetic and functional organization of the nod genes in pRL5JI appears to be similar to that of pRL1JI except for the additional presence of the cultivar extension region.

1.8 Discussion.

A genetic analysis of the nod region of pRL5JI cloned in pIJ1095 (Hombrecher et al., 1984) by extensive transposon (TnphoA) mutagenesis, DNA subcloning and complementation tests has indicated that genes coding for nodulation and cultivar extension functions are clustered and are located on a DNA region of about 15 kb on the Sym plasmid pRL5JI. The organization of nod genes in this DNA region is similar to that in the extensively studied Sym plasmid pRL1JI. For a comparison of these two respective Nod regions, see Figure 1.3. In pRL1JI, nod genes are organized into four transcriptional units, nodABCIJ, nodD, nodFEL and nodMN (Surin and Downie, 1988, Figure 1.3). In this study, the presence of a large transcriptional unit analogous to nodABCIJ of pRL1JI, but also containing the cultivar extension region downstream of nodJ was inferred. Cloned DNA fragments homologous to and complementing the different mutant classes of pIJ1095 could also complement different and well-defined pRL1JI nod mutants, indicating that in these two divergent strains these nod genes are not only similarly arranged, but that their nod functions are also exchangeable. In contrast, the cultivar-extension gene or genes is present in pRL5JI but it or an analogue is not present in pRL1JI. This supports and confirms earlier observations (Hombrecher et al., 1984; Gotz et al., 1985). In R. leguminosarum biovar trifolii and in R. meliloti, the five genes (nodA,B,C,I,J) are also organized into a single operon in the same order as in R. leguminosarum biovar viciae. Interestingly, any gene of this operon in one strain or species of Rhizobium can be functionally replaced by nod genes of Sym plasmids from another strain or species. These genes have therefore been called "common" nod genes. This is also true of nodD. Since a cloned DNA region of pRL5JI can complement the pRL1JI nodD mutant, it can be

inferred that the nodD genes in the two strains have similar functions. Other studies (Djordjevic et al., 1985b; Fisher et al., 1985) have shown that an *R. leguminosarum* biovar trifolii nodD::Tn5 mutant can be complemented by nodD genes from *R. leguminosarum* biovar viciae, *R. meliloti* and *Rhizobium* strain NGR234. This implies that nodD gene in all these species have a common function as well. This function has been inferred to be that of a trans-acting transcriptional activator requiring plant root exudate (Mulligan et al., 1985; Rossen et al., 1985).

A DNA region where class III TnphoA insertion mutants were obtained is involved in proper host recognition. Mutation of this region results in strains showing delayed nodulation on cv. Novella. We reason that genes encoded by this region might be equivalent to nodF and E genes. First, our class III mutants are phenotypically comparable to nodF and E mutants of other *Rhizobium* biovars and species. nodE::Tn5 mutants of pRL1JI exhibited a delay of about 2 days in the onset of nodulation and a reduction (to about 30%) in the number of nodules formed on peas (Downie et al., 1985). Both nodE and F::Tn5 mutants of the Sym plasmid in biovar trifolii showed delayed nodulation on white clover, but not on subterranean clover. Moreover, these mutants acquired the novel capacity to nodulate both commercial and primitive peas (Djordjevic et al., 1985). Secondly, in both *R. leguminosarum* biovar viciae and biovar trifolii, nodFE genes are in the same location relative to nodD gene (Shearman et al., 1986; Schofield and Watson, 1986).

Our results indicate that all four class II mutants are defective in their ability to nodulate cv. Novella peas but not defective in their ability to nodulate cv. Afghanistan. Since the TnphoA inserts giving rise to these class II mutants

mapped between coordinates 4.3 and 6.0 kb, a DNA region which could complement nodD mutant of pRL1JI, we suggest that these four insertions define nodD of pRL5JI. However, these nodD mutants of pRL5JI in strain TOM are phenotypically different from the same type of mutant pRL1JI of the commercial R. leguminosarum biovar viciae and species. In R. leguminosarum biovar viciae and biovar trifolii nodD mutations result in a Nod⁻ phenotype (Downie et al., 1985; Djordevic et al., 1985). In R. meliloti, which contains three nodD genes, insertion mutations in nodD1 or nodD2 result in a leaky Nod⁻ or delayed nodulation phenotype, while a nodD1 and nodD2 double mutant is more severely delayed in nodulation, and deletion of nodD3 has no effect on nodulation (Gottfert et al., 1986; Honma et al., 1986). To determine the reasons why the nodD mutants of pRL5JI show a different behaviour from nodD mutants of pRL1JI, more studies need to be carried out. At present, a few hypothetical models could be suggested. One is that as in some of the other Rhizobium species mentioned, there are two copies of nodD genes on the part of pRL5JI that has been examined here. The two nod genes are different in their ability to determine pea cultivar specific nodulation. Therefore all these four class II mutants probably inactivates one allelic form of the same nodD gene which is more important for cv. Novella pea nodulation whereas a second allelic form of nodD gene which is specific for nodulation of cv. Afghanistan remains unaffected. Supporting this model is the observation made in R. meliloti by Horvath et al. (1987) and Gyorgypal et al. (1988). These researchers demonstrated that nodD gene acts as regulatory genes with a dual function: one to recognize different plant factors (a role in host specific nodulation) and a common function to activate other nod gene expression. For instance, the nodD1 mutants of R. meliloti decreased nodulation on Melilotus alba, Medicago sativa, M. quasifalcata and Trigonella coerulea but not on Medicago

truncatula. Decreased nodulation was observed on Medicago sativa, M. truncatula and Trigonella coerulea but not on Melilotus albas and Medicago quasifalacta when nodD2 mutants were tested. A nodD3 mutant showed only delayed nodulation on Medicago sativa and Melilotus albus. This model requires one to demonstrate the presence of this second hypothetical copy of the nodD gene from pRL5JI. Among others, one way is to use our class II mutants to complement nodD mutants of pRL1JI. A positive result could imply the presence of a second nodD gene in pRL5JI.

An alternative model is that a single nodD gene present in pRL5JI responds to different flavone and flavanone inducers to determine the recognition of the appropriate hosts. Our class II mutants could have TnphoA inserted into one region of nodD coding sequence which is cv. Novella flavonoid-specific, but not cv. Afghanistan flavonoid-specific. Based on the map positions of these TnphoA insertions, this presumed cv. Novella flavonoid-specific region is not less than 0.6 kb in length. Burn *et al*, (1987) have isolated four classes of nodD mutants from pRL1JI which are different in their ability to regulate host nodulation.

Our class IV mutants were characterized by the complete defectiveness in nodulation of both pea hosts. The major difference observed between class IV and class I mutants is that class IV mutants did not block earlier stages like root hair curling and infection thread formation but class I mutants did. A cloned DNA region corresponding to class IV mutants could complement the nodJ::Tn5 mutant of pRL1JI, therefore an analog of this gene (and presumably also nodI) is present in this region of pRL5JI. If this assumption can be proved by DNA sequencing, it is likely that there is a degree of genetic variation in the pRL5JI nodJ gene as

compared to the similar gene of pRL1JI, because the nodJ::Tn5 mutants of pRL1JI only delayed nodulation (by about two days) but did not affect the number of nodules formed (Downie et al., 1985). Comparative sequence analysis of the two nodJ genes would be informative.

Our class V mutants abolished nodulation on cv. Afghanistan, but not on cv. Novella, suggesting that this DNA region represents one cultivar-specific nod gene. These results are in good agreement with that of Gotz et al., (1985). They found that a similar cloned DNA region could confer to a Rhizobium strain containing the Sym plasmid pRL1JI the ability to nodulate cv. Afghanistan. No homologous sequences could be found in the Rhizobium strains carrying pRL1JI or the large majority of other R. leguminosarum biovar viciae field isolates which are unable to nodulate cv. Afghanistan when a 2.4 kb SalI fragment containing the cultivar-extension region or an internal 0.8 kb region defined by the PstI and BglII site (Figure 1.1, coordinates 10.8 to 13.2kb) was used as separate DNA hybridization probes.

CHAPTER 2: THE CULTIVAR EXTENSION REGION OF pRL5JI: ANALYSIS IN AN E. coli MAXICELL SYSTEM AND NUCLEOTIDE SEQUENCING.

