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.

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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).

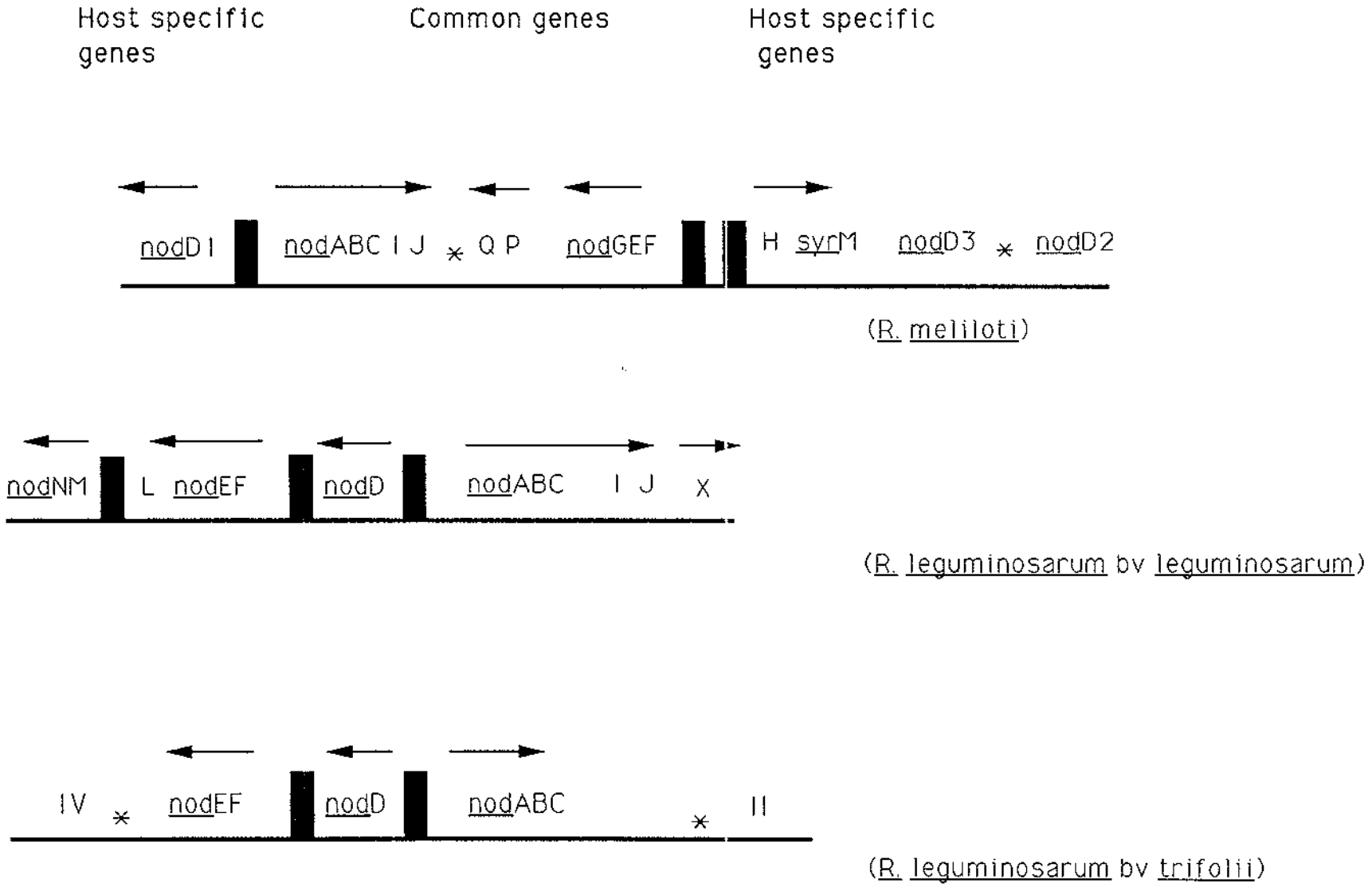
Recent data presented by Surin & Downie (1989) shows that the expression of host specificity in Rhizobium is determined, not by a single gene or operon, but by many of the nod genes that have been identified. Therefore it would appear likely that recognition between Rhizobium and its host legume can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

1.3.1.5 The "nod box".

A number of groups have shown that nod genes are specifically turned on during the symbiotic process. Since mutations in these genes stop the earliest steps of bacterium-plant interaction it seems likely that the nod and hsn genes are co-ordinately regulated. This was further indicated by the demonstration of the existence of a 47bp conserved promoter sequence (the "nod box") in front of the nodABC, nodFEG (hsnABC) and nodH (hsnD) transcription units (Rostas et al., 1986).

This "nod box" is conserved in most Rhizobium species and mutations or deletions in it result in inactivation of the genes located downstream (Kondorosi & Kondorosi, 1988).

The position of the "nod box" is about 200-250bp upstream of the translational start codons of the three nod operons.

Within the "nod box" are a series of subsets of sequences of 25bp, 7bp and 5bp, which are highly conserved between different Rhizobium. The 25bp sequence was found to be essential for the expression of nodE or nodF (Rostas et al., 1986).

In R.leguminosarum three copies of the "nod box" were demonstrated while in different R.meliloti strains six copies were found which may be significant in relation to the induction of a large number of genes (Gottfeit et al., 1986).

The "nod box" has been postulated to be involved in the coordinate regulation of the discrete sets of nod genes. The mechanism by which this coordinate regulation is achieved can be described by a model proposing that the "nod box" serves as a recognition sequence for the binding of some activating factor(s), which directs transcription initiation by RNA polymerase. The simplest model stipulates that the nodD gene product fulfils the role as the activating factor and mediates the effect of the flavonoid inducer.

1.3.1.6 Flavonoids

Recently it has been shown that plants release flavonoids (low molecular weight phenolics) from their roots, which stimulate the transcription of bacterial nodulation genes on the symbiotic plasmid.

Flavonoids can be subdivided into 5 distinct classes; flavones, flavanones, isoflavones, flavonols and coumarins (Djordjevic et al., 1987; Peters & Long, 1988) (Fig. 2).

Compounds from host exudates that induce nodulation genes have been isolated and determined for four Rhizobium species. Flavones and flavanones have been shown to be the inducers for R.meliloti, R.leguminosarum and other Rhizobium species whereas in B. japonicum isoflavones are stimulatory. Horvath et al. (1987) and Spaink et al. (1987b) have shown that the specificity of induction by various flavonoids is correlated with the Rhizobium nodD.

The failure of the compound to induce nodulation gene expression is not necessarily a failure to interact with nodD. Since several compounds such as murin, chrysin, quercetin, fisetin, myricetin, kampferol and umbelliferone have no inducing activity but can antagonise induction by luteolin.

A common feature of these compounds as well as all inducing compounds is a heterocyclic double ring system with a hydroxyl group at the 7 position. The importance of the 7 hydroxyl substitution is illustrated by the structures of umbelliferone and coumarin. Umbelliferone has the 7 hydroxyl substitution and is antagonistic, whereas coumarin does not and is not antagonistic (see Fig. 2) (Peters & Long, 1988).

In general flavones and flavanones which are hydroxylated in both the A and B rings act as inducers. At least one B ring hydroxyl group appears to be necessary for inducing ability. The B ring hydroxyl can be at either or both the 3' or 4' position (Firmin et al., 1986).

1.3.1.7 Summary of our Current Understanding of **nod** Gene Regulation

A model for nod gene activation was proposed by Rossen et al. (1986). They propose that the target site for the stimulatory and inhibitory compounds is the nodD product. Stimulatory compounds may mediate the conversion of NodD to an active regulatory form while inhibitory compounds may compete for and occupy binding sites but fail to activate the nodD product. The activated nodD product may stimulate transcription from the conserved "nod box" sequence which occurs 5' to the all nod operons.(see Fig. 3)

In R.leguminosarum, NodD is autoregulatory since it inhibits the expression of a nodD-lacZ fusion (Rossen et al., 1986).

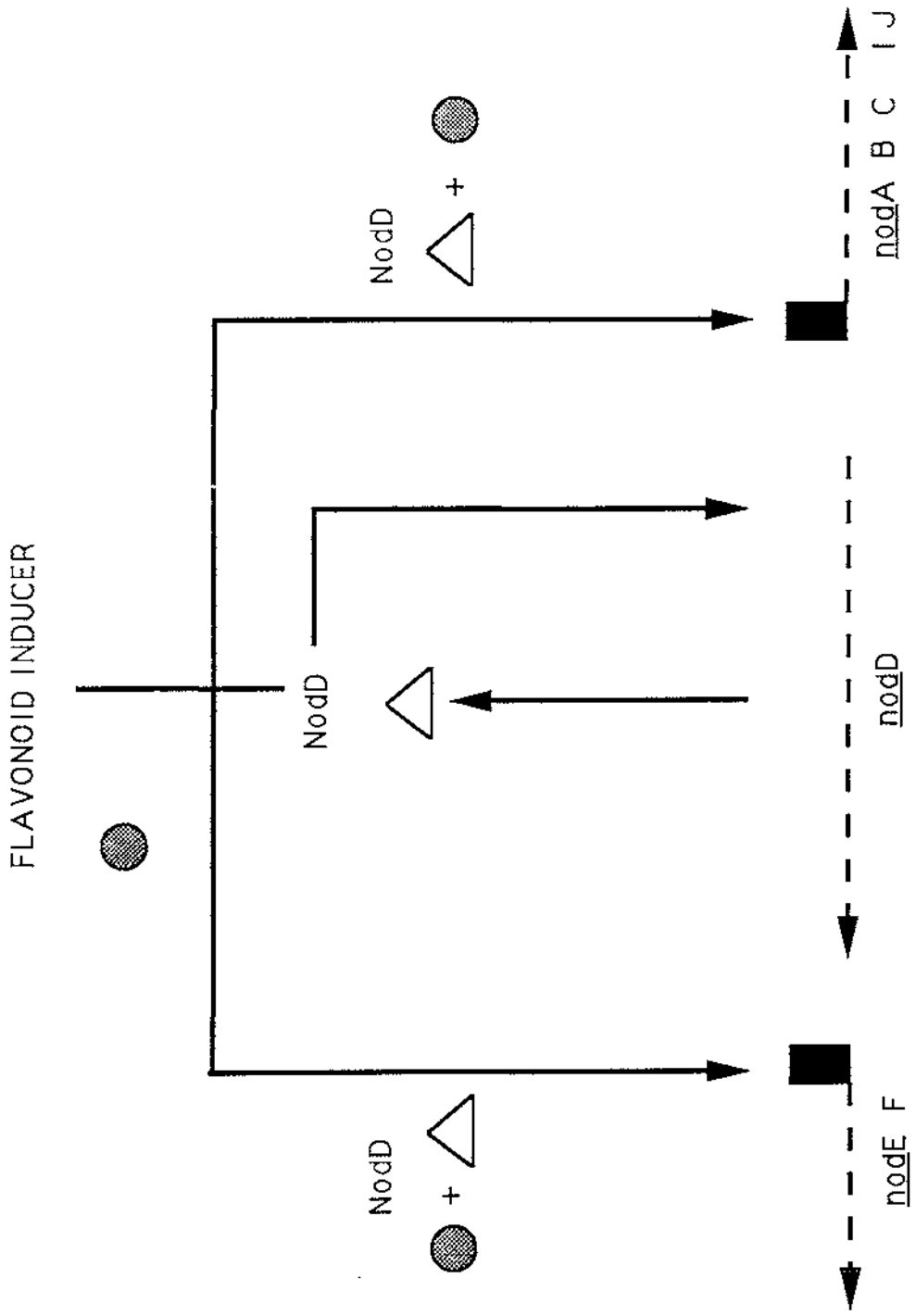
Figure 2. A SUMMARY OF THE MAJOR CLASSES OF FLAVONOIDS TESTED FOR **nod** INDUCTION IN **Rhizobia**.

