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THE USE OF LAC FUSIONS TO ANAYLSE THE REGULATION OF A NOD GENE REGION OF RHIZOBIUM LOTI.

A thesis presented in partial fulfilment of the requirements for the Degree of Masters of Science in Genetics at Massey University, Palmerston North, New Zealand.

MICHELLE ANN GILLMAN

1990

I dedicate this Thesis to my parents.
Thankyou for your love and support.

ACKNOWLEDGEMENTS

I wish to thank Professor Barry Scott for his advice, patience, and supervision, and for providing the facilities which enabled this study to be possible.

I also which to thank the following members of the Molecular Genetics Unit; Dr Eva Rockman for her advice and patience, Carolyn Young for her unending patience, advice and encouragement for which I am indebted to, and all the following poeple for their encouragement, advice and friendship, for which I am forever grateful: Dr Eric Terzaghi, Grant Hotter, David M^{c}Sweeney, Robert Hickson, Julie Emerson, Mike O'Hara, Trish M^{c}Lenachan, Ruth Appleby, Lawrence Ward, George Ionas, Harshani Gunasena, and Sharon Sims. I also wish to thank Laura Medhurst for her help with the construction of this thesis.

Thanks to my friends, and flatmates for your support and encouragement. Special thanks goes to Tina Thomas, Andrea Ede and especially Murray Sarelius for your love and encouragement - Thank you guys!

To my family thank you for your support and love, it is as always appreciated.

Also a special thanks to all the people within the Microbiology and Genetics Department who have encouraged me through their "everyday" happiness.

ABSTRACT

Two approaches where used in the analysis of common and host specific nod gene expression in Rhizobium loti strains NZP2213 and NZP2037.

The first approach using the Tn3-HoHo1 transposon to generate lacZ transcriptional/translational fusions, produced 290 insertions within the 8.3kb EcoRI nod fragment of R.loti strain NZP2213. The position and orientation of all but one of these insertions was determined using restriction enzyme mapping and hybridisation. The sites of the insertion and orientation were generally found to be random.

The lacZ fusions were transferred into R.loti strain NZP2213 where their β-galactosidase activity was measured in the presence and absence of Lotus tenuis seed exudate. All insertions had a low level of β-galactosidase activity that was the same as the controls. This activity was independent of position or orientation. This lack of expression could be a result of the fusions being in regions that are not transcribed ie not downstream of either a nod inducible or other promoter, or that the appropriate conditions for constitutive or inducible activity were not achieved.

The second approach to construct lacZ transcriptional fusions was less random and involved the cloning of three separate nod gene fragments:
i) a 4.1kb SalI fragment that overlaps the nod region of the 8.3kb EcoRI fragment of R.loti strain NZP2213,
ii) a 0.65kb EcoRI fragment isolated from the 4.1kb SalI fragment of R.loti strain NZP2213, and
iii) a 1.4kb SalI fragment isolated from the 7.1kb EcoRI nod region of R.loti strain NZP2037.

These three fragments (4.1kb, 0.65kb and 1.4kb) were isolated and cloned into pMP190, pMP220 and pMP190 respectively, in both orientations. Each lacZ fusion was transferred into the R.loti strains from which the fragment had originated, ie either NZP2213 or NZP2037. The β-galactosidase activity of these transconjugants was measured in the presence and absence of Lotus tenuis seed exudate.

The 4.1kb SalI construct from R.loti NZP2213 was found to have consititutive activity in both orientations indicating that at least two consititutive promoters are located on this fragment. The activity of one orientation, corresponding to pPN38, was twice that of the reverse orientation corresponding to pPN37.

The smaller 0.65kb EcoRI fragment, that lies within the larger 4.1kb SalI fragment, contains a "nod box" and part of a nodD-like gene (Scott et al., In prep.). No significant β-galactosidase activity was observed in either orientation with or without seed extract. These experiments showed that the "nod box" alone was insufficient for plant inducible expression.

The 1.4kb SalI fragment from R.loti NZP2037, that was known to contain a nodA promoter (Emerson-Colins et al., pers. comm.) showed inducible expression for pPN39, corresponding to a fusion between nodA and lacZ. No significant activity was detected in the reverse orientation, pPN40, either with or without plant exudate.

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ABBREVIATIONS

Kan	Kanamycin
Tet	Tetracycline
Amp	Ampicillin
Cam	Chloramphenicol
Str	Streptomycin
kb	kilobase
SDS	Sodium dodecyl sulphate
Nod+Fix+	Rhizobium phenotype characterised by the ability to induce visible nodules (Nod = nodulation) on plant roots which are capable of nitrogen fixation (Fix = Nitrogen Fixation).
Nod+Fix-	Rhizobium phenotype characterised by the ability to induce visible nodules on plant roots which are not capable of nitrogen fixation.

Abbreviations not listed are "accepted" abbreviations.
(Biochemical Journal (1983) 209: 1-27).