It has been concluded (Chapter 1) that there is a striking similarity between the structural and functional organization of nod genes present in pRL1JI and pRL5JI although there also appear to be subtle functional differences between their respective nodD and nodJ genes as indicated by differences in mutant phenotypes when the respective genes are mutated. The outstanding difference is the presence in pRL5JI but not in pRL1JI of a region of about 2 kb which is co-transcribed with some of the other nod genes and which is essential to extend nodulation to cv. Afghanistan. This was already inferred from the studies of Hombrecher et al. (1984) and Gotz et al. (1985) and the observations in Chapter 1 confirmed it. In this chapter, the focus is on this cultivar extension region.

2.1 Detection of a polypeptide specified by the cultivar-extension region.

The 2.4 kb SalI fragment (coordinate 10.8 - 13.2 kb, Figure 1.1) was cloned into same restriction site of the E. coli vector pBR328 with the orientation in which transcription is driven by the promoter of the tetracycline gene of the vector. Coupled transcription and translation was conducted in vivo in the E. coli maxicell-producing strain.

As shown in Figure 2.1, the system originating from the pMA215 carrying strain displayed one additional polypeptide (position shown by arrow) that is absent in the strain containing the plasmid vector without the cloned insert. The

Legend to Figure 2.1: Expression of a gene from the cultivar extension region in an E. coli maxicell system.

³⁵S-labelled polypeptide products were analyzed on SDS-polyacrylamide gels and autoradiographed as described in Materials and Methods. Lane A: CSR603 (maxicells from plasmid-free E. coli); Lane B: plasmid pBR328; Lane C: plasmid pMA215 which contains the 2.4 kb cultivar extension region of pRL5JI in vector pBR328. M = marker (expressed in k α). Arrow indicates the additional polypeptide generated from pMA215.

calculated molecular weight of this polypeptide is 15.5-16.5 kd.

2.2 DNA sequence analysis of the cultivar-extension region.

It has been shown (Chapter 1) that in the plasmid pMA540 (Figure 1.1), the cultivar-extension region is contained in a SalI fragment. This plasmid was used as the source of DNA for sequencing.

Figure 2.2 presents the complete nucleotide sequence of the 2 kb SalI and XhoI fragment (the remaining 0.4 kb sequence is not shown here, because this sequence was found to be not essential for cultivar extension). We searched this sequence for an ORF that would be compatible with the polypeptide detected in the maxicell system. As shown in Figure 1.1, a further consideration arising from previous observations (Gotz et al. 1985) was that the gene was read from left to right (as shown in Figure 1.1) through the BglII site at coordinates 946-951 bp. These two considerations make the coding sequences from 906 to 1340 bp as the most likely candidate for the observed polypeptide. Furthermore, a cloned 0.8 kb fragment (coordinates 142-951) that is internal to the cultivar extension region showed no homology to the DNA of commercial R leguminosarum biovar viciae strains which are unable to nodulate cv. Afghanistan (for details see Chapter 3), implying that at least a portion of the cultivar extension gene is encoded by this region. Sequencing of the junctions of the two TnphoA insertions indicated that TnphoA2 lies immediately the left of position 402 and TnphoA121 to the left of position 547 (Figure 2.2) i.e. in position where they may be expected to disrupt transcription of the gene in the

Legend to Figure 2.2: DNA sequence of the cultivar-extension region.

DNA deletion fragments for sequencing were prepared by the method of Dale et al. (1985). The dideoxynucleotide chain termination method of sequencing was employed, samples being run on 7.5% sequencing gels. The 0.8 kb fragment used as a hybridization probe in the experiment of Chapter 3 is indicated by two arrows. The predicted amino acid sequence is shown below the DNA sequence. The arrows indicate the position of TnphoA insertions that abolish cultivar extension. The Shine-Dalgarno sequences are indicated as SD and the sites of putative initiation of the cv extension gene protein are boxed.

cultivar extension region. The sequence shown in Figure 2.2 is in agreement with that of Davis et al (1988). However, and as will be discussed below, their preferred coding region begins at position 237 in the same reading frame. There are putative ribosome binding sites upstream of both of these initiation codons (Figure 2.2).

2.3: Discussion.

While the sequence determined in this study agrees with the one recently published (Davis et al. 1988), there are differences in its interpretation. There is evidence (Fisher et al. 1987) that polypeptides of Rhizobium genes expressed in the E. coli maxicell system could provide useful and correct measures of polypeptides synthesized in Rhizobium species. In this heterologous system, we could detect only a single polypeptide of 15.5 - 16.5 kd. Inspection of the sequence indicates that a polypeptide initiated at the ATG codon at position 906 and terminating at the TAA codon at position 1340 would have a size (16 - 17 kd) that is consistent with the polypeptide detected in the maxicell system within a small margin of error. Davis et al. (1988) preferred the ATG at position 237 as the initiation codon and called this putative gene nodX. If utilized by Rhizobium, this would yield a polypeptide that is significantly larger than the one detected. We were unable to detect such a polypeptide. A definitive conclusion concerning the choice of the initiation codon can be obtained by site or region-directed mutagenesis of the sequenced region. It would also be desirable to develop and use systems for studying in vivo coupled transcription and translation directly in R. leguminosarum. The observation that there are these two initiation codons in phase with one another also raises the

possibility that one or the other is preferred depending on circumstances; alternatively, both are produced but only one is detectable in the system used. In this event, it is conceivable that one regulates the other.

Figure 2.3 displays a hydropathy plot of the amino acid sequences specified by nucleotides 906 to 1340 and which we believe to be relevant to host extension. It is apparent that the polypeptide is highly hydrophobic. It may be associated with the bacterial membrane. It is relevant that nodI and nodJ, which lie upstream of the cultivar-extension region, may also specify membrane proteins, with nodI being likely to encode a transport protein (Evans and Downie 1986). The molecular mechanism of action of the gene in the cultivar extension region remains to be uncovered. Strains carrying insertion mutations in the region caused root hair curling and infection threads on both pea cultivars, implying that the gene functions at some unknown step after infection thread formation. Most R. leguminosarum bv. viciae strains were also able to cause root hair curling and infection thread formation on cv. Afghanistan although no nodules were formed. Therefore, the polypeptide specified by the cultivar extension region is involved in other steps of the nodulation process. We were unable to detect release of bacteria into the cv. Afghanistan cortical cells when mutant #2 was used. Perhaps, the cultivar extension polypeptide and some specific factor from cv. Afghanistan (the product of sym-2?) are needed together to stimulate the plant.

As reviewed in detail in the Literature Review, in Rhizobium the genetic determinants of legume species or biovar-specificity are a set of nod genes sometimes called hsn genes to distinguish them from the so-called "common" nod genes. Among biovars of R. leguminosarum, the hsn genes

Legend to Figure 2.3: Hydropathy plot of the postulated gene product from the cultivar-extension region.

Analysis of putative cultivar-extension gene product for regions of hydrophobicity and hydrophilicity using the algorithm of Hopp and Woods (1981). Positive values indicate hydrophilic regions of this protein; negative values indicate regions of hydrophobicity.

101 A Ala
102 A Ala
103 A Ala
104 A Ala
105 A Ala
106 A Ala
107 A Ala
108 A Ala
109 A Ala
110 A Ala
111 A Ala
112 A Ala
113 A Ala
114 A Ala
115 A Ala
116 A Ala
117 A Ala
118 A Ala
119 A Ala
120 A Ala
121 A Ala
122 A Ala
123 A Ala
124 A Ala
125 A Ala
126 A Ala
127 A Ala
128 A Ala
129 A Ala
130 A Ala
131 A Ala
132 A Ala
133 A Ala
134 A Ala
135 A Ala
136 A Ala
137 A Ala
138 A Ala
139 A Ala
140 A Ala
141 A Ala
142 A Ala
143 A Ala
144 A Ala
145 A Ala
146 A Ala
147 A Ala
148 A Ala
149 A Ala
150 A Ala
151 A Ala
152 A Ala
153 A Ala
154 A Ala
155 A Ala
156 A Ala
157 A Ala
158 A Ala
159 A Ala
160 A Ala
161 A Ala
162 A Ala
163 A Ala
164 A Ala
165 A Ala
166 A Ala
167 A Ala
168 A Ala
169 A Ala
170 A Ala
171 A Ala
172 A Ala
173 A Ala
174 A Ala
175 A Ala
176 A Ala
177 A Ala
178 A Ala
179 A Ala
180 A Ala
181 A Ala
182 A Ala
183 A Ala
184 A Ala
185 A Ala
186 A Ala
187 A Ala
188 A Ala
189 A Ala
190 A Ala
191 A Ala
192 A Ala
193 A Ala
194 A Ala
195 A Ala
196 A Ala
197 A Ala
198 A Ala
199 A Ala
200 A Ala

cannot be exchanged without altering biovar or species specificity. This occurs despite the fact that there is considerable sequence similarity between the respective genes of biovar viciae and biovar trifolii. Thus in this case host-specificity is not altered by the presence of an additional gene but rather different forms of the same gene. In strain ANU843 of R. leguminosarum biovar trifolii, Tn5 insertions in nodF and E genes resulted in mutants which abolished nodulation ability on clover but acquired the novel ability to nodulate peas which are not nodulated by field isolates of R. leguminosarum biovar trifolii (Djordjevic et al. 1985a). In Chapter 3, we show that the DNA of strain ANU843 hybridizes strongly with the 2 kb cultivar extension probe derived from pRL5JI (see also Davis et al. 1988). These observations are consistent with a hypothesis that requires interaction between products from the cultivar extension region and the nodFE products.