A SUMMARY OF THE MAJOR CLASSES OF FLAVONOIDS

COMPOUNDS	SUBSTITUTION POSITIONS						
	3	5	7	2'	3'	4'	5'
Flavones							
luteolin		OH	OH		OH	OH	
apigenin		OH	OH			OH	
chrysin		OH	OH				
flavone							
Isoflavones							
genistein		OH	OH			OH	
daidzein			OH			OH	
Flavanones							
eriodictyol		OH	OH		OH	OH	
naringenin		OH	OH			OH	
hesperetin		OH	OH			OCH3	
flavanone							
Flavonols							
quercetin	OH	OH	OH		OH	OH	
kaempferol	OH	OH	OH			OH	
myricetin	OH	OH	OH		OH	OH	OH
morin	OH	OH	OH	OH		OH	
fisetin	OH		OH		OH	OH	
flavonol	OH						
Coumarins							
umbelliferone			OH				
coumarin							

Figure 3. A MODEL FOR **nod** GENE ACTIVATION IN **Rhizobia.**

The filled in rectangle represents a "nod box".
The circle represents the flavonoid inducer.
The hatched triangle represents the NodD protein.
The arrows represent the direction of transcription.
The Nod loop represents repression by auto-regulation.
(Rossen et al., 1986).


FLAVONOID INDUCER
NodD
+
NodD
+
NodD
nodA B C I J
nodD
nodE F

1.4) ISOLATION OF nod GENES THAT NODULATE Lotus SPECIES.

1.4.1 Lotus rhizobia

Rhizobia that nodulate Lotus species include both fast and slow growing strains. The fast growing strains (Jarvis et al., 1982) include R.loti, while the slow growing strains, which are more effective on Lotus, are classified as Bradyrhizobium sp. (Jordan, 1982). Most R.loti strains including the type strain NZP2213 form nitrogen fixing (Nod^+ Fix^+) nodules on Lotus corniculatus var cree and Lotus tenuis and ineffective nodules (Nod^+ Fix^-) on Lotus pedunulatus. However, some strains such as NZP2037 are able to nodulate all of these host plants effectively (Greenwood & Pankhurst, 1977).

The nodules formed on L.pedunculatus inoculated with NZP2037 contain a central zone of bacteroid filled plant cells surrounded by cortical cell. In contrast the nodules formed on L.pedunculatus inoculated with NZP2213 contained no rhizobia infected plant cells. However rhizobia were found in localized areas on the nodule surface and between the outer two or three layers of the nodule. In nodules formed on plants inoculated with NZP2037, flavonoids were present in the outer cortical and epidermal cells. In the ineffective NZP2213 nodule flavonoids were present in many of the central nodule cells. The two strains show differential sensitivity to flavonoids (especially prodelphinidin), with NZP2213 being 12 times more sensitive than NZP2037 (Pankhurst et al., 1979; Pankhurst & Jones, 1979a & b). In later research, Jones et al. (1987) found that the resistance of R.loti strain NZP2037 to the prodelphinidin-rich flavolan present in the roots of Lotus pedunculatus was associated with the presence of a peptidoglycan-bound flavolan-binding polysaccharide (FBP) in the outer cell membrane of this bacterium. The outer cell membrane of the flavolan sensitive strain NZP2213 did not contain this polysaccharide.

Unlike other Rhizobium strains examined nif structural genes and nod genes were not localized on a single large indigenous plasmid found in R.loti strains NZP2037 and NZP2213. Isolation of the plasmid cured derivatives of these strains that still nodulate L.tenuis effectively, suggests that nif and nod genes are localized on the chromosome (Chua et al., 1985; Pankhurst et al., 1986).

1.4.2 Isolation of nod genes from R.loti.

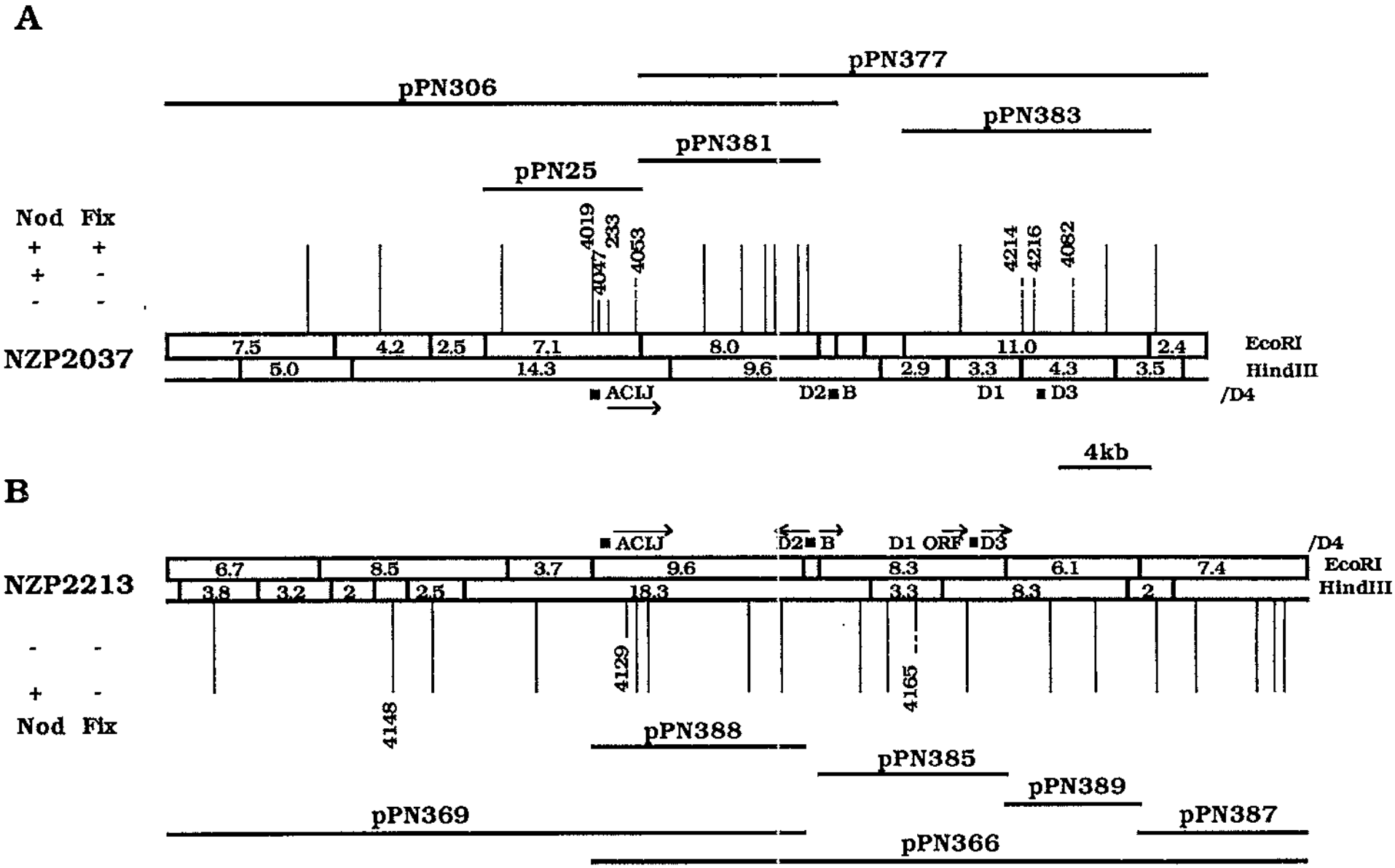
To establish the physical location of related nodulation genes in R.loti transposon mutagenesis was used. The E.coli plasmid pSUP1011 was used as a source of Tn5 and insertion mutations were introduced into the genome of R.loti NZP2037. Mutants defective in a number of separate steps in the nodulation process were isolated, including a mutant defective in nodulation (ie PN233 Nod^-). Using the vector pLAFR1, a cosmid library of R.loti NZP2037 was constructed and DNA that complemented the Nod^- mutant PN233 was identified by in planta complementation (Scott et al., 1985). This resulted in the isolation of pPN306 (Fig. 4). Subsequent to this, Pankhurst et al. (1986) constructed a NZP2213 pLAFR1 library and the nod gene region of this strain was isolated by direct in planta complementation of the NZP2037 Nod^- mutant PN233 (pPN377). Two cosmids, pPN369 and pPN366 (Fig. 4) were isolated by this strategy.

Both cosmids pPN336 and pPN369 were mutagenized in E.coli using the transposon Tn5. The position of the Tn5 insertions were mapped and each of the Tn5 mutations was then homogenotized into R.loti NZP2213. The position and phenotype of the mutations isolated by this method is shown in Figure 4. Also shown for NZP2037 is the extended nod region as defined by Tn5 insertions in pPN377 (Scott et al., unpublished). When the NZP2213 mutants were tested on L.pendunculatus and L.corniculatus two Nod^- mutants were identified; PN4129 and PN4165 corresponding to locus I and II. PN4129 is Nod^- Fix^- on both hosts whereas PN4165 is Nod^+ Fix^+ on L.corniculatus and delayed by 4 weeks) in nodulation of L.pendunculatus (Scott et al., unpublished).

Subsequent work by Scott et al. (unpublished) has shown that PN4129 can be complemented by a 9.6kb EcoR1 fragment (pPN388) and that PN4165 can be complemented by a 8.3kb EcoR1 fragment (pPN385) (Fig. 4). Further work has shown that pPN385 contains two "nod boxes", whereas there is no evidence to date for a "nod box" on the 9.6kb fragment, which is known to complement a R.trifolii nodC mutation. Therefore it is of considerable interest to determine whether the nod genes on the 9.6kb fragment (pPN388) are regulated by a different mechanism than those on the 8.3kb fragment (pPN385). This can be studied by creating gene fusions as was done by: Kahn and Timblin, 1984; Mulligan and Long, 1985; Stachel et al., 1985 and; Martinez et al., 1990.

Figure 4. A SUMMARY OF THE ORGANISATION OF THE **nod** GENES AND COSMIDS PRESENT IN **Rhizobium loti** STRAINS NZP2213 AND ZP2037.

The filled in square represents a "nod box" - a conserved sequence upstream of nod operons and the presumed site of action of NodD.
The + and - signs indicates the influence the specified areas have on Nod and Fix activity on the host; L.pedunculatus.


A
pPN377
pPN306
pPN383
pPN381
pPN25
Nod Fix
+ +
+ -
- -
4019
4047
233
4053
4214
4216
4082
NZP2037
7.5
4.2
2.5
7.1
8.0
11.0
2.4
EcoRI
5.0
14.3
9.6
2.9
3.3
4.3
3.5
HindIII
ACIJ
D2 B
D1
D3
/D4
4kb
B
ACIJ
D2 B
D1 ORF
D3
/D4
NZP2213
6.7
8.5
3.7
9.6
8.3
6.1
7.4
EcoRI
3.8
3.2
2
2.5
18.3
3.3
8.3
2
HindIII
4129
4165
- -
+ -
Nod Fix
4148
pPN388
pPN385
pPN389
pPN369
pPN387
pPN366

1.4.3 The use of lac-fusions as Reporter Genes.

In gene fusions, the control sequences of a gene of interest are placed in front of the coding sequence of a REPORTER gene whose product can be readily assayed. Thus, expression of the gene can be monitored by measuring the reporter gene product, and genetic and environmental factors that effect the expression can be determined and manipulated (Stachel et al., 1985).