Chapter 1 - INTRODUCTION

1.1) SYMBIOSIS.

1.1.1 The Biology of Nitrogen Fixation.

Nitrogen fixation is the process by which gaseous nitrogen from the air is made available for incorporation into organic compounds, and thereby it is brought back into circulation in the nitrogen cycle. Nitrogen fixation can be carried out by only certain bacteria (Raven et al., 1981).

Of the various classes of nitrogen fixing organisms (including approximately one quarter of all blue-green algae species), the symbiotic bacteria are the most important in terms of the amount of nitrogen fixed. The most common genus of nitrogen fixing bacteria is Rhizobium (Rost et al., 1979; Raven et al., 1981).

The genus Rhizobium is of considerable importance because it is responsible for the formation of nitrogen fixing nodules on leguminous plants. The establishment of the Legume-Rhizobium symbiosis involves infection of the host root and the subsequent formation of nodular growths, containing approximately equal masses of root and bacterial cells (Bauer, 1981). As a result, legumes such as alfalfa, soya, peas, beans, lentils, clover, and others may be grown with no requirements for nitrogenous fertilizers.

Worldwide, Rhizobium species reduce about 20×10^6 tonnes of atmospheric nitrogen to ammonia by the nitrogenase catalyzed reaction;

$$3H_2 + N_2 = 2NH_3$$

This reaction converts nitrogen into a form, NH_3, that can be utilized by organisms for growth (reviewed by Beringer et al., 1980).

The beneficial effects on the soil that are derived from growing leguminous plants have been recognised for centuries. The ancient Greeks used broad beans to enrich the soil (Rost et al., 1979).

It was found that where leguminous plants are grown, nitrogen can be released into the soil, becoming available for other plants. But also some detrimental effects can be observed, such as removal of nitrogen from the soil.

In New Zealand, research into the Rhizobium-Legume symbiosis is predominantly on pasture legumes. Research is aimed at making better use of the symbiosis, and in particular should open the way for understanding how bacteria interact with plants, leading to new possibilities for manipulating bacteria-plant associations and help to understand differences between pathogens and symbionts (reviewed by Robertson & Farnden, 1980).

Two of the major problems in agriculture in New Zealand include;
i) loss of symbiotic traits such as infectiveness and effectiveness, and
ii) introduced strains are not competitive with indigenous strains.

1.1.2 Legume-Rhizobium Symbiosis

1.1.2.1 Leguminosae

The family Leguminosae contains approximately 700 genera in several subfamilies. About 1200 of the 12,000-14,000 known species have been tested for nodulation, about 90% of those in the subfamilies Mimosoideae and Papilionideae and 30% of the Caesalioiideae were nodulated (reviewed by Beringer et al., 1979). The Mimosoideae and the Caesalpinoidae consist mostly of trees and shrubs. The Papilionoideae is also called the Lotoideae or bean subfamily because of its composition (reviewed by Robertson & Farnden, 1980).

Although rhizobia nodulate only legumes (with the exception of Parasponia of the family Ulmaceae), it is not a general property of the Leguminosae to form a nitrogen fixing symbiotic interaction with rhizobia.

Because members of all these subfamilies are nodulated, the symbiotic association probably arose in the early evolution of these plants, with most of the genera and species that have lost the ability to form an association arising after the divergence of the three subfamilies. Although nodulation is not essential for legume growth it has selective advantages, although this depends on the nitrogen content of the soil (reviewed by Beringer et al., 1979).

1.1.2.2 Lotus Species in Agriculture

The genus Lotus is represented in New Zealand by five species; Lotus pedunculatus cav, L.corniculatus L, L.angustissimus L, L.hispidus Dest and L.tenuis (Pankhurst et al., 1979).

Of these Lotus species there are two which are agriculturally important in New Zealand: Lotus pedunculatus cav (big trefoil) and;
Lotus corniculatus L (bird's foot trefoil).

Rhizobium strains that nodulate these hosts fall into 2 groups; R.loti (fast growers) and Bradyrhizobium sp.(Lotus) (slow growers). Within the R.loti group some strains are effective on both hosts whereas others are ineffective on L.pedunculatus (Pankhurst et al., 1979 and, Pankhurst and Jones, 1979b).

1.1.2.3 Lotus rhizobia

Micro-organisms of the genus Rhizobium are aerobic, gram negative soil bacteria (reviewed by Robertson & Farnden, 1980).

The recognition of six Rhizobium species is based on the work of Fred et al. (1932), who introduced the idea of cross inoculation groups with species in the family Leguminosae.

However, the assumption that each species of Rhizobium nodulates only plants within a specified cross-inoculation group has lost some credibility, as the strains within each group vary considerably in their basic biochemical and physiological properties. The ability of these very different strains to nodulate the same host presumably arose as a consequence of plasmid transfer of nodulation ability. Recently it has been shown that even different genera are able to nodulate the same plant.