CHAPTER 3: SURVEY OF FIELD ISOLATES OF R. leguminosarum BIOVAR viciae FOR NODULATION OF PRIMITIVE AND COMMERCIAL PEA CULTIVARS, FOR THE PRESENCE OF SEQUENCE SIMILAR TO THE CULTIVAR EXTENSION REGION OF pRL5JI AND FOR STRUCTURAL VARIATIONS IN THEIR nod REGION(S).

In Chapters 1 and 2, the plasmid pRL5JI-associated root nodulation (nod) region was characterized and compared to the nod region of pRL1JI, the plasmid that confers a nodulation host-range limited to commercial pea cultivars. This chapter presents results from a broad survey of R. leguminosarum biovar viciae field isolates and from a search for new isolates that have a cultivar specificity extending to cv. Afghanistan. The questions raised were:

- 1) Are isolates with the ability to nodulate cv. Afghanistan rare in nature?
- 2) Can new isolates with this ability be identified?
- 3) Are nodulation and cv extension regions on these strains associated with plasmids? If so, are they conjugative plasmids?
- 4) Do field isolates with this cultivar-extension capacity have a region homologous to the 2 kb cultivar extension region identified in plasmid pRL5JI of strain TOM; i.e., is there any evidence for the horizontal transmission of the region?
- 5) Is there an absolute correlation between the presence of the cultivar extension region and the ability to nodulate cv. Afghanistan?

6) What is the structural organization of the nod regions in field isolates of R. leguminosarum biovar viciae as compared to that of the nod and the cv extension region of strain TOM?

Experiments bearing on these questions are described in this chapter. Also included are the results of some limited experiments involving other Rhizobium species. At the outset, it is emphasized that there is evidence in the literature for an overall structural and functional similarity in the relative organization of the genetic regions determining root nodulation by not only different biovars of a species but also different species of Rhizobium. This and the observed differences between species was reviewed in the Literature Review (see Figure 0.3 for a summary presentation).

3.1 Survey of field isolates for their nodulating ability on commercial (cv. Novella) and primitive (cv. Afghanistan) peas.

One of our objectives was to examine the geographic distribution of field isolates which nodulate cv. Afghanistan in addition to or instead of commercial peas. A total of 56 field isolates of R. leguminosarum biovar viciae, with widely different geographical origins, were tested for their ability to nodulate both cultivars (Table 0.4). All strains tested were able to form abundant pink-coloured nodules on the roots of cv. Novella and also on two other commercial cultivars cv. Alaska and cv. Trapper but majority failed to nodulate cv. Afghanistan pea [Pisum sativum cv. Trapper seeds were obtained from Prairie Regional Laboratory, National Research Council, Saskatoon, Canada. P. sativum cv. Alaska seeds were obtained from Stokes Seeds Inc., Buffalo, New York, USA.]. Occasionally, some of these non-nodulating strains formed a

few small white nodules on roots of cv. Afghanistan. However, it was not possible to isolate from such nodules a strain that stably infected cv. Afghanistan. In addition to strain TOM, 12 strains were identified in this study to form effective nodules on cv. Afghanistan. Five were isolated from nodules of cv. Afghanistan in Denmark, one from a soil sample from central India, three from nodules of Viciae sp. in the northwest of the People's Republic of China, one from soils of the northwestern part of the USSR and one from nodules of a field pea in Yugoslavia. A number of strains isolated from England were unable to nodulate cv. Afghanistan. Twenty-seven strains isolated from Canada (most from the province of Ontario) also failed to nodulate cv. Afghanistan. Surface-sterilized seeds from accessions of Afghanistan and Turkish origin (but not cv. Afghanistan) were also sown directly in a plot at the Central Experimental Farm, Ottawa. Those pea lines (126341, 212112 and 169600) which were phenotypically close to cv. Afghanistan and characterized by small flowers and pods and brown marbled seeds, were not nodulated. On the other hand, some pea lines which were phenotypically less similar to cv. Afghanistan (125839, 125840, 135846, 167363, 171812, 212112, 223527) were effectively nodulated. The bacteria isolated from such nodules failed to nodulate cv. Afghanistan. In this survey, no isolate was identified that would nodulate cv. Afghanistan but not cv. Novella.

3.2 Comparative description of nodulation patterns induced by strains that have extended host specificity.

When the 12 isolates identified as being cv. Afghanistan positive were inoculated onto a commercial pea cv. Novella, they all formed abundant nodules with no significant difference between them in terms of nodule appearance, size

and number of nodules, and the visibly observed patterns of nodule arrangement in their root system. However, the nodulation patterns on the roots of cv. Afghanistan induced by some of these 12 "broad" host-nodulating strains were different. Strain D52, originally isolated from the root nodules of Vicia faba, exhibited a unique nodulation pattern. The earliest-induced nodules were always clustered on the uppermost part of the root system. Only after two weeks, the lower parts of the roots became infected and occupied with young-developing nodules. This sequential nodulation makes the same root system have nodules in different developmental stages and sizes (Figure 3.1). The mature nodules that occurred first in the uppermost part of the roots were deep rose-coloured. They were elongated in shape ranging from 1 - 1.5 to 3 - 4.5 mm in size. The number of root nodules per plant was up to 65.

Inoculation of cv. Afghanistan with the Danish strain A6 resulted in a few and late-appearing nodules (>25 days). The feature of delayed nodulation was stable and could not be reversed phenotypically by pre-incubating the bacteria in cv. Afghanistan root-exudate prior to inoculation of seedlings. Examination of roots by light microscopy showed that the appearance of the infection threads is delayed as compared to that induced by TOM. It should be emphasized that strain A6 induces a normal nodulation phenotype on cv. Novella.

Nodulation patterns of the remaining strains on cv. Afghanistan were similar to that induced by strain TOM (i.e. nodules were small and distributed around the primary and main lateral root system). However, the nodules induced by strains A1, A7, A8, A10 and USSR A-3 were generally larger in size (2-3 mm in diameter) than those induced by strains I9, USDA 2386 and T14. Figure 3.2 shows the nodulation of cv. Afghanistan which has been inoculated by strain USDA 2386.

Legend to Figure 3.1: Light micrograph of nodulation patterns of cv. Afghanistan inoculated by strain D52.

A. Nodules from the roots of cv. Afghanistan peas, three weeks after inoculation with strain D52. The large arrow indicates where the mature nodules first occurred on the uppermost part of the root. The small arrow indicates the emerging nodules from the lower part of the root.

B. Nodules from the roots of cv. Afghanistan peas, four weeks after inoculation with strain D52. The arrows indicate the nodules which have developed on the lower part of the root.

A

B

Legend to Figure 3.2: Light micrograph of the nodulation response of cv. Afghanistan peas after inoculation by strain USDA 2386.

The nodules were photographed from cv. Afghanistan peas, three weeks after inoculation with strain USDA 2386. Nodules are generally small, and were well-distributed around the primary and main lateral roots.

The number of nodules scored on strain A1-induced cv. Afghanistan roots is higher than the number induced by strain TOM.

3.3 Structural variations in *nod* regions of field isolates of Rhizobium leguminosarum biovar viciae using subclones of the *nod* region of strain TOM as a standard.

As the information about the structure and organization of *nod* genes in strain TOM had been determined and various subclones of these regions were available (Chapters 1 and 2), they could be used as a reference to study the presence and organization of similar *nod* regions in the new field isolates. We used the subclones derived from pIJ1095 as DNA hybridization probes to hybridize against the total DNA isolated from the different field isolates of R. leguminosarum biovar viciae, also included were a few isolates of other species related to R. leguminosarum. The major features of these clones are listed in Tables 0.6 and 3.1 and the *nod* regions present in them are shown in Figure 3.3. Total DNA isolated from each of 41 natural R. leguminosarum biovar viciae isolates of different geographical origin and a few strains of some other Rhizobium species was digested to completion with endonuclease EcoRI, electrophoresed through a 0.7% agarose gel and blotted to nylon membranes. The membranes were then hybridized with ³²P labelled probes that had been generated by nick-translation (for details, see Materials and Methods).