The reporter gene which is most often used in gene fusions is the E.coli β-galactosidase structural gene. This is mainly because several indicator media can be used, eg Lactose-MacConkey agar, lactose-tetrazolium agar and eosin-methylene blue medium, to differentiate between Lac^- (interrupted lac genes) and Lac^+ (non-interrupted lac genes). Analogues can also be used to detect β-galactosidase activity, for example X-gal, which is a substrate of β-galactosidase, and yields a blue colour upon hydrolysis (Silhavy & Beckwith, 1985).

There are a variety of reasons for using lac fusions, these include the following; to study the regulation of another gene or operon, to detect genes which are subject to a particular regulatory signal, to study the mechanism of localization of a protein, to detect and assay a protein which has not been identified or for which no assay exits (Silhavy & Beckwith, 1985).

A recent method of obtaining both transcriptional and translational lacZ gene fusions is the use of the transposon Tn3-HoHo1. In these fusions the lacZ gene product is placed under the control of the gene into which the Tn3-HoHo1 has inserted and the expression of the gene can thus be analysed by monitoring β-galactosidase activity. This activity is only observed if Tn3-lacZ is in the correct orientation, as shown in Figure 5 (Stachel et al., 1985).

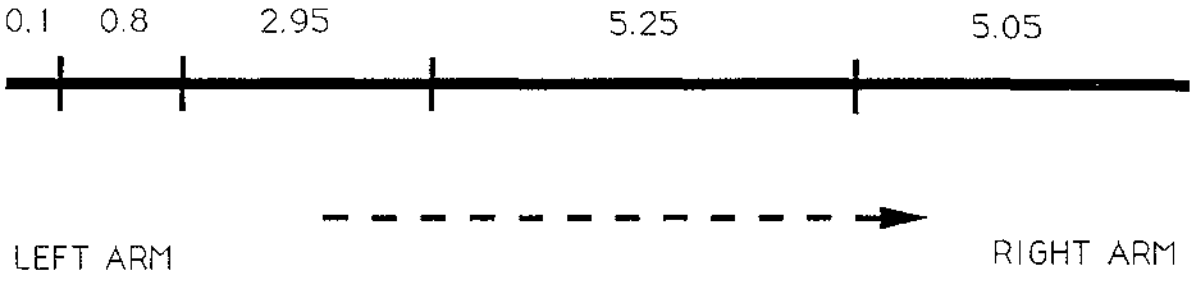
Tn3 is the best studied of a class of related transposons that are medically important because they confer antibiotic resistance to many pathological bacteria. Tn3 encodes Amp resistance, is 4957kb in length and has repeated ends of 38bp in length, it preferentially transposes into plasmid versus chromosomal DNA and may have a preference for A T-rich DNA (Heffron, 1983; Stachel et al., 1985).

The lacZ coding sequence within Tn3-HoHo1 is open and in frame to the end of the Tn3 left terminal repeat. When Tn3-HoHo1 inserts into a gene so that the coding sequences of the gene are in frame to the lacZ coding sequences, expression of the gene will result in the production of a hybrid β-galactosidase protein.

Tn3-HoHo1 carries a non-functional transposase gene, consequently it can transpose only if transposase activity is provided in trans, usually by pSShe, and is stable in the absence of this activity (Stachel et al., 1985).

Figure 5. EcoRI Restriction map of Tn3-HoHo1.

This figure is a diagrammatic representation of the Tn3-HoHo1 (14.25kb) region in the plasmid pHoHo1 (=PN1134).
The arrow represents the direction of transcription, and defines for the purpose of this work the left arm and the right arm of the transposon (Stachel et al., 1985).


0.1
0.8
2.95
5.25
5.05
LEFT ARM
RIGHT ARM

AIMS:

To study the regulation of common and host specific nodulation genes in R.loti strains NZP2213 and NZP2037. This will be done by constructing lac fusions using Tn3-HoHo1 (Stachel et al.,1985) and by a directed approach using vectors containing a promoterless lacZ (Spaink et al.,1987a).

Chapter 2 - METHODS AND MATERIALS

2.1) Bacterial Strains and Plasmids

These are described in Table 1.

2.2) Preparation of Culture Media

2.2.1 Liquid Media

E.coli Growth Medium:

Luria Broth (LB). (Miller, 1972). Composition (g l^{-1}): NaCl, 5; Bacto Tryptone (Difco), 10; Yeast Extract (Difco), 5; pH to 7-7.5 with 0.5ml 5M NaOH.

R.loti Growth Medium:

Tryptone Yeast Extract Media. (TY; Beringer, 1974). Composition (g l^{-1}): Bacto Tryptone (Difco), 5; Yeast Extract (Difco), 3; Autoclave then add 5ml 30% $CaCl_2.6H_2O$.

S20 (M9 modified S10 media; Chua et al., 1985). Composition (g l^{-1}): Na_2HPO_4, 6; KH_2PO_4, 3; NaCl, 0.5; NH_4Cl, 1; make up to 500ml autoclave. Make up Agar in 500ml (2.3.2). Combine. Then add (ml l^{-1}): 10% $MgSO_4.7H_2O$, 2.5; 1% $CaCl_2.2H_2O$, 1.5; 20% Sucrose, 12; 500µg/ml Biotin, 1.

M⁻ Media (M^-; Pankhurst et al., 1982). Stock solutions (g $100ml^{-1}$) autoclaved:

(1) Phosphate salts: KH_2PO_4, 3; Na_2HPO_4, 3 (2) $MgSO_4.7H_2O$, 1 (3) $CaCl_2$, 0.5 (4) $FeCl_3$, 0.1 (5) Sodium glutamate, 20g $200ml^{-1}$ (6) Vitamins (mg $20ml^{-1}$): Thiamine hydrochloride, 10; Biotin, 10; calcium pantothenate 200 were added to sodium glutamate and sterilised .

TABLE 1. BACTERIAL STRAINS AND PLASMIDS

Strain or Plasmid	Relevant Characteristics	Source or Reference
Strains		
E. coli		
HB101	pro leu thi lacY Str^R recA endA hsdR hsdM	Boyer and Roulland-Dussoix (1969).
MC1022	ara139 (ara leu) 7697 (lacZ) ΔM15 galU galK strA	Casadabam and Cohen (1980).
PN341	C2110 polA Nal^R	Leong et al (1982).
PN1134	HB101/pHoHo1 pSShe	Stachel et al (1985).
PN362	HB101/RK2013	Ditta et al (1980).
PN627	HB101/pPN385	Scott et al (unpublished).
PN1168	MC1022/pPN29	This study.
PN1165	MC1022/pPN30	Scott et al (unpublished).
PN1175	DH5/pMP220	Spaink et al (1987a).
PN1229	HB101/pMP190	This study.
PN464	HB101/pPN366	Scott et al (unpublished).
PN1232	MC1022/pPN36	This study.
PN1233a	HB101/pPN37	This study.
PN1233b	HB101/pPN38	" "
PN1234a	HB101/pPN39	This study.
PN1234b	HB101/pPN40	" "
PN1235a	HB101/pPN41	This study.
PN1235b	HB101/pPN42	" "

Strain or Plasmid	Relevant Characteristics	Source or Reference
Strains		
Rhizobium loti		
NZP2213	Nod$^+$ Fix$^-$ (L. pedunculatus) Nod$^+$ Fix$^+$(L. tenuis)	DSIR culture collection.
PN4115	Str^R derivative of NZP2213	Pankhurst et al (unpublished).
NZP2037	Nod$^+$ Fix$^+$ (L. pedunculatus) Nod$^+$ Fix$^+$ (L. tenuis)	DSIR culture collection.
D95	NZP2037/pLAFR1 - 7.5kb EcoRI fragment of inv region containing a Tn3 HoHol insertion	D. G. McSweeney. Personal Comm.
D71	NZP2037/pLAFR1 - 7.5kb EcoRI fragment of inv region containing a Tn3 HoHol insertion	D. G. McSweeney. Personal Comm.
MG1	NZP2037 containing pPN39	This study.
MG2	NZP2037 containing pPN40	This study.
MG3	NZP2037 containing a recombinant plasmid of pMP190 (colony #11)	This study.
MG4	NZP2213 containing pPN37	This study.
MG5	NZP2213 containing pPN38	This study.
MG6	NZP2213 containing pPN41	This study.
MG7	NZP2213 containing pPN42	This study.
MG8	NZP2037 containing pMP190	This study.

Strain or Plasmid	Relevant Characteristics	Source or Reference
Strains		
Rhizobium loti cont...		
MG9	NZP2037 containing pMP220	This study.
MG10	NZP2213 containing pMP190	This study.
MG11	NZP2213 containing pMP220	This study.
MG12	NZP2213 containing pLAFR3	This study.
Plasmids		
pHoHo1	17.2 kb pMB8 derivative tnpA-EcoRI fragment sizes: 8.2, 5.25, 2.95, 0.8kb	Stachel & Nester (1985).
pSShe	8.9kb pACYC184 derivative tnpA$^+$ Cam^R	Stachel & Nester (1985).
pRK2013	Rep colEI Kan^R RK2 derivative 60kb Mobility plasmid	Ditta et al (1980).
pPN385	pLAFR1 (21.6kb) containing an EcoRI 8.3kb fragment from the nod region of NZP2213 (Fig 4). Tet^R EcoRI fragment sizes: 21.6, 8.3kb. HindIII fragment sizes: 26.2, 3.6kb	Scott et al (unpublished).
pUC18	2.68kb Amp^R α peptide of lacZ, a multiple cloning site	Yanisch-Peron et al (1985)
pUC118	3.2kb Amp^R ori site α peptide of lacZ containing sites: EcoRI SstI KpnI SmaI BamI XbaI SalI PstI SphI and HindIII	Vieria and Messing (1987)

Strain or Plasmid	Relevant Characteristics	Source or Reference
Plasmids Cont...		
pLAFR1	Tet^R 21.6kb RK290 containing Lambda cos. Single sites for: SalI BstEII and EcoRI	Friedman et al (1982)
pLAFR3	Tet^R 22kb Single sites for: EcoRI PstI BamHI and SalI	Staskawicz et al (1987)
pBR322	4.363kb Amp^R Tet^R Single sites for: EcoRI PstI and SalI	Bolivar et al (1977)
pBR328	4.907kb Amp^R Tet^R Cam^R	Soberon et al (1980)
pMP220	Tet^R 10.5kb IncP plasmid containing a promoter-less lacZ and a polylinker with sites for: HindIII BglII EcoRI KpnI XbaI PstI SphI	Spaink et al (1987a)
pMP190	Cam^R Str^R 15kb IncQ plasmid containing a promoter-less lacZ and a polylinker with sites for: HindIII SalI XbaI KpnI BglII	Spaink et al (1987a)
pPN366	Tet^R a pLAFR1 (21.6kb) cosmid from a nod region of NZP2213 (Fig 4.) EcoRI fragment sizes; 21.6kb, 9.6kb, 8.3kb and 0.65kb	Scott et al (unpublished).
pPN29	Amp^R pUC118 (3.2kb) containing a 8.3kb EcoRI fragment from pPN385 (PN627)	This study.
pPN30	Amp^R pUC119 (3.2kb) containing a 0.65kb EcoRI fragment from pPN366 (PN464)	Scott et al (unpublished).

Strain or Plasmid	Relevant Characteristics	Source or Reference
Plasmids Cont..		
pPN36	Amp^R pUC118-4.1kb SalI fragment from pPN366 (PN464).	This study.
pPN37	Cam^R Str^R pMP190 containing a 4.1kb SalI fragment from pPN366 (PN464). In left to right orientation with respect to Fig. 16b.	This study.
pPN38	Cam^R Str^R pMP190 containing a 4.1kb SalI fragment from pPN366 (PN464). In right to left orientation with respect to Fig. 16b	This study.
pPN39	Cam^R Str^R pMP190 containing a 1.4kb SalI fragment from pPN25 (PN1154) In left to right orientation with respect to Fig. 17	This study.
pPN40	Cam^R Str^R pMP190 containing a 1.4kb SalI fragment from pPN25 (PN1154) In right to left orientation with respect to Fig. 17	This study.
pPN41	Str^R pMP220 containing a 0.65kb EcoRI fragment from pPN385 (PN627) In left to right orientation with respect to Fig. 16b	This study.
pPN42	Str^R pMP220 containing a 0.65kb EcoRI fragment from pPN385 (PN627) In right to left orientation with respect to Fig. 16b	This study.