To account for these differences rhizobia have now been classed into two genera;

1) The fast growing Rhizobium species;
2) The slow growing Bradyrhizobium species (Jordan, 1982).

1.2) NODULATION.

1.2.1 The Biology of Nodulation.

1.2.1.1 The Process of Nodulation.

Nitrogen fixation in the root nodules of legumes requires specific interactions between plants and compatible rhizobia (Bisseling et al., 1986).

The rhizobia which invade temperate legumes tend to exhibit a narrow plant host range. In contrast, Rhizobium strains that infect tropical legumes tend to exhibit a broader host range (Rolfe, 1984).

Serological analysis of rhizobia has shown that individual nodules generally contain only one Rhizobium strain, although adjacent nodules on a plant, given a mixed inoculum, may contain different strains. Specificity in Legume-Rhizobium interactions may be exerted at several different levels;

i) By control of rhizobial growth in the rhizosphere of the legumes; an effect of low specificity which is controlled by both partners in the symbiosis.

ii) By controlling rhizobial binding to root hairs. Specificity in this reaction would vary depending on the nature of the substance(s) responsible for mediating binding.

iii) Through root-hair curling; an effect of high specificity that is controlled by both symbiotic partners and;

iv) Through root-hair penetration and infection thread formation; a process which is normally quite selective (reviewed by Broughton, 1978).

1.2.1.2 Rhizosphere Effects.

Adaption of a micro-organism to a developing rhizosphere may begin as early as the time of seed germination by the plant partner. As the root develops and extends in contact with soil, soil micro-organisms in the immediate area respond selectively, so that growth of some is enhanced (Schmidt, 1979). Although it is well known that root exudates promote growth of rhizobia, little is known about the nature of the chemical interactions. Recently Murphy et al. (1987) have shown that there may be a mechanism whereby rhizobia gain a competitive advantage. This may be through the use of substances such as rhizopines which benefit the rhizobial system but not any other bacteria. This situation is analogous to the production of opines in the crown gall tissue following invasion by Agrobacterium tumefaciens. Another report by Triplett (1988) has also shown that competitiveness may result from the production of substances such as trifolitoxins, which are potent bacteriocides to other rhizobia (Triplett, 1988).

1.2.1.3 Attachment.

After establishment in the rhizosphere, rhizobia attach themselves to the root hair surface, particularly near the tips. It appears that some moiety on the root hair surface and the same or complementary compound on rhizobia is responsible for joining the two symbiotic partners. The exact nature of these compounds is unknown, but it has been proposed that lectins and cellulose fibrils may have some role (Reviewed by Broughton, 1978).

Rhizobia must be in close contact with the host cell in order to exert the specific morphological responses that are required for multiplication of the bacteria. It seems that the initial binding of the rhizobia to the host plant surface is rather loose and probably the result of non-specific ionic interactions. Tight irreversible binding, however, may only occur in certain compatible interactions that lead to nodulation.

Tight binding of rhizobia to the surface of the root hairs appears to be accompanied by the formation of cellulose fibrils which apparently help anchor the bacteria to the surface of the host cell. Since rhizobia appear to attach in an end-on fashion, it must be assumed that the cellulose fibrils are produced at one pole (Sequira, 1978).

There is considerable evidence for polar attachment of rod shaped rhizobia to various surfaces, including host roots. It seems likely that polar attachment is mediated by "Polar Bodies". The biochemical nature of these polar bodies and the environmental conditions that govern their formation and loss have not yet been ascertained.

The capsules of rhizobia, which are probably distinct both chemically and morphologically from polar bodies, may also be instrumental in attachment of the rhizobia to the root hairs, along with the polysaccharides (Bauer, 1981).

1.2.1.4 Polysaccharides.

Bacteria of the genus Rhizobium are Gram negative, hence they have the usual polysaccharides consisting of extracellular, capsular, and lipopolysaccharides (Carlson et al., 1987). Surface polysaccharides of the bacteria act as immuno-chemical determinants, lectin receptors, phage receptors as well as involvement in many other interactions of the cell and its environment (Ugalde et al., 1986).

It has been proposed that polysaccharides play a role in the symbiotic infection process (Carlson et al., 1987). Exopolysaccharide (Finan et al., 1985; Leigh et al., 1985), lipopolysaccharide (Noel et al., 1986), cellulose and βD(1-2) glucan (Dylan et al., 1986) have all been implicated (Zorreguieta and Ugalde, 1986).

Leigh et al. (1985) isolated an extensive set of exopolysaccharide deficient (Exo^-) mutants of R. meliloti that were genetically diverse but phenotypically homogeneous (ie. Nod^+ Inf^- and Calcofluor dark). These results suggest that the R.meliloti acidic calcofluor binding EPS is involved in the formation of an effective nodule (Leigh et al., 1985). Finan et al. (1986), on the basis of resistance to several phages and complementation analysis with cloned DNA, have divided the Exo mutants into 7 phenotypic groups (exoA to exoF and exoH). They showed that the loci for two of the groups (exoC & exoD) are chromosomal, the remaining five (exoA, B, E, F, & H) are located on a megaplasmid (Finan et al., 1986).