Table 3.1: Characteristics of plasmids used for DNA hybridization.

Subclone:	Vector:	DNA from the <u>nod</u> region of pIJ1095 ^a :	<u>nod</u> gene known to be present in probe:
pMA510	pKT210	6.0 - 8.8	<u>nod</u> (A)BC
pMA520	pKT230	4.3 - 6.0	<u>nod</u> D
pMA540	pKT230	10.5 - 12.9	cv. extension region
pMA550	pKT230	0 - 4.3	<u>nod</u> FE
pMA560	pBR328	8.8 - 10.6	<u>nod</u> (I)J
pMA570	pGV1106	10.6 - 11.4	cv. extension r e g i o n (i n t e r n a l part).

^a: kb coordinates, see Figure 1.1.

(): indicates presence of the nod gene is an inference and has not been unequivocally demonstrated.

Legend to Figure 3.3: Illustration of subclones used as hybridization probes against EcoR1-treated genomic DNA isolated from field Rhizobium isolates.

Nod regions present in subclones which were used as hybridization probes against EcoR1-treated total genomic DNA isolated from various R. leguminosarum biovar viciae field isolates and a few other Rhizobium species. These subclones represent different nodulation functions of pRL5JI. For detailed information of these probes see Table 3.1 and the text.

The letters above the three arrows indicate the nod genes and the arrows indicate their direction of transcription. (), as in Table 3.1.

3.3.1 Hybridization with pMA510 (containing nodABC sequences).

As inferred from the results and discussion in Chapter 2, plasmid pMA510 contains nod A, B and C, a group of genes sometimes called "common nod genes". When used in hybridization experiments, homology was detected in all of the R. leguminosarum biovar viciae strains studied (Figure 3.4; Table 3.2), only a single band of hybridization was detected in all cases. The size of this hybridizing fragment appeared to be the same for groups of strains. Thus, out of 40 strains tested, 17 showed a single 4.5 kb hybridizing fragment; 11, a 3.1 kb fragment; 5, a 6.0 kb fragment; 3, a 2.8 kb fragment and 2, 6.6 kb fragment. Each of the remaining 2 strains had the hybridizing fragments of distinctive sizes. As expected, hybridizing bands were missing in the Sym plasmid-cured strain of R. leguminosarum biovar viciae (strain B151), but the same fragment as in strain TOM was found in the Agrobacterium tumefaciens transconjugant MS136 containing pRL5JI.

3.3.2 Hybridization with pMA520 (containing nodD sequence).

pMA520 has been inferred to carry a nod D sequence (Chapter 1). As summarized in Table 3.2, homology to this sequence was found in all strains tested. Interestingly, all 4.5 kb fragments that hybridized to probe pMA510 in 17 strains also hybridized to pMA520 (Figure 3.4), strongly suggesting that in all these strains nodABC and nodD are located on the same EcoR1 DNA fragment. The other strains showed a hybridization fragment with different sizes, suggesting that nodD and nodABC genes in these strains are separated by a single EcoR1 restriction site. The size of the hybridizing

Legend to Figure 3.4; A,B,C,D,E and F: Southern blot analysis of genomic DNA isolated from different field isolates of R. leguminosarum biovar viciae using subclones of nod region of pIJ1095 as probes.

A. Autoradiographs resulting from hybridization of Southern blots with the ³²P-labelled plasmid pMA510 containing the nodABC sequences of prL5JI. Lane 1: TOM (control); Lane 2: A1, A7, A8, A10, JIM1104, JIM1201, S1, I2, I7, I8, I9, USDA2397, USDA2426, USDA2449, D38B, D52, USSRA-3; Lane 3: USDA2429; Lane 4: JISP18, JISP24, JISP48, JISP75, JIM1121, JIM1122, JIM1325, JIM1418, JIM1726, S2, S10; Lane 5: JISP22, JISP36, JIM1331, 128C53, 300; Lane 6: 248, A6; Lane 7: S13, JIM1934, USDA2386; Lane 8: USDA2478.

B. Autoradiographs resulting from hybridization of Southern blots with the ³²P-labelled plasmid pMA520 containing nodD sequence of prL5JI. Lane 1: TOM (control); Lane 2: A1, A2, A7, A8, A10, D38B, D52, JIM1104, JIM1201, I2, I7, I8, I9, S1, USDA2397, USDA2426, USSRA-3; Lane 3: JISP22, JISP36, JISP1331, 128C53, 300; Lane 4: JISP18, JISP24, JISP75, S13; Lane 5: JISP48; Lane 6: JIM1121, JIM1122; Lane 7: JIM1325, S2, S10; Lane 8: JIM1418, JIM1724; Lane 9: JIM1934; Lane 10: 248, A6; Lane 11: USDA2478; Lane 12: B151.

C. Autoradiographs resulting from hybridization of Southern blots with the ³²P-labelled plasmid pMA550 containing nodFE sequence of prL5JI. Lane 1: TOM (Control); Lane 2: JISP18, JISP75, JISP1104, JIM1201, JIM1325, JIM1934, S1, S2, S10, S13, I2, I7, I8, I9, USDA2386, USDA2397, USDA2426, USDA2449, USDA2478; Lane 3: A1, A7, A8, A10, USSRA-3; Lane 4: A6; Lane 5: JISP22, JISP36, JIM1331, 300; Lane 6: JISP48, JISP1418, JIM1726; Lane 7: USDA2429; Lane 8: JIM1121, JIM1122; Lane 9: JISP24, 248; Lane 10: B151.

D. Autoradiographs resulting from hybridization of Southern blots with ^{32}P -labelled plasmid pMA560 containing nodIJ sequence of pRL5JI. Lane 1: TOM (control); Lane 2: A1, A7, A8, A10, JIM1934, I9, USSRA-3; Lane 3: A6, USDA2386, USDA2478, D388; Lane 4: JISP18, I2, I7, I8; Lane 5: JIM1418, JIM1726; Lane 6: JISP48; Lane 7: S1, S2, S10; Lane 8: JISP22, JISP24, JISP36, JIM1121, JIM1122, JIM1331; Lane 9: JIM1201; Lane 10: JISP75, JIM1104, JIM1325, S13, USDA2397; Lane 11: USDA2429; Lane 13: 248; Lane 14: B151.

E. Autoradiographs resulting from hybridization of Southern blots with the ^{32}P -labelled plasmid pMA540 containing the 2 kb cultivar extension region (nodX) of pRL5JI. Lane 1: TOM (control); Lane 2: A1, A7, A8, A10, JIM1934, I9, USSRA-3; Lane 3: USDA2386, USDA2478, D388, T14; Lane 4: USDA2429; Lane 5: no hybridization was detected with any of the remaining strains.

F. Autoradiographs resulting from hybridization of Southern blots with the ^{32}P -labelled plasmid pMA570 containing the internal 0.8 kb region of the cultivar extension region. Lane 1: TOM (control); Lane 2: A1, A7, A8, A10, JIM1934, I9, USSRA-3; Lane 3: D388, USDA2386, USDA2478, T14; Lane 4: USDA2429; Lane 5: no hybridization was detected with any of the remaining strains.

A

Table 3.2: EcoRI fragments (sizes in kb) from the total DNA of various R. leguminosarum biovar viciae strains that hybridized to various nod region probes.