To make up media add 5g Mannitol to 1 litre of H_2O and autoclave, then add (ml l^{-1}): phosphate stock, 10; $MgSO_4.7H2O$, 10; $FeCl_3$, 1; $CaCl_2$, 10; vitamins, 13.

2.2.2 Solid Media

This was prepared by adding 15g l^{-1} agar (Difco) to liquid media.

2.3) Maintenance of Cultures

E.coli cultures were maintained on LB agar plates at room temperature.
R.loti cultures were maintained on S20 agar plates at 4°C.

2.4) Growth of Bacteria

E.coli were grown at 37°C on solid media or at 30°C in liquid media supplemented with appropriate antibiotics. R.loti were grown at 30°C in solid or liquid media supplemented with appropriate antibiotics.

2.5) Antibiotic Concentrations

2.5.1 In E.coli:

Ampicillin	(Amp)	100µg/ml
Chloramphenicol	(Cam)	25µg/ml
Kanamycin	(Kan)	25µg/ml
Nalidixic Acid	(Nal)	25µg/ml
Streptomycin	(Str)	200µg/ml
Tetracycline	(Tet)	15µg/ml

N.B. For pMP190 containing cultures 20µg/ml of chloramphenicol was used instead of 25µg/ml.

2.5.2 In R.loti

Streptomycin	(Str)	200µg/ml
Tetracycline	(Tet)	2µg/ml
Chloramphenicol	(Cam)	10µg/ml

2.6) Isolation of Plasmid DNA

Plasmid DNA was isolated by the two methods described below.

2.6.1 Alkaline Lysis Method

The method of Ish-Horowitz and Burke (1981) was scaled up for the preparation of the plasmids from E.coli.

Materials

(1) Solution I: 50mM glucose; 25mM Tris-HCl, pH8; 10mM Na_2 EDTA.
(2) Solution II: 0.2M NaOH; 1% (w/v) SDS or Sarkosyl (prepared fresh).
(3) Solution III: Potassium acetate pH5.8; 5M potassium acetate; acetic acid, 11.5ml/100ml final volume.
(4) Lysozyme (3ml of 50mg/ml).
(5) CsCl.
(6) Ethidium Bromide (10mg/ml).
(7) TE 10:1 buffer pH 8.0: 10mM Tris-HCl; 1mM Na_2EDTA.
(8) Isopropanol.
(9) 70% (v/v) ethanol.
(10) TES (10/1/100) buffer pH8: 10mM Tris; 1mM Na_2EDTA; 100mM NaCl.

Method

E.coli cells (250ml), grown overnight in LB plus the appropriate antibiotics, were harvested by centrifugation (10,400 **g**, 10min, 4°), washed by resuspension in 100ml TE (10/1) followed by centrifugation (10,400 g, 10min, 4°). Then resuspended in 30ml of solution I. Lysozyme was added and the mixture was incubated at room temperature for 10min. Solution II (60ml) was

added, mixed by inversion, and incubated on ice for 10min. Solution III (45ml) was added, mixed by inversion, incubated for 10min on ice, and centrifuged (10,400 **g**, 10min, 4°C). DNA was precipitated by adding 0.6 volume of ice cold isopropanol to the supernatant, followed by incubation for 20min at room temperature and centrifugation (23,300 **g**, 10min, 4°C). The pellet (DNA + RNA) was washed once with 70% ethanol (75ml) at room temperature, vacuum dried and resuspended in TE 10:1 buffer (3.75ml). CsCl (1.05g/ml) and ethidium bromide (75µl/ml of 10mg/ml stock) were added, mixed and left in the fridge (5°C) overnight. The mixture was centifuged (17,300 **g**, 10min 4°C), the refractive index adjusted to between 1.3860 - 1.3920 and ultracentrifuged (Beckman vertical rotor, TLV100, 70,000rpm, 5hrs, 18°C). The plasmid DNA was removed and purified by NaCl-saturated isopropanol extraction and dialysed in TES (10/1/100). The purity and concentration of the DNA was determined spectrophotometrically by a A260/A280 ratio.

2.6.2 Rapid boiling method

The method of Holmes and Quigley (1981) was used.

Materials

(1) STET buffer: 8% (w/v) sucrose; 5% (v/v) Triton X-100; 50mM Na_2 EDTA pH8; 50mM Tris-HCl pH8.
(2) Lysozyme (10mg/ml) in 10mM Tris pH8.
(3) Isopropanol.

Method

E.coli cells (1.5ml), grown up to late exponential phase, were harvested in a microcentrifuge tube and resuspended in 350µl of STET buffer. Lysozyme (25ul) was added, and the cells were boiled for 40sec and immediately centrifuged (microcentrifuge, top speed, 10min). The supernatant (300-350µl) was collected and an equal volume of isopropanol was added, incubated at 20°C for 20min and centrifuged (microcentrifuge, top speed, 10min). The pellet (DNA + RNA) was washed with 95% ethanol, dried in vacuo and resuspended in 50µl of sterile water.

2.7) Purification of DNA Preparation

The following method was used to purify small quantities of DNA.

Materials

(1) Tris equilibrated phenol
(2) 3M sodium acetate
(3) 95% ethanol or isopropanol.

Method

A measured volume of DNA was transferred to a microcentrifuge tube and an equal volume of phenol-chloroform (1:1; v/v) was added, mixed thoroughly and centrifuged (microcentrifuge, top speed, 3min). The aqueous phase was collected and pooled, an equal volume of sterilized water was added to the phenol-chloroform mixture and back extracted. The aqueous phases were collected and pooled, an equal volume of chloroform was added, mixed thoroughly and centrifuged (microcentrifuge, top speed, 1min). The DNA was then precipitated by adding 2.5 volumes of ethanol (or 0.6 volumes of isopropanol), and 0.1 volumes of sodium acetate, followed by incubation in the freezer for 1-2hr. The sample was then centrifuged (microcentrifuge, top speed, 10min) vacuum dried and resuspended in sterile water to recover the DNA. E.coli transfer-RNA (20mg/ml) was added to a final concentration of 20µg/ml when the concentration of DNA was less than 1-5µg/ml.

2.8) Digestion of DNA with Restriction Enzymes

DNA was digested with restriction enzymes as described below.

Materials

(1) 1M NaCl
(2) Universal Buffer (x10): 60mM Tris pH 7.6; 100mM $MgCl_2$; 100mM β-mercaptoethanol
(3) SDS dye mixture: 20% (w/v) sucrose; 5mM Na_2EDTA; 1% (w/v) SDS; 0.2% (w/v) bromophenol blue
(4) Restriction enzymes (New England Biolabs).

Method

All DNA digestions were carried out in universal buffer (1x) with the salt concentration adjusted as recommended by the manufacturer, using 1M NaCl. Digestions were performed at 37°C for 1-2hrs. An aliquot of the digest was checked on a minigel by adding 0.2 volume of the SDS dye mixture. If the DNA digestion was incomplete, more enzyme was added and the mixture was incubated further, or the DNA was further purified and the experiment repeated.

2.9) Horizontal Agarose Gel Electrophoresis of DNA

The horizontal slab gels (150x200x5mm) used contained 0.7% (w/v) agarose (Sigma, Type 1). The TBE electrophoresis buffer pH8.3, contained 89mM Tris, 1mM Na_2EDTA and 89mM Boric acid. Electrophoresis was usually carried out at 1.5V/cm for 16hrs. Gels were stained with ethidium bromide and photographed. For rapid electrophoresis of DNA, small samples were analysed on a minigel (93x68x1.5mm) run at 13V/cm for 1hr.

For Eckhardt electrophoresis, the DNA samples were analysed on a small gel (120x80x10) run at 1.5V/cm for 1hr then at 3V/cm for 16hrs.

2.10) Determination of Molecular Weights

A HindIII digest of lambda DNA was used as a standard to determine the molecular weight of DNA fragments. The relative mobilities of DNA fragments were measured and molecular weights determined graphically from a plot of relative mobility versus $\log_{10}$ molecular weight (Sanger et al., 1982).

2.11) Extraction of DNA From Agarose

This method is based on that described by Ausubel et al. (1987).

Freeze-thaw method

Preparative 1% Seaplaque agarose gels were stained with ethidium bromide and DNA was visualised under long wave (350nm) UV light. The band from the 1% Seaplaque gel, containing the DNA fragments was cut into small pieces, placed in a microcentrifuge tube and melted at 65°C. Tris equilibrated phenol was added to cover the melted gel fragments, vortexed vigorously and then placed at -20°C overnight, to freeze the gel fragments. The sample was centrifuged (microcentrifuge, top speed, 10mins, room temperature) and the aqueous phase was collected, extracted using phenol-chloroform, and precipitated using ethanol. The DNA was resuspended in water and used directly for cloning or preparation of a [^{32}P]-labelled probe.

2.12) Preparation of [^{32}P]-Labelled DNA Probes

The method described by Taylor et al. (1976) and Whitfeld et al. (1982) was used.

Materials

(1) Random primers (25mg/ml) (prepared by Professor D B Scott (Massey University, NZ) from herring sperm DNA)
(2) Deoxycytidine 5'[^{32}P] triphosphate (Amersham PB, 10205 3,000 Ci/mmol)
(3) DNA polymerase I (Klenow fragment from Boehinger Mannheim)
(4) Deoxyribonucleoside triphosphates (Sigma): dATP (20mM), dGTP (20mM), dTTP (20mM)
(5) HaeIII restriction enzyme (New England Biolabs)
(6) Universal Buffer 10x (see 2.8)
(7) 0.25M Na_2EDTA, pH8.0
(8) Tris equilibrated phenol
(9) Chloroform
(10) Sephadex G-50 (Phamacia, fine grade)
(11) TES (10/1/100) buffer pH8: 10mM Tris; 1mM Na_2EDTA, 100mM NaCl.

Method

DNA (0.25-1.0μg) to be labeled, was digested with HaeIII restriction enzyme a 25μl reaction mixture for 30min at 37°C. Random primers (100μg) were added and the mixture was boiled for 2min, then chilled rapidly on ice. Reagents were added in the order listed below: Sterilized distilled water, 2.5μl; Universal (x10) buffer, 1.5μl; dTTP, 1μl; dATP, 1μl; dGTP, 1μl; [^{32}P] dCTP, 3μl; DNA polymerase I, 1μl. The mixture was then incubated at 37°C for 30min, and the reaction was stopped by adding 2μl of 0.25M Na_2EDTA. The reaction mixture was extracted with phenol-chloroform and the aqueous phase was loaded onto a Sephadex G-50 (packed in a 1ml syringe) column equilibrated with TES buffer pH8.0. The syringe column was spun (1,085 g, 2min) and the DNA peak was collected and stored at -20°C. DNA probes were usually labelled to a specific activity of 1-5x10^7 cpm per μg of DNA.

2.13) DNA Transfer and Hybridization

A modified method of Southern (1975) was used.

Materials

(1) Solution I: 0.25M HCl
(2) Solution II: 0.5M NaOH; 0.5M NaCl
(3) Solution III: 0.5M Tris, pH7.4; 2.0M NaCl
(4) 20x SSC (3M NaCl, 0.3M sodium citrate)
(5) 2x SSC
(6) Denhardts (10x) 500ml: 1M Hepes buffer pH7.0, 25ml; 20x SSC, 75ml; herring sperm DNA (3mg/ml) (Sigma D2251), 3ml; E.coli tRNA (10mg/ml), 1ml; 20% (w/v) SDS, 2.5ml; Ficoll (Sigma 70), 1g; bovine serum albumin (BSA) (Sigma A4503), 1g; polyvinylpyrolidone (PVP) (Sigma PVP-10), 1g.
(7) Nitrocellulose filter (Schleicher and Schuell BA 85).