Other workers using random Tn5 mutagenesis isolated mutants of R.leguminosarum bv phaseoli that also formed empty nodules, but these mutants had altered lipopolysaccharide (LPS) rather than exopolysaccharide (Noel et al., 1986).

A third group, Dylan et al. (1986), isolated two sets of genes from R.meliloti that are required relatively early in the symbiotic process. Because mutations in these genes lead to abnormal nodule development they were designated ndv genes (Dylan et al., 1986). Geremia et al. (1987) found that the ndv mutants of R.melioti are equivalent to the chvB mutants of A.tumefaciens; both loci being involved in the synthesis of β(1-2) glucans.

Another polysaccharide proposed to be involved in the infection is the teichoic acid fraction. Geremia et al. (1987) found that R.meliloti mutants selected for phage resistance lost competitiveness and the ability to form this polysaccharide. Further work is needed to clarify the role of each of these polysaccharides in the infection process.

1.2.1.5 Susceptibility to Infection.

A study of soybean nodule formation showed that nodules fail to develop in the zone where mature (fully elongated) root hairs were present at the time of inoculation. Nodules formed occasionally in the zone where developing root hairs were present at inoculation, but the most frequent nodulation occurred in the zone where no root hairs were present at inoculation (Bauer, 1981).

There are two general mechanisms of penetration of legume roots by rhizobia. For most temperate strains, binding of the bacteria at the surface of the root hair is followed by involution of the plant cell wall to form an infection thread containing rod-shaped bacteria. It should be noted that these bacteria are not truly intracellular because they are enclosed in an invagination of the plant cell wall (reviewed by Beringer et al., 1979).

The rhizobia then penetrate via the infection thread into the cortex and induce dedifferentiation of the cortical cells resulting in the formation of meristematic tissue from which the root nodule grows (Callaham and Torrey, 1981; Bisseling et al., 1986). Induction of cortical cell division happens without the need for infection thread formation, or even root hair curling, as shown by using Exo^- strains which induce nodules (Nod^+ Inf^-).

The ability of these mutants to initiate nodule development, yet not invade, suggests that a signal is transferred from the bacteria to the inner cortical cells to initiate cell division.

This signal has recently been identified as a glucosamine tetrasaccharide for R.meliloti (Lerouge et al., 1990).

The alternative mode of infection, the crack entry method, is more common among the tropical Rhizobium species. This mode of entry is characterized by the absence of infection threads passing from one cell to another: instead access is gained by intercellular penetration, particularly where the root epidermis is damaged, for example at the point of emergence of lateral roots. Once bacteria have penetrated the root, the cortical cells are stimulated to divide and the bacteria are taken up by the dividing cells (presumably by involution of the plant cell membrane). Subsequently, intracellular bacteria are dispersed in the developing nodule by the division of root cells (reviewed by Beringer et al., 1979).

For bacteria that invade by infection threads the next step in development is root hair curling.

1.2.1.6 Root Hair Curling.

The root hair curling process is very specific. Four conditions are characteristic of the early stages of the root hair reaction to invasive rhizobia; branching, moderate curling, marked curling (curvature >360 degrees) and the formation of infection threads (reviewed by Broughton, 1978).

A theoretical model of root hair curling has been proposed by Bauer (1981):

A hair emerges from the apical end of an epidermal cell. The flexible α-cell wall layer of the hair bulges outward as the result of turgor pressure against an area of localized removal of the rigid β-layer. The localized removal of the β-layer is presumed to be a consequence of host induced disintegration of the microfibrillar matrix or inhibition of β-layer synthesis. It is also presumed that deposition of new α-layer material is heaviest at the apex of the swelling. As a consequence, any bacterial cell attached to the emerging tip will gradually be displaced from the apex to the edge. The model proposes that curling results from a localized inhibition of the β-layer deposition in the emerging hair, induced by an attached cell. The rigid cylinder of β-layer material thus does not develop past the attached rhizobia but continues to be deposited inside the α-layer opposite the attached rhizobia. As the hair continues to elongate, the

flexible tip gradually pivots around the attached bacterium, resulting in a tight coil that envelopes the cells. The envelopment and enclosure of attached rhizobia between the cell walls is likely to be a function of root hair curling. The enclosing wall also provides something solid to push against as the developing infection thread progresses inward against the turgor pressure of the root hair cell (Bauer, 1981).

1.2.1.7 Infection Thread Formation.

Infection threads are tubular structures that carry rhizobia from the root surface into the root cortex. Rhizobia are released from the end of the infection thread in host membrane envelopes, establishing the bacterial symbiont in the host cortical cell cytoplasm (although they are still effectively extracellular) (Bauer, 1981).