Hybridization probes used:

Strain 510 520 550 560 540 570 Symbols^b
 (nodABC)(nodD) (nodFE) (nodIJ)(cv extension)

TOM	4.5	4.5	4.3	6.9	6.9	6.9	<u>AA</u> <u>B</u> <u>CC</u>
A1	4.5	4.5	2.5+ 2.0	6.9	6.9	6.9	<u>AA</u> X <u>CC</u>
A6	6.7	6.7	11	5.5	-	-	DD Y F
A7	4.5	4.5	2.5+ 2.0	6.9	6.9	6.9	<u>AA</u> X <u>CC</u>
A8	4.5	4.5	2.5+ 2.0	6.9	6.9	6.9	<u>AA</u> X <u>CC</u>
A10	4.5	4.5	2.5+ 2.0	6.9	6.9	6.9	<u>AA</u> X <u>CC</u>
JISP18	3.1	1.5	4.3	7.5	-	-	GE <u>B</u> M
JISP22	6.0	6.0	6.0+ 2.2	3.7	-	-	LL O U
JISP24	3.1	1.5	6.7	3.7	-	-	GE D U
JISP36	6.0	6.0	6.0+ 2.2	3.7	-	-	LL O U
JISP48	3.1	5.2	5.2	2.3	-	-	GS S Q
JISP75	3.1	1.5	4.3	9.5	-	-	GE <u>B</u> H
JIM1104	4.5	4.5	4.3	9.5	-	-	<u>AA</u> <u>B</u> H
JIM1121	3.1	4.3	4.3+ 2.0+ 1.3	3.7	-	-	GB K U
JIM1122	3.1	4.3	4.3+ 2.0+ 1.3	3.7	-	-	GB K B
JIM1201	4.5	4.5	4.3	2.5	-	-	<u>AA</u> <u>B</u> T
JIM1325	3.1	2.7+1.2	4.3	9.5	-	-	GR <u>B</u> H
JIM1331	6.0	6.0	6.0+ 2.2	3.7	-	-	LL O U
JIM1418	3.1	5.2+ 2.7	5.2	3.1	-	-	GI S G
JIM1726	3.1	5.2+ 2.7	5.2	3.1	-	-	GI S G
JIM1934	2.5	2.5+ 1.2	4.3	6.9	6.9	6.9	TR <u>B</u> <u>CC</u>
SYM8501	4.5	4.5	4.3	10.0	-	-	<u>AA</u> <u>B</u> N
SYM8502	3.1	2.7+ 1.2	4.3	10.0	-	-	GR <u>B</u> N
SYM8510	3.1	2.7+ 1.2	4.3	10.0	-	-	GR <u>B</u> N
SYM8513	2.5	1.5	4.3	9.5	-	-	TE <u>B</u> H
I2	4.5	4.5	4.3	7.5	-	-	<u>AA</u> <u>B</u> M

(continued ...)

Table 3.2 (continued).

Hybridization probes used:							
Strain	510	520	550	560	540	570	Symbols
	(nodABC)(nodD) (nodFE)(nodIJ)(cv extension)						
I7	4.5	4.5	4.3	7.5	-	-	<u>AA</u> <u>B</u> M
I8	4.5	4.5	4.3	7.5	-	-	<u>AA</u> <u>B</u> M
I9	4.5	4.5	4.3	6.9	6.9	6.9	<u>AA</u> <u>B</u> <u>CC</u>
USDA:							
2386	2.5	nt	4.3	5.5	5.5	5.5	T? <u>B</u> FF
2397	4.5	4.5	4.3	9.5	-	-	<u>AA</u> <u>B</u> H
2429	14	nt	8.5	7.1+	11*	-	V? P J
				3.5			
2478	12	25	4.3	5.5	5.5	5.5	ZW <u>B</u> FF
2426	4.5	4.5	4.3	nt	-	-	<u>AA</u> <u>B</u> ?
2449	4.5	4.5	4.3	nt	-	-	<u>AA</u> <u>B</u> ?
D38B	4.5	4.5	6.0	5.5	5.5	5.5	<u>AA</u> O FF
D52	4.5	4.5	nt	nt	-	-	
T14	4.5	4.5	6.0	5.5	5.5	5.5	<u>AA</u> O FF
USSRA-3	4.5	4.5	2.5+ 2.0	6.9	6.9	6.9	<u>AA</u> X CC
300	6.0	6.0	6.0+ 2.2	nt	-	-	LL O ?
128C53	6.0	6.0	nt	nt	-	-	LL ? ?
248	6.7	6.7	6.7	3.0	-	-	DD D Y

Explanatory notes to Table 3.2:

*: Symbols are used for comparison of hybridization patterns as suggested by J.P.Young (personal communication). The hybridization pattern shown by homologous fragments of TOM is expressed as "AA B CC". The first two AA indicate that the hybridizing fragments are the same (4.5 kb) when probes nodABC and nodD are used. The appearance of the same symbol or different symbol for other strains indicate that the fragment length is the same or different (larger or smaller or more than one fragment). The third letter "B" indicates a hybridizing band with probe nodFE that has different size (4.3 kb); the fourth "C" indicates a homologous fragment with probe nodIJ, the size being 6.9 kb; the fifth "C" indicates a homologous fragment with probe cultivar extension region (both pMA540 and pMA570), being the same 6.9 kb fragment.

nt: not tested; -: no homology; *: see text.

?: hybridization pattern for a particular probe is not known.

bands in them was variable, ranging from 1.2 to 6.7 kb. It is of interest that in six of the 41 strains two hybridizing bands were present. Based on size differences, they could be classified into three groups: one group (strains JIM1418 and JIM1726) had 5.2 and 2.7 kb fragments; the second group (strains JIM1325, S1 and S10) had 2.7 and 1.2 kb fragments and the third group (strain JIM1934) had 2.5 and 1.2 kb fragments.

3.3.3 Hybridization with pMA550 containing the *nodFE* gene sequence).

Insertion mutagenesis (Chapter 1) has shown that plasmid pMA550 contains essential *nod* genes similar to *nodE*, F of pRL1JI in the phenotypes they confer. Homology to pMA550 was clearly detected in all isolates studied. The pattern of hybridizing bands (Figure 3.4; Table 3.2) for the different isolates varied from a single band to triple bands. Among 27 "single band" strains, 19 had a hybridization fragment of 4.3 kb which is identical to that displayed by strain TOM and the remaining 8 strains exhibited a higher molecular weight than that displayed by strain TOM, ranging from 5.2 to 11 kb. In 11 strains, two homologous bands were present. Among them, four strains (JISP22, JISP36, JIM1331 and strain 300) showed two bands of 6.0 kb and 2.2 kb and the remaining strains (A1, A7, A8, A10 and USSR A-3) had hybridization bands of 2.5 kb and 2.0 kb.

Two strains from the UK, JIM1121 and JIM1122, exhibited three distinct bands with molecular weights of 4.3, 2.0 and 1.3 kb respectively. These are not due to partial digestion because when the amount of endonuclease used was increased. The hybridization patterns remained unchanged. They represent

another group of *R. leguminosarum* strains with respect to this particular probe.

3.3.4 Hybridization with pMA560 (containing *nodIJ* sequence).

pMA560 contains a 1.7 kb *Eco*R1-*Pst*I fragment of pRL5JI encoding at least one *nod* gene equivalent to *nodJ* of pRL1JI (it is quite likely that it includes *nodI* as well but this has not been demonstrated unequivocally). As shown in Figure 3.4, in almost all strains tested, except USDA2429, only one hybridizing band was observed. In seven of them, the size of the band was identical to that in strain TOM (6.9 kb), suggesting that in these strains the *nodIJ* genes may have an organization similar to that of strain TOM. It will be mentioned in the next section, that in the majority of these strains, the cultivar extension gene is located on the same 6.9 kb *Eco*R1 fragments, because when hybridized with probe pMA540 (or pMA570), the hybridizing fragment was also identified to be 6.9 kb. Interestingly, other strains, the DNA of which showed no hybridization to the cultivar extension probe, showed a greater variability in size (ranging from 2.3 to 10 kb) with pMA560 (*nodIJ*) as the probe. Strain USDA2429 exhibited two hybridization bands with molecular weight of 7.1 and 3.5 kb.

3.3.5 Hybridization with plasmids pMA540 and pMA570 (containing the cultivar-extension region).

pMA540 has an insert containing the complete cultivar-extension region of pRL5JI of strain TOM whereas pMA570 contains an 0.8 kb internal subset of the region present in pMA540. This subset includes part of the coding sequences of

cultivar extension gene (confirmed by sequencing data). When using pMA540 as the hybridization probe, a unique hybridizing fragment was observed in 11 strains. Of these 11 hybridizing strains, 7 had a 6.9 kb hybridizing band which is identical to that exhibited by strain TOM. The remaining 4 strains (USDA2478, an isolate from Morocco; USDA2386, an isolate from Yugoslavia; and two PRC strains D38B and T14, an isolate from China), had a smaller hybridizing band, approximately 5.5 kb in size.

In a few cases, a weakly hybridizing band was found. However, these weakly hybridizing bands could be removed by more stringent washing. Since there was no such weak hybridization bands detectable with the subset probe pMA570, it is likely that a small part of the nodJ sequence extending into pMA540 is responsible for this display of weak hybridization. As pMA540 and pMA570 gave the same hybridization patterns, the data with pMA570 is not shown in Figure 3.4. One exception is strain USDA2429, which, as shown in Figure 3.4 (Lane 4, panel E), exhibited a hybridization band of 11 kb to pMA540 but not to pMA570. This strain does not nodulate cv. Afghanistan. The observed 11 kb band, unlike other weak hybridization bands, could not be removed by stringent washing.