Method

Gels were stained with ethidium bromide and photographed. DNA was subjected to partial depurination by shaking the gels in Solution I for 15min (Wahl et al., 1979), then denatured by shaking for 30min in Solution II, followed by shaking for 60-90min in Solution III to neutralize the gel. The gel was washed in 2x SSC for 5min and placed on a blotting stand for DNA transfer to nitrocellulose filter. The nitrocellulose

filter was removed after 16hrs, washed in 2x SSC for 5mins, blotted dry and baked in vacuo at 80°C for 2hrs. The filter was sealed in a tube containing 20ml (10x) Denhardts solution and pre-hybridized at 65°C for at least 2hrs. Most of the liquid in the tube was drained off, and the boiled probe (10^7 cpm) was added. The tube was sealed and incubated at 65°C overnight. After incubation the filter was removed from the tube, washed in three changes of 2x SSC, blotted dry, covered in "Gladwrap" and exposed to Ilford Curex X-ray film in the presence of Cronex intensifying screens for 1-5 days at -70°C. When filters were re-used, the probe DNA was removed by washing for 20min in 20mM NaOH, 15min in Solution III and 15mins in 2x SSC.

2.14) Alkaline Phosphatase Treatment of Vectors

The vector DNA was routinely treated with alkaline phosphatase to prevent self ligation as described below.

A measured volume of cut vector DNA was transferred to a microcentrifuge tube and 2.2 units of calf intestine alkaline phosphatase / 5µg DNA was added, mixed thoroughly and incubated at 37°C for 20min. After incubation 5-10mM EDTA was added. The reaction was stopped by incubating the mixture at 65°C for 10min, then phenol extracted (2.7).

2.15) DNA Ligation

A modified method of King & Blakesley (1986) was used.

Material

(1) Ligation buffer (5x): 250mM Tris pH7.6; 50mM $MgCl_2$; 25% (w/v) polyethylene glycol 8000; 5mM ATP; 5mM dithiothreitol
(2) T4 DNA Ligase (New England Biolabs).

Method

All DNA ligations were carried out in ligation buffer (1x) with mixtures containing 20ng of vector DNA, 20-50ng of insert DNA, 1µl of ligase (400 units) and an appropriate volume of sterile water to make up the final volume (10µl), were incubated at 4°C for 16hrs. An aliquot (1µl) was checked on a 0.7% minigel before and after the ligation. The ligated DNA was used directly for transformation.

2.16) Transformation of E.coli

A modified method of Cohen et al. (1972) was used.

Material

(1) 60mM $CaCl_2.2H_2O$
(2) TEC buffer pH8.0: 10mM Tris pH8.0; 0.25mM Na_2 EDTA pH8; 30mM $CaCl_2$.

Method

A stationary phase culture of E.coli strain HB101 was diluted 1/100 into 25ml of LB and incubated at 37°C. The cells (OD_{600} = 0.4) were harvested by centrifugation (3,020 **g**, 5min, 4°C). All subsequent steps were carried out at 4°C. The cells were resuspended in 10ml of calcium chloride solution (60mM) and incubated on ice for 20min. These cells were then harvested (as above) and resuspended in 250µl of calcium chloride solution (60mM) and used in transformation experiments. The transformation mixture containing 50µl of competent cells, 5µl ligated DNA and 45µl of TEC buffer was incubated at 4°C for 1hr, heat shocked at 42°C for 2.5min, then diluted 1/10 in LB (2.2.1), containing 20mM glucose, 10mM $MgSO_4.7H_20$ and 10mM $MgCl_2.6H_20$, and incubated at 37°C for 1.5hr. The culture (100µl) was plated on LB medium supplemented with the appropriate antibiotics and incubated at 37°C overnight.

2.17) Conjugation

Triparental crosses (Ditta et al., 1980) were performed by dispensing 50µl of a late log culture of the recipient as a spot on a LB plate (for crosses between E.coli) or on a TY plate (for crosses between E.coli and Rhizobium). After allowing to dry, a mixture (50µl) of the donor plus the helper (50:50 v/v) was dispensed on top of the recipient spot and dried. The plate was incubated overnight at 37°C or 30°C depending on strains.

2.18) Production of Plant Exudate

A modified method of Mulligan and Long (1985) was used.

Plant exudate was prepared using Lotus tenuis (Grasslands #S1036, source: F Smith & Co. Inc., USA) seeds. The seeds (1g) were sterilized by soaking in a mixture (50:50 v/v) of 30% peroxide and 95% ethanol for 2min, drained and soaked in 95% ethanol for 1min. Washed several times in sterile water, then imbibed overnight in 2-3 volumes of sterile water to yield seed exudate. Root exudate was collected 3hr to several days after the addition of water to the sprouted seeds. Both seed and root exudates were centrifuged (12,100 **g**, at 4°C, 10min) to remove any precipitants, filter sterilized and tested for the presence of bacteria by plating on LB media.

2.19) β-Galactosidase Enzyme Assay

β-Galactosidase activity in permeabilized cells was assayed using the chromogenic substrate o-nitro-phenyl-β-D-galactosidase (ONPG) by a modified method of Miller (1972).

Materials

(1) Sodium phosphate stock (10x): 0.6M Na_2HPO_4; 0.4M $NaH_2PO_4.2H_2O$; adjust to pH7.0
(2) Z Buffer pH7.0: sodium phosphate stock (10x) 100ml/l; 10mM KCl; 1mM $MgSO_4$; 50mM β-mercaptoethanol
(3) o-nitro-phenyl-β-D galactosidase (ONPG): 4mg/ml in (1x) sodium phosphate
(4) Sodium dodecyl sulphate (SDS) 0.1% (w/v)
(5) 1M Na_2CO_3
(6) Chloroform.

Method

All assays were performed in 1.5ml microcentrifuge tubes at room temperature, unless otherwise stated.

The cells were grown in M^- media (2.2.1) until the OD_{600} was >0.6. Then the cells (0.5ml) were added to an equal volume of Z-buffer; two drops of chloroform and one drop of sodium dodecyl sulphate (SDS) were added to permeabilize the cells. The mixture was vortexed for 10sec and incubated for 5min at 28°C. ONPG (200µl) was added and the assays were timed until a pale yellow colour developed. The reaction was stopped by the addition of 450µl of 1M Na_2CO_3. The reactions were then spun in a microcentrifuge (top speed, room temperature, 5min) to remove the cells. The amount of o-nitro-phenyl (ONP) produced was determined by measuring the absorbance at 420nm. The activity was measured, as units of β-galactosidase = 1000 x OD_{420} / time x vol x OD_{600}.

Induction was performed by adding 1/10th volume of plant exudate to early logarithmic phase cultures 16hrs before the samples were assayed.

2.20) The Eckhardt Gel Procedure

To determine the sizes of large plasmids a modified method of Eckhardt (1978) was used.

Materials

(1) 0.1% Sarcosine (g/10ml TBE) 0.01.
(2) TE 10:1 buffer pH 8.0: 10mM Tris-HCl; 1mM Na_2EDTA.
(3) Solution I (g/10ml TBE): Ficoll 400,000 (Sigma) 1.0; Bromophenol blue (Sigma) 0.005; RNAase 0.01; Immediately prior to use add 1/10 of 2mg/ml Lysozyme solution.
(4) Solution II (g/10ml TBE): Ficoll 400,000 1.0; SDS 0.02.
(5) Solution III (g/10ml TBE): Ficoll 400,000 0.5; SDS 0.02.

Method

Liquid samples were used for this method. The samples were grown up overnight (for 16hrs) and 0.2 to 1ml aliquots (depending on the density of the culture) were removed and added to microcentrifuge tubes. Centrifuged (microcentrifuge, top speed, 3min), drained, using a drawn out Pasteur pipette attached to a vacuum, and resuspended in 1ml 0.1% sarcosine. Centrifuged (microcentrifuge, top speed, 3min), drained and resuspended in 1ml TE (10:1). Centrifuged (microcentrifuge, top speed, 5min), drained and resuspended in 20µl of Solution I. These samples were then transferred to the appropriate wicked wells in the 0.7% Eckhardt gel (2.9). After 10min, 20µl of Solution II was added to each well and stirred once with a sealed Pasteur pipette. Solution III was then added as a layer on top to fill the wells, then the TBE buffer was added to the gel box and run at 1.5V/cm for 1hr then at 3V/cm for 16hrs.

Chapter 3 - RESULTS

In order to construct Rhizobium loti nod promoter-lacZ fusions two approaches were used in this study, (i) the random Tn3-HoHo1 mutagenesis approach and (ii) the directed cloning of nod promoters into a promoterless lacZ gene.

3.1) The Random Tn3-HoHo1 Mutagenesis Approach to Constructing lacZ Transcriptional/Translational Fusions.

3.1.1 Construction of a physical map of the 8.3kb **nod** region of NZP2213.

Prior to the mapping of Tn3-HoHo1 inserts into the 8.3kb EcoRI nod region of NZP2213, (Fig. 4), a more detailed restriction map of this fragment was made in the following way. First the 8.3kb EcoRI fragment was subcloned from pPN385 (Fig. 6b) into pUC118 (Fig. 6a). A culture of PN627 was grown over night and pPN385 plasmid DNA was isolated by the rapid boil method (section 2.6.2), and the DNA purified by phenol-chloroform extraction (section 2.7). This DNA (30ng) was digested with EcoRI and then ligated (section 2.14) with 40ng EcoRI digested pUC118 DNA. The ligation mixture was transformed (section 2.15) into MC1022 cells, and transformants selected by plating on LB media supplemented with Amp and X-gal. To check the transformation was successful a number of white colonies were picked and grown overnight. The plasmid DNA was isolated by the rapid boil method, digested with EcoRI and run on a minigel alongside an EcoRI digest of pPN385. All seven clones tested contained an 8.3kb insert, and one of these strains, containing pPN29 (Fig. 7), was selected for further work. The pPN29 plasmid was isolated from PN1168 by the rapid boil method and digested with various combinations of EcoRI, BamHI, and HindIII. The various digests were then run on an overnight gel, as shown in Figure 8.

Figure 6. RESTRICTION MAPS OF PLASMIDS USED FOR MAPPING ENZYME SITES IN THE 8.3kb **nod** FRAGMENT OF **R.loti** NZP2213.

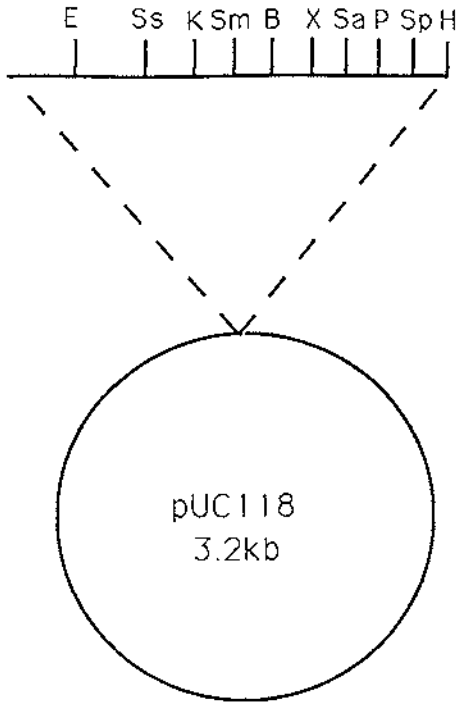
(A) Restriction Map of pUC118.