Initiation of the infection thread is first observed as a swelling and appearance of callose in the host cell wall at the site of infection and an increase in both opacity and cytoplasmic streaming of the associated cytoplasm. The nucleus of the infected root hair cell, which is generally close to the point of initiation of the thread, swells to almost twice the normal size and moves towards the base of the root hair cell just in front of the advancing infection thread tip (reviewed by Robertson & Farnden, 1980).

The infection thread grows through the cells of the root cortex by continued extension of its primary cell walls and division of the enclosed bacteria within a slimy matrix (reviewed by Beringer et al., 1980).

Dense cytoplasm surrounds the tip of the infection thread which grows at a rate of approximately 7μm/hr. If the nucleus moves too far ahead of the infection thread growth stops. Only bacteria in the tip of the thread continue to divide, presumably because the growth of the tip allows bacterial cell division to continue, while those behind the tip are constrained by the walls of the infection thread.

During the period of growth of the infection thread through 3 to 6 layers of root outer cortex cells, meristematic activity is initiated in a small group of root cortical cells directly in front of the tip of the infection thread. Growth of the infection thread continues into this meristematic region where rhizobia are released from the tip into the inner most meristematic cells.

The cells of the meristematic zone (which are infected by rhizobia) in indeterminate nodules are predominantly polyploid (reviewed by Robertson & Farnden, 1980).

Rhizobia are released from the tips of the infection thread by endocytosis and are surrounded by envelopes of host plasma membrane (Bauer, 1981), the so called peribacterioid membrane.

After the rhizobia have been released from the tip of the infection thread, although still surrounded by a membrane of plant origin, they continue to divide until the cytoplasm of the plant cell is filled with bacteroids (Reviewed by Robertson & Farnden, 1980).

Nodules are of two types; determinate and indeterminate.

In indeterminate nodules (eg clover and alfalfa) infection threads continue to penetrate the cortical cells in the nodule meristem, and thus provide a continuous release of rhizobia into the plant cells, as the nodule increases in size.

In determinate nodules infection threads are a transient feature of nodule development and an increase in nodule size is by the division of a few cortical cells containing rhizobia (reviewed by Beringer et al., 1979).

1.2.2 Bacteroid Differentiation.

The rod shaped rhizobia differentiate into bacteroids when released from infection threads and surrounded by a peribacteroid membrane. In some cases they divide further before they swell and differentiate. Their size and shape is largely determined by the plant (reviewed by Sutton et al., 1981).

For some rhizobial species, such as R.leguminosarum and R.trifolii differentiation involves an increase in volume of up to 40 fold and significant changes in morphology, while bacteroids of other species, such as B.japonicum, are morphologically very similar to free-living bacteria (reviewed by Beringer et al., 1979)

When discussing bacteroids, it is convenient to recognise three developmental stages;

i) Immature bacteroids which lack nitrogenase activity and are present in nodule tissue that has not yet produced significant quantities of leghaemoglobin. Immature bacteroids depend on the plant cytoplasm for energy and combined nitrogen.

ii) Mature bacteroids are characterized by high nitrogenase activity and are normally found in tissue with high leghaemoglobin content. Mature bacteroids depend on the plant cytoplasm for energy, but they excrete substantial quantities of combined nitrogen in the form of ammonia.

iii) Senescent bacteroids represent the terminal stage of nodule symbiosis when nitrogenase activity and leghaemoglobin content decline and the peribacteroid membrane disintegrates (Reviewed by Sutton et al., 1981).

Bacteroid differentiation is accompanied by a switch from aerobic to micro-aerobic metabolism, resulting in the shut down of many aerobic pathways and induction of proteins specifically required for nitrogen fixation, ie. synthesis of nitrogenase and new electron transport components.

A continuous supply of metabolic energy and the protection of the oxygen-labile nitrogenase enzyme system are two main requirements for the reduction of nitrogen. Nitrogen is reduced to ammonia (by nitrogenase) which is excreted by bacteriods into the cytoplasm of the plant where the ammonia is assimilated into glutamate and glutamine, which are subsequently converted into translocatable products. Nitrogen compounds exported from the nodule can be divided into two groups; the ureides and the asparagine type. The ureides, allantoin and allantoic acid, are the primary nitrogen containing compounds exported from soybean, cowpea and bean nodules. Other legumes export fixed nitrogen as amides, such as asparagine and glutamine (reviewed by Verma & Long, 1983).

1.2.3 Nodule Cytosol Metabolism.

At least 30 polypeptides are specifically synthesised in root nodules by the plant. The predominant nodule specific proteins are the leghaemoglobins, which comprise up to 20% of the total proteins in the nodule (Stougaard et al., 1987). The leghaemoglobins

are produced in large amounts, giving the root nodules a characteristic red-brown colour, but in L.pedunculatus this colour is due not to leghaemoglobins but to flavolans (reviewed by Bisseling et al., 1986).