3.3.6 Hybridization to Sym plasmid DNA.

In order to determine whether the observed hybridizing bands from the total DNA of the strains were present on indigenous plasmid(s), hybridization experiments were done wherever possible with the isolate carrying only the Sym plasmid.

To do this, we labelled the Danish strains A7, A8 and A10 with Tn5 using the procedure described in Materials and Methods. Tn5-labelled Sym plasmids were identified by transferring a single labelled plasmid into the Sym plasmid-cured strain B151, and then inoculating transconjugants onto cv. Afghanistan seedlings. On the roots of inoculated plants nodules appeared from which bacteria were re-isolated, purified and plasmid DNA extracted. This plasmid DNA was cleaved by EcoRI and used for Southern blotting analysis using each of the six probes.

In all these cases, the hybridization patterns obtained were identical to those observed in probings with the total DNA of the field isolates. This indicates that at least in these three field isolates the nodulation and cultivar extension region are present in a single plasmid. We do not know that this is the case for all other field isolates.

3.4 Hybridization with the DNA of different Rhizobium species.

The results of limited hybridization experiments with the DNA of other Rhizobium species and with the DNA of Bradyrhizobium and Agrobacterium are summarized in Table 3.3. Homology was found in R. leguminosarum biovar trifolii although in some cases hybridizing bands were rather weak with strains of the former species (Figure 3.5).

Table 3.3: EcoRI restriction DNA fragments from other Rhizobium species and from Bradyrhizobium and Agrobacterium sp. that hybridize to the nod region probe made from pIJ1095 (pRL5JI).

Species:		Hybridization probes:				
		520	550	560	540	570
		(<u>nodD</u>)	(<u>nodFE</u>)	(<u>nodIJ</u>)	(cv ext)	(cv ext)
<u>R. meliloti</u>						
USDA	1027	4.6	3.0	2.1	-	-
	1030	4.0	-	2.1	-	-
	1066	nt	6.7	2.1	-	-
	1096	3.5	-	2.1	-	-
	1098	2.6	9.9	2.1	-	-
	1171	3.0	9.5	2.1	-	-
<u>R. trifolii</u>						
ANU843		7.0	nt	7.0	2.4	2.4
<u>Bradyrhizobium</u> sp.						
IC3554		-	-	-	-	-
<u>A. tumefaciens</u>						
315		-	-	-	-	-

cv ext. - cultivar extension region.

n.t. - not tested.

Six natural isolates of R. meliloti were used for homology analysis. When EcoRI-treated DNA from these strains was hybridized with pMA520, a homologous fragment was observed from all of them, ranging from 2.6 to 4.6 kb. The pMA550 (nodFE)-hybridizing bands were also found in four out of six strains. The pattern was different depending on the individual strains studied. Interestingly, when plasmid pMA560 (nodIJ) was used as a probe, the hybridizing band appeared to be well conserved in all these strains, being 2.1 kb. There was no homology found with either pMA540 or pMA570 in these strains.

One R. leguminosarum biovar trifolii strain, ANU843, was examined for nod homology. A fragment of 7 kb hybridized with both pMA520 and pMA540, suggesting that genes nodD, I and J are located on the same fragment. Surprisingly, both the cultivar-extension gene probes, pMA540 and pMA570, could hybridize with a 2.4 kb fragment in this strain although the hybridization intensity was not the same as that observed in some R. leguminosarum biovar viciae strains.* In both Bradyrhizobium sp. IC3554 and A. tumefaciens strain 315 the total DNA exhibited no homology to any of the probes used.

: After this thesis was submitted, Cremers et al.^{}, (1988) demonstrated the existence of a new cv. Afghanistan-nodulating gene in R. leguminosarum bv. trifolii ANU843. When Sym plasmid pRL1JI from R. leguminosarum bv. viciae was transferred to wild type ANU843, the resulting strain nodulated cv. Afghanistan pea. This finding, together with my observations, indicates that the 2.4 kb hybridizing fragment contains a functional gene necessary for nodulating cv. Afghanistan.

* J. Plant Physiol. 132: p. 398.

Legend to Figure 3.5; A, B, C and D: Southern blots analysis of the genomic DNA isolated from different Rhizobium species using the subclones of nod regions of pIJ1095 as hybridization probes.

A. Autoradiographs resulting from hybridization of Southern blots with ^{32}P -labelled plasmid pMA520. Lane 1: TOM (control); Lane 2: RmeUSDA1027; Lane 3: RmeUSDA1096; Lane 4: RmeUSDA1030; Lane 5: RmeUSDA1098; Lane 6: RmeUSDA1171; Lane 7: R. leguminosarum biovar trifolii ANU843; Lane 8: Bradyrhizobium IC3553; Lane 9: A. tumefaciens 315.

B. Autoradiographs resulting from hybridization of Southern blots with plasmid pMA550 as a probe. Lane 1: TOM; Lane 2: USDA1027; Lane 3: USDA1066; Lane 4: USDA1098; Lane 5: USDA1171; Lane 6: IC3554; Lane 7: 315.

C. Autographs resulting from hybridization of Southern blots with plasmid pMA560 as a probe. Lane 1: TOM; Lane 2: USDA1027, 1030, 1066, 1096, 1098, 1171; Lane 3: ANU843; Lane 4: IC3554; Lane 5: 315.

D. Autoradiographs resulting from hybridization of Southern blots with plasmid pMA540 as a probe. Lane 1: TOM; Lane 2: ANU843; Lane 3 and 4 indicated no hybridization with Pm strains or IC3554 and 315.

3.5 Discussion.

All of the 56 field isolates studied formed abundant and healthy nodules on three commercial cultivars of pea (cv. Novella, cv. Alaska and cv. Trapper). On the other hand, only 11 of these nodulated cv. Afghanistan. To the best of our knowledge, field isolates that will nodulate cv. Afghanistan but not commercial cultivars have not been found. It is to be noted that the fraction of strains (12/56) nodulating cv. Afghanistan is probably a considerable overestimate of their occurrence in nature. This is because 6 of these 12 strains were acquired as strains already known to nodulate cv. Afghanistan (Jensen *et al.*, 1986; Chetkova *et al.*, 1986). Nevertheless, it is now clear that however rare, such strains are not limited to regions of the Middle East. It is possible that particular local conditions favour the maintenance of such strains and as suggested before (Nash *et al.*, 1988), that such conditions may include legume species or cultivars. This is suggested by the observation that all 3 isolates from *Vicia* nodules in a region of Northwest China could nodulate cv. Afghanistan. Of the remaining strains of this type, five originated from one field in Denmark (Jensen *et al.*, 1986), one from Russia (Chetkova *et al.*, 1986), one from Yugoslavia (U.S. Dep. of Agriculture accession) and one from India. No strains with this phenotype could be isolated from Canada (Province of Ontario) or England. The rare and uneven distribution of strains nodulating cv. Afghanistan has been noted before (Lie, 1978; Kneen *et al.*, 1984; Jensen *et al.*, 1986).

In strain TOM, the first of such strains described to have the extended cultivar specificity (Lie, 1978), nodulation and nodulation specificity determinants are located on a conjugatively transmissible plasmid called pRL5JI (Hombrecher

et al., 1984.)). This is also the case for three of the remaining 11 strains (Danish strains A7, A8 and A10). It may therefore turn out to be a general phenomenon implying that there is the potentiality for the horizontal transfer of these genes.

Different subclones of the nod region of pRL5JI were used as hybridization probes against the total DNA of these strains in an effort to determine whether there was an absolute correlation between the presence of a sequence similar to the 2 kb cultivar extension sequence of pRL5JI and the ability of strains to nodulate cv. Afghanistan. Some potentially interesting observations were made that invite more detailed investigation:

- 1) Two of the strains did not reveal the presence of such a sequence and yet nodulated cv. Afghanistan well; these strains originated from Denmark and from China and their occurrence raises the possibility of alternative mechanisms of extending cultivar specificity;

- 2) The remaining strains that nodulated cv. Afghanistan showed a sequence that hybridized to the cultivar extension region of pRL5JI. They originated from China, Denmark, India, Russia and Yugoslavia. We presume that some of these strains are clonally derived and that others by horizontal plasmid transmission. This is suggested by the absence or presence of polymorphisms in their restriction fragment length (summarized in Table 3.2). For example, all of the Danish isolates (A1 to A10, except A6) and the Russian isolate (USSR A-3) show a similar pattern suggesting clonal derivation. On the other hand, these isolates differ from strain TOM, I9 and the isolate from Yugoslavia (USDA2386).