The polylinker region of pUC118 has been drawn separately to show the unique cloning sites.
The map is not drawn to scale.
The restriction enzyme sites shown include: E, EcoRI; H, HindIII; Sa, SalI; Ss, SstI; K, KpnI; Sm, SmaI; B, BamHI, X, XbaI; P, Pst; Sp, SphI.

(B) Restriction Map of the cosmid pPN385.

The cosmid, pPN385, is a pLAFR1 (21.6kb) recombinant cosmid containing the 8.3kb nod fragment (see Fig. 4) from R.loti strain NZP2213.
Shown on the map are the cos fragment (heavy line) from lambda, the rlx site and the 8.3kb EcoRI fragment.
The map is not drawn to scale.
The restriction enzyme sites shown include: E, EcoRI; H, HindIII; Sa, SalI; Bg, BglII; Bs, BstEII.

A



B

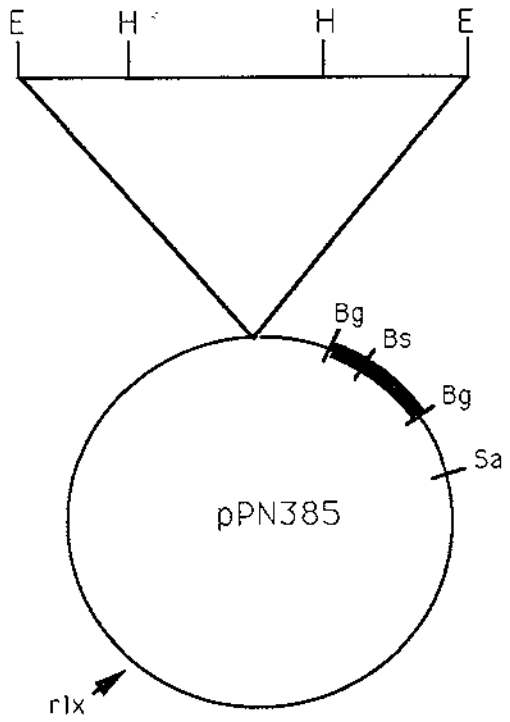


Figure 7. RESTRICTION MAP OF pPN29.

The plasmid pPN29 is a pUC118 derivative containing the 8.3kb EcoRI Fragment from pPN385.

While pUC118, and the polylinker are not drawn to scale, for the 8.3kb insert 1cm = 1kb.

Restriction enzyme sites shown include: E, EcoRI; H, HindIII; Sa, SalI; Bg, BglII; Bs, BstEII; Ss, SstI; K, KpnI; Sm, SmaI; B, BamHI, X, XbaI; P, Pst; Sp, SphI.

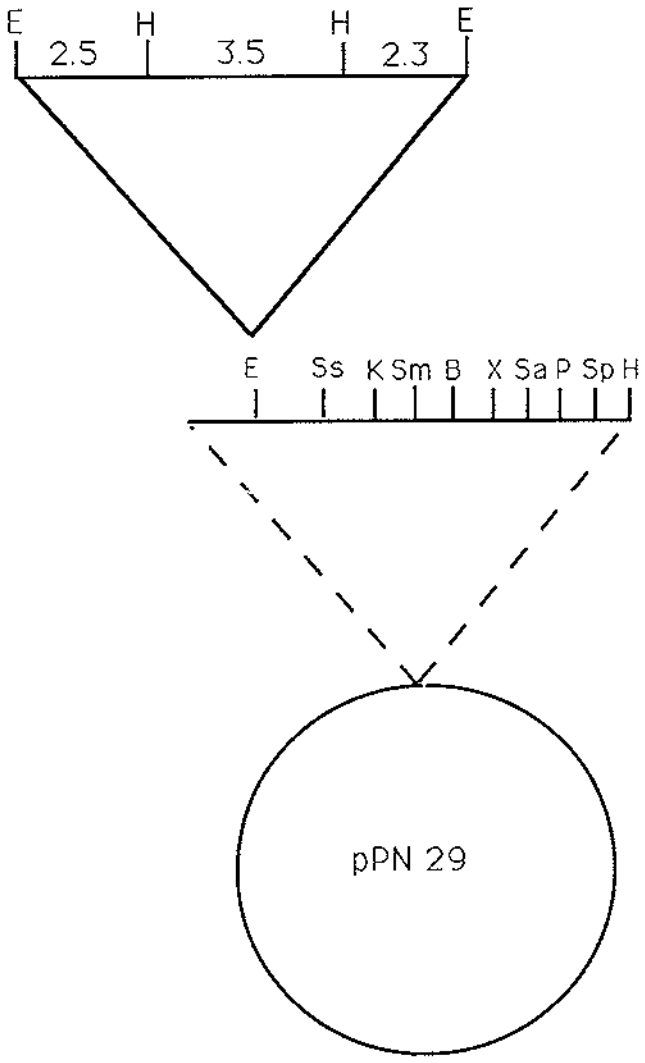
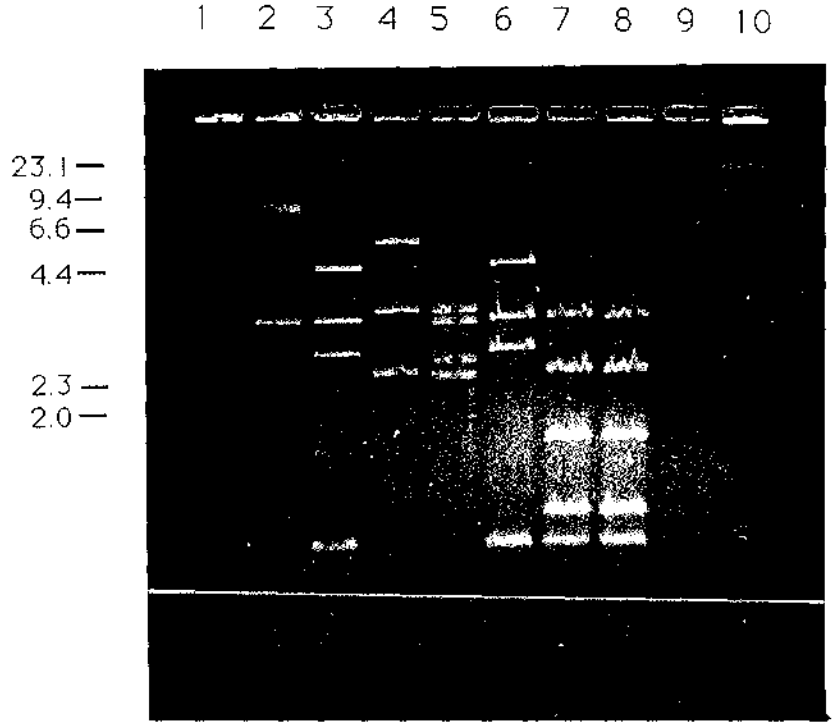

E
H
H
E
2.5
3.5
2.3
E
Ss
K
Sm
B
X
Sa
P
Sp
H
pPN 29

Figure 8. AGAROSE GEL ELECTROPHORESIS OF SINGLE, DOUBLE, AND TRIPLE DIGESTS OF pPN29.

Lanes 1 and 10 contain lambda HindIII size markers. The enzymes used to digest pPN29 were;
Lane 2 EcoRI,
Lane 3 BamHI,
Lane 4 HindIII,
Lane 5 EcoRI + HindIII,
Lane 6 EcoRI + BamHI,
Lane 7 HindIII + BamHI,
Lane 8 EcoRI + BamHI + HindIII.


1 2 3 4 5 6 7 8 9 10
23.1
9.4
6.6
4.4
2.3
2.0

The physical map of the 8.3kb fragment was constructed using the single, double and triple enzyme digestion data and by the following deductions:

(1) The EcoRI digest of pPN29 (lane 2) shows two bands, one of 8.3kb, representing the subcloned fragment from pPN385, and one of 3.2kb, representing the vector, pUC118

(2) The BamHI digest of PN1168 (lane 3) shows four bands; These correspond to two vector containing fragments of 4.9kb and 3kb and two internal fragments of 2.6kb and 0.8kb (c.f. lane 6);

(3) The HindIII digest (lane 4) shows three bands; These correspond to a central insert fragment of 3.6kb and two vector containing fragments of 5.7kb and 2.3kb (c.f. lane 5);

(4) The EcoRI/HindIII double digest (lane 5) by definition (compared with lanes 2 and 4) must produce five fragments. However, only four are visible as bands on the gel. The five fragments represent; The bands of 2.3kb, 3.6kb, and 2.5kb correspond to fragments from the 8.3kb EcoRI insert and the 3.2kb to the vector. The very small polylinker sequence (36bp) will not be visible under these conditions;

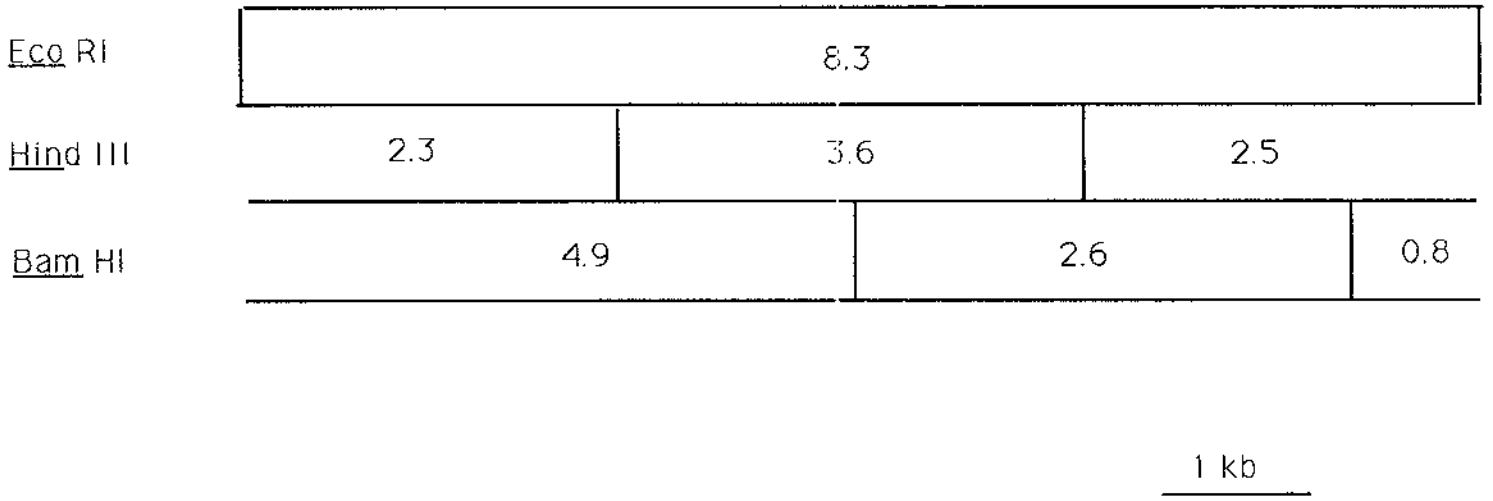
(5) The EcoRI/BamHI double digest (lane 6) must produce by definition (compared with lanes 2 and 3) six fragments, but only four are visible on this gel. The six fragments represent; four internal fragments from the 8.3kb insert, of 4.9kb, 2.6kb, 0.8kb and a very small fragment of unknown size. The remaining two fragments are derived from the vector; the smaller fragment (about 20bp) being part of the polylinker and the larger fragment being the rest of the vector (about 3.2kb);

(6) The HindIII/BamHI double digest (lane 7) by definition (compared with lanes 2 and 3) produces seven fragments, of which six are visible as bands on this gel. The seven fragments represent; five internal fragments from the 8.3kb insert; 0.8kb, 1.4kb, a doublet of 1.6kb and a fragment of about 2.3kb by definition containing a small region from the vector polylinker region. The remaining two fragments are; a small 36bp fragment of polylinker DNA and a larger fragment representing the remaining vector (pUC118) DNA;

(7) The triple digest produces nine fragments, six of which are visible as bands on the gel. These represent; six internal fragments from the 8.3kb insert, (of 2.3kb, a doublet of 1.6kb, 1.4kb, 0.8kb and a very small fragment of unknown size). The remaining three fragments belong to the vector; one large piece and two 20bp and 30bp fragments from the polylinker region.