The leghaemoglobin present in the host cell cytoplasm plays a role in transport of oxygen by maintaining a sufficiently high $p0_2$ in the host cell cytoplasm for oxidative phosphorylation, while providing a sustained low level of oxygen to the bacteriods (reviewed by Verma & Long, 1983).

1.3) NODULATION GENETICS

1.3.1 Rhizobium Genetics.

1.3.1.1 Location of Genes Essential for Effective Nodulation.

Formation of nitrogen fixing nodules on plant roots is a consequence of expression of symbiotic genes from both rhizobia and the plant.

It is now clear that a number of genes from both partners code for different steps in the nodulation process. Recognition and nodulation induction are controlled by a nod regulon, in the bacterium, that consists of several operons containing both common and host specific nodulation genes, which are all regulated by plant factors.

Development of the symbiosis can be followed by analysing Rhizobium mutants that are defective at various stages of symbiotic nodule formation. Phenotypically, symbiotic mutants fall into two categories; Nod^- Fix^- or Nod^+ Fix^-.

Nod^- mutants fail to form nodules due to blocks prior to visible nodule meristem induction.

Fix^- mutants are inhibited in later steps in that they nodulate the host plant but nitrogen fixation either does not occur at all or it occurs at a very reduced level (Kondorosi & Kondorosi, 1986).

In Agrobacterium and many species of Rhizobium large plasmids have been shown to determine infectivity and host range. However, there is no evidence that part of any Rhizobium plasmid might be transferred to the genome of the host plant in a manner analogous to the T-DNA of Agrobacterium (reviewed by Beringer et al., 1980)

In many Rhizobium species symbiotic genes including nod and nif are located on indigenous plasmids. R.meliloti, for example, harbours 2 megaplasmids each of at least 1000 megadaltons in size. One megaplasmid carries nod, nif and fix genes, the other carries genes for exopolysaccharide synthesis. Further fix genes are located on the bacterial chromosome, as are genes for β-glucan (ndv) and exopolysaccharide (exo) synthesis (Kondorosi & Kondorosi, 1986).

1.3.1.2 Common Nodulation Genes

In the fast growing rhizobial strains such as R.meliloti, R.leguminosarum bv leguminosarum, bv. viciae, bv trifolii and bv phaseoli (which specifically nodulate alfalfa, peas, clover and Phaseolus beans respectively) the nod genes are present on one indigenous (symbiotic) megaplasmid. The nod genes fall within one region of approximately 14kb in R.leguminosarum bv viciae and bv trifolii, and within two regions separated by 12kb in R.meliloti (Gyorgypal et al., 1988).

Mutations in nodA, nodB or nodC of the common nod genes block induction of nodulation and can be complemented by homologous genes from other Rhizobium species. For example, there is a 69-72% nucleotide sequence homology between the nodABC genes of R.leguminosarum and R.meliloti (Torok et al., 1984; Rossen et al., 1984).

In R.leguminosarum six common nod genes, nodABCDI & J, have been located on the symbiotic plasmid pRL1JI (Fig. 1). Genes equivalent to nodDABC have been identified in R.meliloti, and R.trifolii and these strains presumably have nodI and J as well (Rossen et al., 1986).

The biochemical role of the nodABC gene products is not known. The NodC protein has a hydrophobic carboxyl terminal end associated with the bacterial outer membrane. The nodABC genes are in one transcriptional unit which in R.leguminosarum also contains nodI & J (reviewed by Downie & Johnston, 1986). Computer analysis of NodI & J shows that their structure is consistent with their being membrane bound proteins. The predicted polypeptide specified by nodJ is extremely hydrophobic and the nodI product bears a striking resemblance to gene products in enteric bacteria which are involved in the transport of low molecular weight compounds such as phosphates, maltose and histidine.

Given these observations, it is possible that the products of the nodABCIJ genes (which are transcribed as a single unit) form a multiprotein complex associated with the Rhizobium membrane (Rossen et al., 1986).

When wild type Rhizobium strains were inoculated on to their host plants in the presence of antibodies directed against the NodC protein, nodule formation was strongly inhibited (John et al., 1988). These experiments suggest that the NodC protein is located on the cell surface of Rhizobium so that extracellular antibodies would be able to bind to it, thereby causing a reduction in nodulation. Because of its transmembrane location, NodC may play an important role in the signal transduction from bacteria to host plants. The function of the highly conserved NodC protein is essential because mutations within the nodC gene completely abolish root hair curling and nodule formation.

It has also been recently shown that the NodC protein is also present in mature nodules induced by R.meliloti on Medicago sativa. During nodule development the NodC protein appears to be processed to a smaller molecule.

John et al. (1988) have detected the processed NodC in the nodules of various legumes and have shown that the amount of this protein is increased during development (John et al., 1988).

While nodD is classified as a common nod gene on the basis of cross complementation experiments, it also has an important role in the initial stages of host recognition and so is discussed below.