The difference in hybridization patterns between Danish and Russian strains and strains TOM, I9, and USDA2386 could be due to the genetic rearrangement of the Sym plasmid. In nature, plasmid transfer occurs frequently. In R. leguminosarum biovar phaseoli, different structural changes of the Sym plasmid have been found and related to the conjugative transfer (Soberon-Chavez et al., 1986). Sue Denes found that after transferring of Sym plasmid pRL5JI into different R. leguminosarum biovar viciae strains, it undergoes different rearrangement frequently which leads to different DNA restriction digestion patterns from the parent plasmid, different sizes and in some cases leads to modification of host range (personal communication);

3) One strain from England (JIM1934) exhibited strong homology to the cultivar extension region but it was unable to nodulate cv. Afghanistan. This strain could have a small but significant change in its cultivar extension gene. Alternatively, it may carry a functional gene for nodulating cv. Afghanistan, but its expression is inhibited by the function of another nod gene. This hypothesis is based on the observation that in R. leguminosarum biovar trifolii strain ANU843, a 2.4 kb EcoRI fragment was shown to hybridize to the cultivar extension gene probe of pRL5JI. However, the wild-type ANU843 strain does not nodulate this or any other pea host. Recently, Tn5-insertion mutants have been isolated by Djordjevic et al. (1985) in the nodF and E genes of this same strain. These mutants displayed an extended host range including cv. Afghanistan pea. We would suppose here that like ANU843, in strain JIM1934, the normal expression of nodE or F gene suppresses the function of the cultivar extension gene.

Inspection of Table 3.2 indicates that in all strains tested, nodABC genes are not only well conserved, but also carried on a single EcoR1 fragment. In as many as 17 of these strains, the fragment size is identical to that in strain TOM and the some fragment hybridizes the nodD probe pMA520. These three genes have been detected with a high degree of homology at both nucleotide and amino acid sequence levels even in different Rhizobium species (Gottfert et al., 1986; Rossen et al., 1984; Schofield et al., 1986). The smallest hybridizing fragment detected with probe pMA510 is about 2.5 kb, comparable to the size of the nodABC region in R. leguminosarum biovar viciae pRL1JI (Johnson et al., 1984). This might imply that the length of the coding region for any one of these three genes in different field isolates is more or less the same as that of pRL1JI.

The nodD sequences of pRL5JI also exhibited strong homology with these tested R. leguminosarum field isolates. In most strains, a single hybridizing band was observed. This result may be because, unlike some other Rhizobium species, it has been observed that there is only one copy of the nodD sequence in R. leguminosarum biovar viciae (Rodriguez-Quinones et al., 1987). Interestingly, in seven out of 41 strains, two hybridizing bands were detected. This may represent the reiteration of nodD gene in these strains as has been observed in other Rhizobium species (three copies of nodD genes in R. meliloti; two copies in R. leguminosarum biovar trifolii). Alternatively, it may represent a single nodD gene with an EcoR1 restriction site splitting the sequences into two fragments. To test these two possibilities, more experiments need to be done. One can clone the two hybridizing fragments separately and test their nod functions. If the two fragments indeed represent two copies of nodD, either should be functional; if they are the result of internal EcoR1 digestion

of one gene, neither subclone is expected to be functional. pMA550 was inferred to carry nodFE genes. All strains showed extensive hybridization to this probe. 19 strains showed a hybridization fragment identical to that displayed by strain TOM. This might imply that the molecular structure of nodFE genes in these strains are also identical to that in TOM. The fragment that hybridize in the remaining strains show considerable polymorphism, especially among the strains that do not nodulate cv. Afghanistan. Hybridization with pMA560 (containing the nod(I)J sequence) indicates that these two genes are present in all strains tested. In almost all cases, there is a single hybridizing band, implying that nod(I)J is present in a single copy in these strains. Strain USDA 2429 is an exception. The nod(I)J region in this strain is characterized by two hybridization fragments. The meaning of this needs to be further explored.

All nod genes of pRL5JI used for sequence homology analysis except the host-extension gene are clearly present in all R. leguminosarum biovar viciae strains tested. This is not surprising if we consider that commercial R. leguminosarum biovar viciae tends to have common legume hosts. On the other hand, they showed so much divergence in DNA hybridization patterns that restriction site maps corresponding to these patterns can not be deduced from the known map of pRL5JI. Divergent nod hybridization patterns in these field isolates may reflect the differences in the fine structure and organization of individual nod genes or the regions that contain them. In nature, such diversity in terms of structure and organization of nod genes might ensure the virulence, competitiveness and effectiveness of a given strain that harbour them in entering into a successful symbiosis with local leguminous hosts. Our observations tend to support this reasoning. The degree of diversity is less in strains

isolated from the same geographical locations, implying that uniform or closely-related nod patterns may represent the most effective bacterial strains in nodulating the plants cultivated or grown in a given environment. For instance, four out of five strains isolated from the same geographic region in India showed identical nod hybridization patterns. The same is true of all Danish strains except A6. On the other hand, even without comparing the cultivar extension gene, there is a significant difference in the hybridization pattern of other nod genes between the Indian and Danish strains.

MAJOR CONCLUSIONS

1. Analysis of the nod region of sym plasmid pRL5JI of strain TOM of R. leguminosarum biovar viciae present in the cosmid pIJ1095 by transposon mutagenesis, subcloning and complementation indicated that the overall structural and functional organization of the genes in this region are similar to those in pRL1JI except that pRL5JI has an additional region of about 2 kb that is absent in pRL1JI.

The presence and expression of a gene from this region is necessary for the ability of the strain to nodulate the primitive pea cv. Afghanistan. Additionally and as suggested by the mutant phenotypes, there are detectable differences in the nodD and nodJ genes of the respective plasmids.

2. In an in vivo coupled transcription-translation system of E. coli, the presence of this 2 kb region leads to the production of a single additional polypeptide of molecular weight 15.5 - 16.5 kd. A sequence that could code for this polypeptide was identified. It is contained but is not identical to a sequence proposed earlier (Davis et al. 1988).
3. Field isolates with the ability to form nodules on cv. Afghanistan are not geographically limited to the Middle East. They are however rare and may be geographically limited.
4. In some, but not all of this class of strains, a region homologous to the 2 kb cultivar extension region

is present and presumably is functional. In one rare strain it is present (as detected by hybridization) but not functional.

5. Analysis of polymorphism in restriction fragment length suggests that strains from the same geographical region vary less than strains from different geographical regions. There is also relatively less polymorphism among the strains that nodulate both primitive and commercial pea cultivars. There is evidence that some strains from different geographical regions may be clonally derived and that others have arisen by horizontal transmission.

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Appendix 1: Derivatives of bacterial strains constructed in this study.

Strain:	Derivation and relevant characteristics:	Reference:
<u>R. leguminosarum</u> biovar <u>viciae</u> :		
WA 600	Rif ^r derivative of strain TOM	This study
WA 701	Rif ^r derivative of Danish strain A1	This study
WA 702	Rif ^r derivative of Danish strain A6	This study
WA 702	Rif ^r and Sm ^r derivative of Danish strain A7	This study
WA 704	Sp ^r derivative of Danish strain A8	This study
WA 705	Rif ^r derivative of Danish strain A10	This study
WA 706	Cm ^r derivative of strain USSR A-3	This study
WA 707	Sp ^r derivative of strain D38	This study
WA 708	Sp ^r derivative of strain T14	This study
WA 709	Cm ^r derivative of strain D52	This study
WA 710	Rif ^r derivative of strain USDA 2386	This study
WA 711	Sp ^r derivative of strain 1934	This study
WA 712	Rif ^r derivative of strain 19	This study
WA 713	Rif ^r derivative of strain USDA 2478	This study
B151	128C53 cured of its Sym plasmid, Sm ^r	Brewin et al., (1982)
WA801	Sp ^r derivative of B151	This study
WA8011	WA801 containing pIJ1095, <u>nod2::TnphoA</u>	This study
	<u>nod18::TnphoA</u>	This study
WA8012	<u>nod24::TnphoA</u>	This study
WA8013	<u>nod27::TnphoA</u>	This study
WA8014	<u>nod28::TnphoA</u>	This study
WA8015	<u>nod29::TnphoA</u>	This study
WA8016	<u>nod37::TnphoA</u>	This study
WA8017	<u>nod42::TnphoA</u>	This study
WA8018	<u>nod44::TnphoA</u>	This study
WA8019	<u>nod51::TnphoA</u>	This study
WA8020	<u>nod57::TnphoA</u>	This study
WA8021	<u>nod58::TnphoA</u>	This study
WA8022	<u>nod63::TnphoA</u>	This study
WA8023	<u>nod66::TnphoA</u>	This study
WA8024	<u>nod78::TnphoA</u>	This study
WA8025	<u>nod81::TnphoA</u>	This study
WA8026	<u>nod87::TnphoA</u>	This study
WA8027	<u>nod94::TnphoA</u>	This study
WA8028	<u>nod99::TnphoA</u>	This study
WA8029	<u>nod100::TnphoA</u>	This study
WA8030	<u>nod101::TnphoA</u>	This study
WA8031	<u>nod110::TnphoA</u>	This study
WA8032	<u>nod115::TnphoA</u>	This study
WA8033	<u>nod117::TnphoA</u>	This study
WA8034		This study

(continued...)