Figure 9. RESTRICTION ENZYME MAP OF THE 8.3kb REGION OF pPN29

Positions of enzyme sites were determined by analysing the sizes of the fragments produced in single, double and triple digests as shown in Figure 8.


Eco RI
8.3
Hind III
2.3
3.6
2.5
Bam HI
4.9
2.6
0.8
1 kb

From the analysis of this gel the precise positions of the digestion sites where determined and mapped. A summary of the map is shown in Figure 9.

3.1.2 Tn3-HoHo1 Mutagenesis

The initial step in Tn3-HoHo1 mutagenesis was the introduction of pPN385 plasmid DNA into strain PN1134, which contains the pHoHo1 and pSShe plasmids, (Maps shown in Figure 10). Plasmid DNA was purified from PN627 by the rapid boil method followed by phenol-chloroform extraction (sections 2.6.2 and 2.7).

Purified pPN385 (70ng) DNA was then transformed into PN1134, (section 2.16), using pBR328 as a positive control for transformation. Transformants were selected on LB agar containing Cam and Amp. The frequency of transformation was calculated to be 1.9×10^{-4} transformants / μg of pPN385 DNA. Eight transformants were grown up overnight, the plasmid DNA isolated, digested with BamHI and analysed on agarose gels (Fig. 11).

Digestion of a strain containing pHoHo1, pSShe and pPN385 should give BamHI fragments of 26kb (pPN385 plus insert), 17.2kb (pHoHo1), 8.9kb (pSShe), 2.4kb and 0.79kb. In lanes 4, & 6-10 (Fig. 11) only four of these five bands are visible; 26kb, 17.2kb, 8.9kb, and 2.4kb. The largest fragment corresponds to pLAFR1 plus the large EcoRI/BamHI 4.4kb fragment of the insert and a very small fragment from the opposite end of the insert. The 17.2kb fragment corresponds to pHoHo1, the 8.9kb fragment corresponds to pSShe, the 2.4kb fragment is the middle BamHI fragment within the 8.3kb insert, and the last fragment of 0.79kb corresponds to the small BamHI fragment within the 8.3kb insert.

The 8.9kb fragment band has been lost, in lanes 2 and 5, but a band of 6.5kb appears, suggesting that these isolates have a deleted pSShe plasmid.

Clone number seven (Fig. 11) was chosen for further analysis. To select for Tn3-HoHo1 inserts within pPN385 a triparental cross (section 2.17) was carried out using PN341 (polA) as a recipient and RK2013 as the helper plasmid. Transconjugants from this cross were selected on LB agar containing Nal, Tet and Amp.

Figure 10. PHYSICAL MAPS OF pSShe AND pHoHo1.

(A) Map of the pSShe (8.9kb) plasmid.

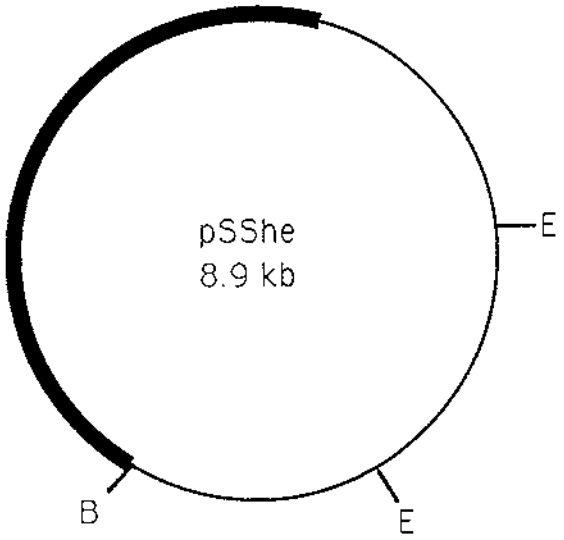
The pSShe plasmid contains the tnpA coding region of Tn3 (the black area); the pACYC184 origin of replication and the chloramphenicol resistance gene.

(B) Map of the pHoHo1 (17.2kb) plasmid.

The pHoHo1 plasmid contains the Tn3-lac transposon.
The black area represents the EcoRI region of the Tn3-lac transposon.

The plasmids have not been drawn to scale.
E, EcoRI; B, BamHI.

A



B

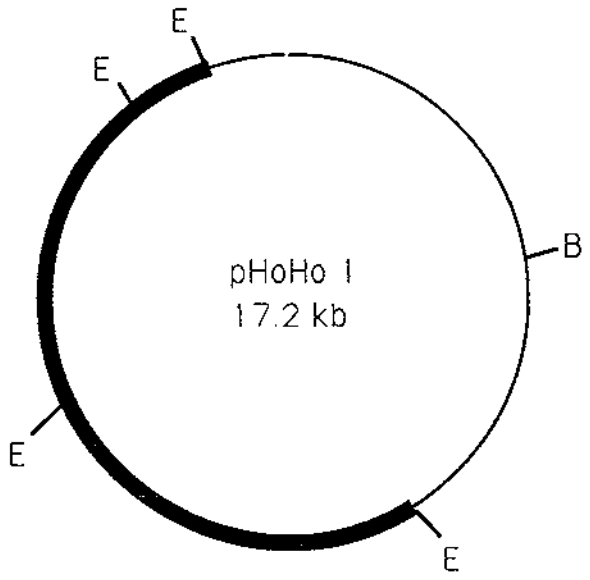


Figure 11. AGAROSE GEL ELECTROPHORESIS OF A **Bam**HI DIGEST OF PLASMID DNA ISOLATED FROM STRAIN PN1134 TRANSFORMED WITH pPN385.

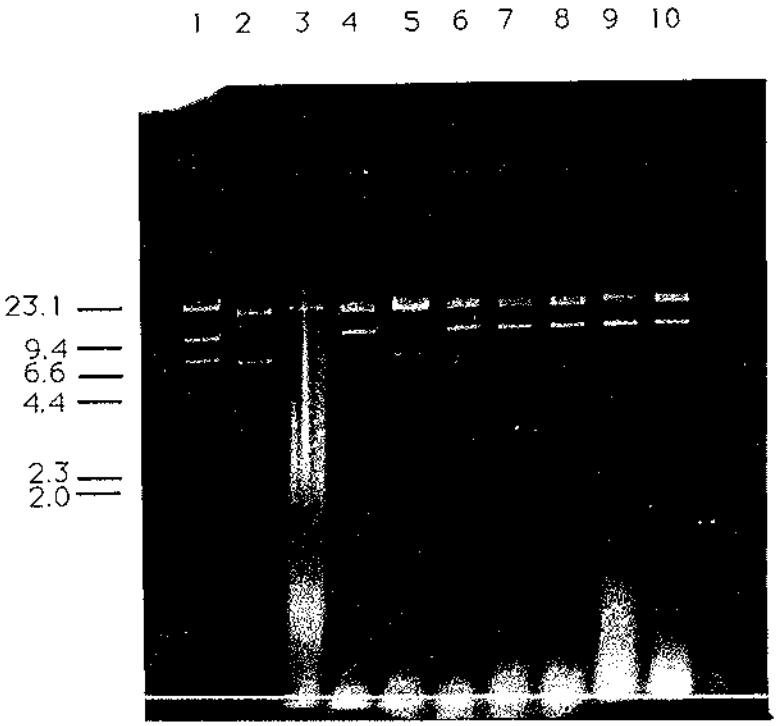
Lane 1 contains the lambda HindIII size markers.

Lane 2 contains BamHI digested PN1134 DNA missing a piece of DNA from the pSShe plasmid.

Lane 3 to 10 contain the BamHI digested transconjugants.

Notes: i)The difference in banding patterns observed in lanes 2, 3 & 5 is due to a deletion in the pSShe plasmid reducing it to about 6.5kb from the normal 8.9kb.

ii) The standard BamHI digestion pattern for PN1134 DNA is not shown in this gel but gives bands of 17.2kb and 8.9kb which is the pattern observed in lanes 4 and 6 to 10.


1 2 3 4 5 6 7 8 9 10
23.1
9.4
6.6
4.4
2.3
2.0

As previous experiments in our laboratory had shown, (M^{c}Sweeney, Honours Thesis, 1987), that transconjugants of strain PN341 gave poor yields of plasmid DNA using the rapid boil method, the pPN385::Tn3-HoHo1 (Tet^{R} Amp^{R}) plasmids were transferred to HB101 by another triparental cross, and transconjugants selected on LB agar containing Str, Tet and Amp.

In total three crosses were carried out and four hundred transconjugants were colony purified on LB agar containing Amp and Tet, and two hundred and ninety of these where analysed further.

The next step was to map the position of each Tn3-HoHo1 insert within pPN385, using EcoRI. The purpose of this analysis was to determine whether Tn3-HoHo1 had inserted within the pLAFR1 vector or within the 8.3kb insert from pPN385. Plasmid DNA isolated from transconjugants by the rapid boil method was digested with EcoRI and separated by agarose gel electrophoresis.

A typical digestion profile is shown in Figure 12.
Bands corresponding to the internal EcoRI fragments of HoHo1 (5.25kb, 2.95 & 0.8kb) were observed in all lanes. The other fragments varied depending on the position of Tn3-HoHo1.

Lanes in which the 8.3kb band was present were assumed to represent Tn3-HoHo1 inserts into pLAFR1, whereas lanes in which the 8.3kb band had moved were assumed to contain Tn3-HoHo1 inserts in the 8.3kb region. Overall, 32% (93) of the 290 transconjugants analysed were found to have Tn3-HoHo1 inserts in pLAFR1, and 68% (197) contained inserts in the 8.3kb fragment.

When the 8.3kb region of pPN385 was digested with the enzymes EcoRI and HindIII, three fragments were formed (as seen in Fig. 13), ie 2.3kb, 3.6kb and 2.5kb in size. The next step was to determine into which of these areas the Tn3-HoHo1 had inserted.

The plasmid DNA was isolated from the transconjugants by the rapid boil procedure, digested with both EcoRI and HindIII and run out on a gel.

As shown in Fig. 6b and Fig. 10 neither pLAFR1 (Friedman et al., 1982) nor pHoHo1 (Stachel et al., 1986) contain HindIII restriction sites. Bands (Fig. 13) corresponding to pLAFR1 (21.6kb) and the internal EcoRI fragments of

Figure 12. AGAROSE GEL ELECTROPHORESIS OF EcoRI DIGESTS OF pPN385::Tn3-HoHo1 PLASMID DNA ISOLATED FROM A SELECTION OF HB101 TRANSCONJUGANTS.

Lane 1 contains the lambda HindIII size markers.

Lane 2 contains an EcoRI digest of pPN385 DNA producing bands of 21.6kb and 8.3kb.

Lanes 3 to 11 contain nine different transconjugants.

Notes: i) The high band present in lanes 4, 5, 6, 7, & 11 is due to the presence of RK2013 (the helper plasmid) DNA.

ii) The DNA used in lane 2 was obtained from a bulk plasmid preparation (2.6.1).