1.3.1.3 Host Specific Nodulation Genes

While nodD plays an important role in the initial stage of host recognition by responding to inducers (flavonoids) from the plant, other sets of genes, the so called host specific nod genes, are then required for signalling back to the plant for root-hair curling and infection to proceed (Gyorgypal et al., 1988; Horvath et al., 1987; Spaink et al., 1987b; Faucher et al., 1988).

Putnoky and Kondorosi (1986) have shown that at least two nod gene regions are involved in the transfer of host specific nodulation. Two nod regions from R.meliloti were identified that would enable R.leguminosarum bv trifolii to nodulate alfalfa; one region carried the hsnABCD (≡nodFEGH) whilst the other contained undefined genes (Putnoki and Kondorosi, 1986; Surin & Downie, 1989).

Davis et al. (1988) have identified an additional gene, nodX, which allows R.leguminosarum bv viciae strain TOM to nodulate both commercial and Afghanistan peas. There is no homologue of this region in normal strains of R.leguminosarum bv viciae, thus the ability to nodulate Afghanistan peas is due to an extra gene or genes. nodX is located in a 2.0kb region of the Sym plasmid pRL5JI downstream from nodJ, as illustrated in Figure 1.

nodX specifies a hydrophobic protein which may be associated with the bacterial membrane. The function of nodX is not known but it is possible that its product forms a complex with the membrane proteins specified by other genes in the nodABCIJX operon (Davis et al., 1988).

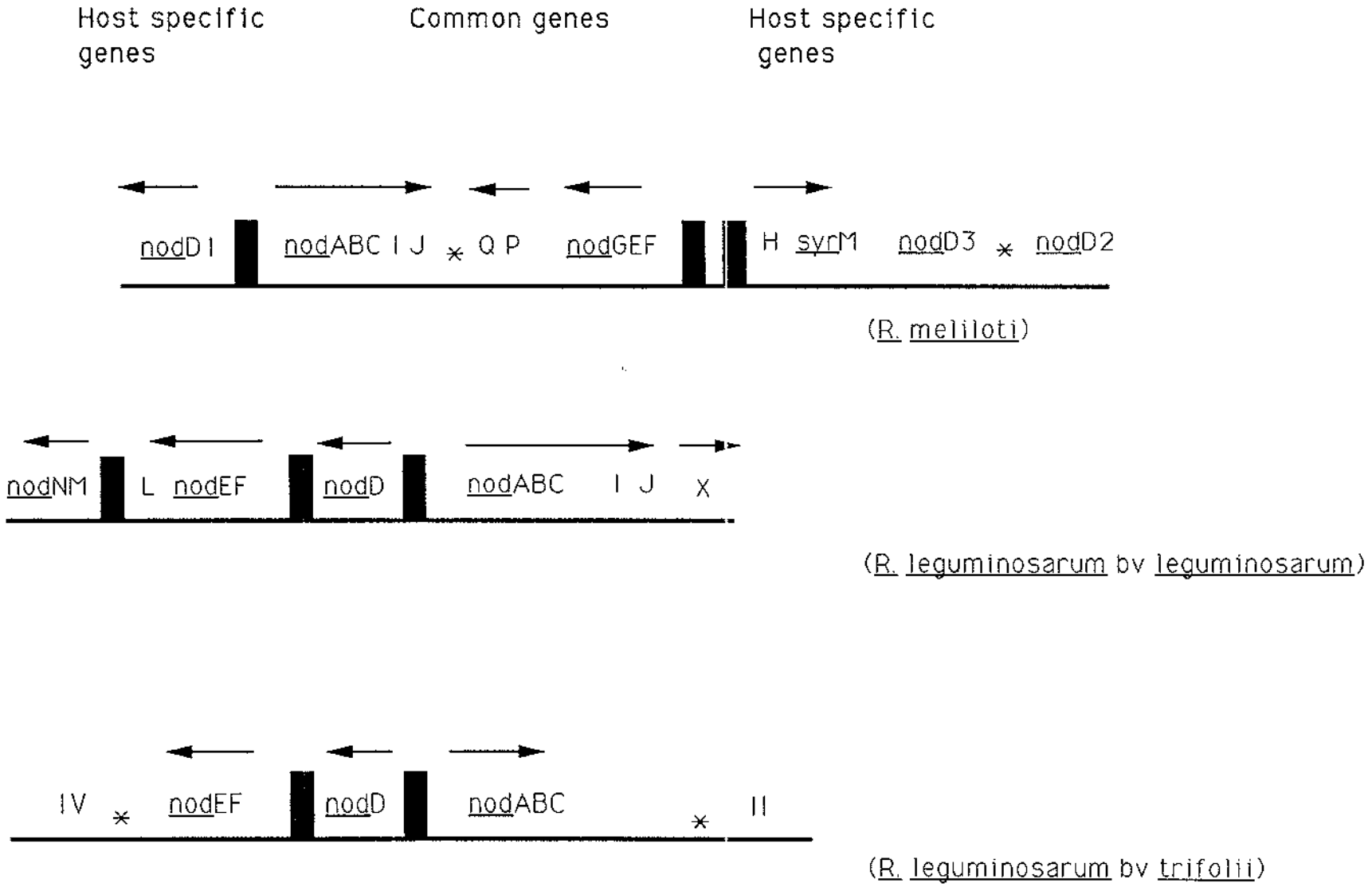
Recent data presented by Surin & Downie (1989) shows that the expression of host specificity in Rhizobium is determined, not by a single gene or operon, but by many of the nod genes that have been identified. Therefore it would appear likely that recognition between Rhizobium and its host legume can occur as a series of parallel events (Surin & Downie, 1989).