(... continued)

Strain:	Derivation and relevant characteristics:	Reference:
<u>R. leguminosarum</u> biovar <u>phaseoli</u>		
8401	strain 8002, cured of its Sym plasmid, Sm ^r	Lamb <i>et al.</i> , (1982)
WA8402	8401(<u>nod2</u> ::TnphoA) containing pMA525	This study
WA8405	8401 containing pMA500	This study
WA8418	8401(<u>nod18</u> ::TnphoA) containing pMA500	This study
WA8424	8401(<u>nod24</u> ::TnphoA) containing pMA525	This study
WA8429	8401(<u>nod29</u> ::TnphoA) containing pMA525	This study
WA8444	8401(<u>nod44</u> ::TnphoA) containing pMA500	This study
WA8458	8401(<u>nod58</u> ::TnphoA) containing pMA500	This study
WA8463	8401(<u>nod63</u> ::TnphoA) containing pMA500	This study
WA8499	8401(<u>nod99</u> ::TnphoA) containing pMA550	This study
WA84117	8401(<u>nod117</u> ::TnphoA) containing pMA550	This study
5535	8401 containing Tn1831-marked <u>R. leguminosarum</u> biovar <u>viciae</u> Sym plasmid pRL1JI	This study
	5535 containing <u>nod 14</u> ::TnphoA	
WA5650	5535 containing <u>nod 120</u> ::TnphoA	This study
WA5651	5535 containing <u>nod 121</u> ::TnphoA	This study
WA5652	5535 containing <u>nod 122</u> ::TnphoA	This study
WA5653	5535 containing <u>nod 124</u> ::TnphoA	This study
WA5654	8401 containing pRL1JI <u>nodB</u> ::Tn5	This study
8401(<u>nod41</u>)	8401 (<u>nod41</u>) containing pMA500	A.W.B. Johnston
WA6250	8401 (<u>nod41</u>) containing pMA510	This study
WA6251	8401 (<u>nod41</u>) containing pMA520	This study
WA6252	8401 (<u>nod41</u>) containing pMA530	This study
WA6253	8401 containing pRL1JI <u>nodC</u> ::Tn5	This study
8401(<u>nod128</u>)	8401 (<u>nod128</u>) containing pMA500	A.W.B. Johnston
WA6260	8401 (<u>nod128</u>) containing pMA510	This study
WA6261	8401 containing pRL1JI <u>nodD</u> ::Tn5	This study
8401(<u>nod32</u>)	8401 (<u>nod32</u>) containing pMA500	A.W.B. Johnston
WA6270	8401 (<u>nod32</u>) containing pMA520	This study
WA6271	8401 containing pRL1JI <u>nodJ</u> ::Tn5	This study
8401(<u>nod29</u>)	8401 (<u>nod29</u>) containing pMA500	A.W.B. Johnston
WA6280	8401 (<u>nod29</u>) containing pMA530	This study
WA6281		This study

(continued...)

(... continued)

Strain:	Derivation and relevant characteristics:	Reference:
<u>E. coli</u> strains		
WA400	HB101 containing pMA100	This study
WA401	CSR603 containing pMA215	This study
WA402	HB101 containing pMA500	This study
WA403	HB101 containing pMA510	This study
WA404	HB101 containing pMA520	This study
WA405	HB101 containing pMA521	This study
WA406	HB101 containing pMA525	This study
WA407	CC118 containing pMA530	This study
WA408	HB101 containing pMA535	This study
WA409	HB101 containing pMA540	This study
WA410	HB101 containing pMA545	This study
WA411	HB101 containing pMA550	This study
WA412	HB101 containing pMA560	This study
WA413	HB101 containing pMA570	This study
WA414	HB101 containing pMA580	This study
WA415	HB101 containing pMA585	This study
WA416	JM103 containing pMA600	This study
WA417	JM103 containing pMA605	This study

Appendix 2: Restriction enzyme analysis of nodulation region (present in pIJ1095) of Sym plasmid pRL5JI and the physical mapping of TnphoA insertions on the nod region of pIJ1095

Panels A through D were selected examples to show the the principles of mapping the restriction cleavage sites of the nodulation region of pIJ1095. Panel A showed the restriction cleavage patterns of pIJ1095 by different enzymes: lane 1, the digestion products of DNA produced by cleavage with Hind III (fragment size marker); lane 2, the digests of pIJ1095 by SstI; lane 3, the digests of pIJ1095 by EcoRI; lane 4, the digests of pIJ1095 by a double digestion with EcoRI and SstI. The difference in fragment number and size were seen between lane 2 and lane 3. The double digestion was attempted to define the relative cleavage positions of the two enzymes. However, in this example, it is hard to define their positions because too many new fragments were generated.

Panel B showed the restriction cleavage patterns of the isolated 7 kb SstI insert DNA (from a subclone, pMA530) by a double digestion with EcoRI and SstI: lane 1, the the SstI digests of pIJ1095 DNA (control); lane 2, the SstI digests of pMA530 clone; lane 3, the digests of pMA 530 by a double cleavage with EcoRI and SstI. The generation of the two fragments (4.3+2.7 kb) revealed the presence of a single EcoRI site in the cloned fragment.

Panel C showed the restriction cleavage patterns of the isolated 4.5 kb EcoRI insert DNA (from clone pMA500) by a double digestion with EcoRI and SstI: lane 1, the EcoRI digests of pMA500; lane 2, the digests of pMA500 by a double cleavage with EcoRnI and SstI. The generation of the two fragments (2.7+1.8 kb) revealed the presence of

a single SstI site in the cloned fragment.

Panel D showed the restriction cleavage patterns of the isolated 6.8 kb insert DNA (pBR 328 used as a vector) by a double digestion with EcoRI and SstI: lane 1, the EcoRI digests of the clone; lane 2, the digests of the same clone by a double cleavage with EcoRI and SstI. The generation of the two fragments (4.3+2.5 kb) revealed the presence of a single SstI site in this fragment.

As shown in Panels B and C, a 2.7 kb overlapping DNA fragment was generated from a single cut of both 4.5 kb EcoRI fragment and 7 kb SstI fragment. This allowed the fragments to be positioned with respect to each other. As shown in Panels B and D, the relative positions of the fragments generated by a single cut from 7 kb SstI fragment and 6.8 kb EcoRI fragment was determined by the use of a 4.3 kb overlapping fragment.

Panels E and F were selected examples to show the physical mapping of TnphoA insertions in pIJ1095 nod::TnphoA and pMA530::TnphoA. Panel E showed the restriction cleavage patterns of pIJ1095 nod::TnphoA DNA by EcoRI: lane 1, the EcoRI digests of pIJ1095 (control); lane 2, the EcoRI digests of pIJ1095 nod44::TnphoA DNA. The absence of a 4.5 kb EcoRI fragment in lane 2 (compared to lane 1) implied the approximate position of TnphoA on this 4.5 kb nod region. For the further detailed mapping of TnphoA location on this 4.5 kb region, other restriction enzymes were used (data not shown).

Panel F showed the restriction cleavage patterns of pMA530::TnphoA DNA: lane 1, the HindIII digests of λ DNA; lane 2, the double digests of pMA530 DNA by EcoRI and SstI; lane 3, the SstI digests of pMA530; lane 4, the SstI digests of pMA530 nod::TnphoA DNA (two bands were migrated closely,

the upper band representnig a new band); lane 5, the EcoRI + SstI digests of pMA530 nod121::Tnp_{hoA} DNA; The absence of 7 kb fragment in lane 4 implied the insertion of Tnp_{hoA} on this region; the absence of 4.3 kb fragment in lane 5 (compared to lane 2) further suggested that Tnp_{hoA} was located within the 4.3 kb EcoRI + SstI region of the cloned 7 kb fragment in this mutants. The mapping data with other enzymes are not shown.

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