It has been determined that the nodFEGH genes are involved in infection thread development induced by R.meliloti, the nodFELMN gene region has also been shown to be involved in infection thread development (Surin & Downie, 1989). Therefore many of the nod genes are important in the determination of host specificity: the nodE plays a key (but not essential) role (Surin & Downie, 1989); nodF resembles acyl carrier proteins and therefore may have a role during symbiosis in LPS or fatty acids biosynthesis (Shearman et al., 1986); the nodH and nodQ genes as well as nodABC genes were shown recently, Lerouge et al. (1990), to be involved in the production of an extracellular Nod signal which controls recognition, infection and nodulation (this signal was identified as a glucosamine tetrasaccharide). The efficiency of transfer of host specific nodulation increases with additional genes such as nodL < nodLMN (Putnoki & Kondorosi, 1986; Surin & Downie, 1989).

In addition to the nodLMN genes, which function in conjunction with nodFEGH, there are other nod gene(s) downstream of the nodC which are involved in host specificity (an assumed 2kb region of DNA immediately downstream of nodJ).

Figure 1. A SUMMARY OF THE ORGANISATION OF **nod** GENES IN **Rhizobium.**

nodP and Q are two open reading frames identified by Long et al. (1988).
nodX is present in R.leguminosarum bv viciae strain TOM (Davis et al., 1988).
The filled in rectangle represents a "nod box" - a conserved sequence upstream of nod operons and the presumed site of binding of NodD.
The arrows represent the direction of transcription.
The asterisk indicates a distance between the nod genes as the diagram is not to scale.
Regions II and IV for biovar trifolii represent additional nodulation regions identified by Tn5 mutagenesis (Djordjevik et al., 1985).


Host specific genes
Common genes
Host specific genes
nodD1
nodABC I J
*
Q P
nodGEF
H syrM
nodD3
*
nodD2
(R. meliloti)
nodNM
L nodEF
nodD
nodABC
I J
X
(R. leguminosarum bv leguminosarum)
IV
*
nodEF
nodD
nodABC
*
II
(R. leguminosarum bv trifolii)

It is now thought that the host specific genes are involved in attaching different side chain groups to the nodulation induction signal molecule. The identification of the R.meliloti signal now allows this idea to be directly tested.

1.3.1.4 NodD Functions

Unlike other nod genes NodD is expressed constitutively. The other nod genes are not expressed unless a plant root factor(s), now identified as a flavonoid(s), is present in the media. This induction does not occur in strains mutated in nodD suggesting that NodD regulates the induction of other nod transcriptional units in response to the specific flavonoids from the host. In R.leguminosarum NodD has been shown to be autoregulated (see Fig. 3) since it inhibits the expression of lacZ in nodD-lacZ fusions (Downie & Johnston, 1986).

The nodD genes of different Rhizobium sp. are conserved and interspecies complementations with nodD genes have been reported. It has recently been shown that nodD genes of different rhizobia can interact with different flavonoids and a role of nodD in host specific nodulation has been demonstrated.

In R.meliloti 3 copies of nodD were detected. The involvement of at least two of these (nodD1 & nodD2) in the nodulation of alfalfa has been shown. Gyorgypal et al. (1988) showed that all three nodD copies present in R.meliloti are involved in nodulation to an extent depending on the specific plant. This suggests that these allelic forms of nodD have evolved to provide R.meliloti with the ability to interact with the divergent flavonoid compounds exuded by the different natural host plants.

Expression of nodulation genes can be influenced by a number of factors; such as the number of nodD genes as well as the flavonoid range, the "nod box", (a conserved sequence found in the promoter region of several nod operons), affinity and expression level of the indigenous nodD alleles. From experimental data it seems that Rhizobium sp MPIK3030, R.leguminosarum, and R.trifolii adapted to their hosts by evolving one nodD allele which is responsive to a broad host range of flavonoids and/or has higher nod gene inducing ability and/or high expression levels. Whereas in R.meliloti the number of nodD alleles has been increased. The natural divergence of the indigenous nodD alleles may have contribute to the ability of Rhizobium to nodulate new hosts during evolution (Gyorgypal et al., 1988).