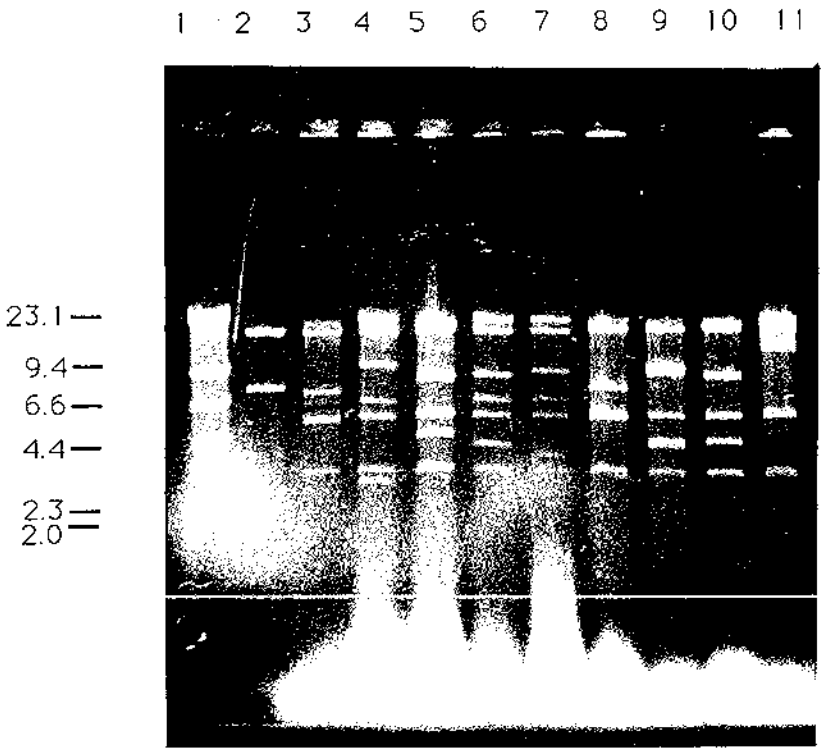
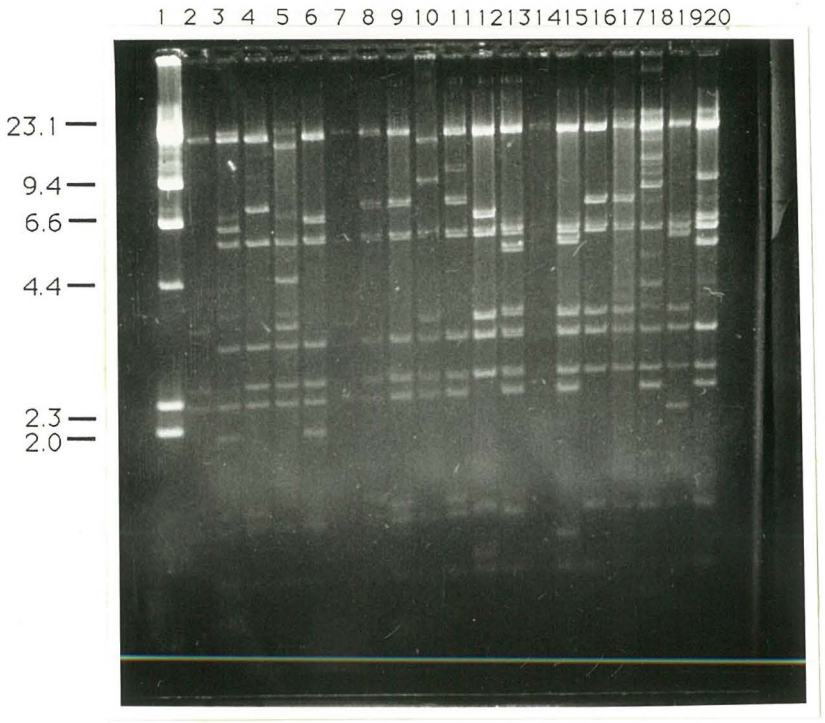

1 2 3 4 5 6 7 8 9 10 11
23.1
9.4
6.6
4.4
2.3
2.0

Figure 13. AGAROSE GEL ELECTROPHORESIS OF AN EcoRI/HindIII DOUBLE DIGEST OF pPN385 DNA CONTAINING DIFFERENT Tn3-HoHo1 INSERTS.

Lane 1 contains the lambda HindIII size markers.
Lanes 2 to 20 contain EcoRI + HindIII double digests of different pPN385 Tn3-HoHo1 inserts.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
23.1
9.4
6.6
4.4
2.3
2.0

HoHo1 (5.25kb, 2.95kb & 0.8kb) were observed in all lanes, while bands corresponding to the 2.3kb, 3.6kb & 2.5kb fragments of the 8.3kb EcoRI fragment varied depending on where Tn3-HoHo1 had inserted. If one band was absent then the other two would be present (except if the insert was in pLAFR1), and the missing band would give rise to two new fragments of differing sizes.

From these observations the following deductions could be made:

(1) Lanes containing two of the three fragments (2.3kb, 3.6kb or 2.5kb) were assumed to contain the Tn3-HoHo1 insert in the absent region;
(2) Lanes containing all three fragments were assumed to contain the Tn3-HoHo1 within pLAFR1.

Thus the region into which Tn3-HoHo1 inserted can be determined. Overall, of the 290 inserts analysed, 114 were located in the 2.3kb region, 80 in the 3.6kb, 3 in the 2.5kb region and 93 in the pLAFR1 vector.

The last step in this analysis was to determine the orientation of the inserts within the 8.3kb fragment and their precise position on the map. The plasmid DNA was isolated from the transconjugants by the rapid boil procedure, digested with EcoRI, and run on a 0.7% overnight gel.

A typical set of results is shown in Figure 14a.
Bands corresponding to pLAFR1 (21.6kb) and the internal EcoRI fragments of HoHo1 (5.25kb, 2.95 & 0.8kb) were observed in all lanes. Other fragments varied considerably in their banding positions.

This suggested that:

(1) Any band < 5.15kb (the size of one end fragment of Tn3-HoHo1, see Figure 5) contained the left arm of Tn3-HoHo1;
(2) Any band > 8.4kb (the size of the EcoRI insert in pPN385 plus the other end fragment of Tn3-HoHo1, see Figure 9) contained the right arm of Tn3-HoHo1.

From this analysis the position and orientation of inserts in the 2.3 and 2.5kb fragments could be mapped, but because the EcoRI/HindIII fragments are very similar in length (2.3 and 2.5kb respectively), the orientation of the Tn3-HoHo1 inserts within the 3.6kb region was difficult to determine. To solve this

Figure 14. HYBRIDIZATION OF THE 2.5kb **EcoRI/HindIII** FRAGMENT FROM pPN385 TO PLASMIDS CARRYING THE Tn3-HoHo1 INSERTS IN THE 3.6kb **HindIII** REGION.

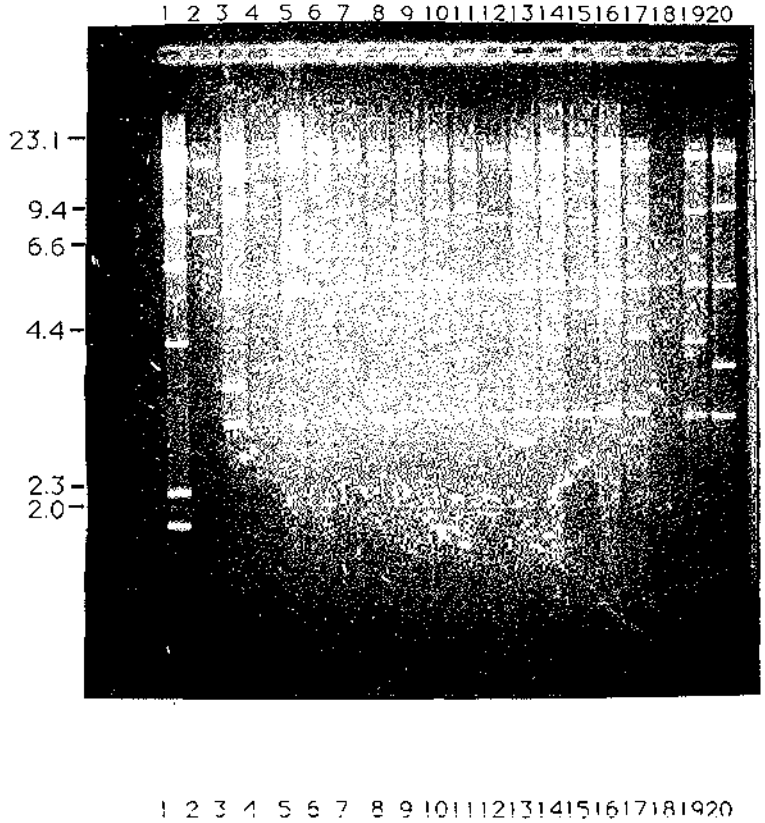
(A) Agarose gel of EcoRI digests of pPN385::Tn3-HoHo1 DNA containing inserts within the 3.6kb region of the 8.3kb fragment.

Lane 1 contains the lambda HindIII size markers.
Lane 2 contains an EcoRI digest of pPN385 DNA.
Lanes 3 to 20 contain EcoRI digests of pPN385::Tn3-HoHo1 DNA.
Note: The high molecular weight band present in lanes 3 to 20 corresponds to the helper plasmid RK2013.

(B) Autoradiograph of the same gel hybridized with the [^{32}P] labelled 2.5kb EcoRI/HindIII region from pPN385 to determine the orientation of the pPN385::Tn3-HoHo1 inserts into the 3.6kb HindIII fragment.

Lane 1 contains the lambda HindIII size markers.
Lane 2 contains an EcoRI digest of pPN385 DNA.
Lanes 3 to 20 contain EcoRI digests of pPN385::Tn3-HoHo1 DNA.

A


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
23.1
9.4
6.6
4.4
2.3
2.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

B


23.1
9.4
6.6
4.4
2.3
2.0

problem EcoRI digested plasmid DNA was run on a gel, Southern blotted on to nitrocellulose, (section 2.13), and probed (section 2.12) with the 2.5kb EcoRI/HindIII fragment from pPN385.

A typical set of results is shown in Figure 14b.
Bands which have shown up on the blot (excluding lane 1) identify the EcoRI band homologous to the EcoRI/HindIII 2.5kb fragment. Comparison of the position of the hybridizing band (Fig. 14b) to the original digestion pattern (Fig. 14a) shows that the probed bands corresponded to either the left arm or the right arm of Tn3-HoHo1 but not both (Table 2).

From these observations the following deductions could be made:

(1) Any band < 5.15kb (the size of one end fragment of Tn3-HoHo1, see Figure 5) contained the left arm of Tn3-HoHo1;
(2) Any band > 8.4kb (the size of the EcoRI insert in pPN385 plus the other end fragment of Tn3-HoHo1, see Figure 9) contained the right arm of Tn3-HoHo1.

Hence the orientation and position of the Tn3-HoHo1 inserts within the 8.3kb EcoRI fragment could be determined. All these inserts (with the exception of one within the 2.5kb region) were grouped and mapped to thirty specific points within the 8.3kb fragment, as shown in Figure 15.

In order to study the activation of the Rhizobium promoters within the plasmid pPN385, a selected number (28) of the pPN385::Tn3-HoHo1 inserts were transferred into a Rhizobium loti wild type background and selected for transfer by plating on S20 media containing Str and Tet.

This was achieved by a triparental cross (section 2.17) involving a range of Tn3-HoHo1 inserts within the pPN385 plasmid. Crosses of pLAFR1 and pPN385 alone were also carried out to use as controls for assaying lacZ β-galactosidase activity (section 3.3).

Table 2. SUMMARY OF THE FRAGMENT SIZES OF pPN385::Tn3-HoHo1 PLASMIDS DIGESTED WITH EcoRI.

Each number represents in most cases several insertions.
The continuous line stretching from some numbers represents a common band in all of the lanes of the gel.
The underlined numbers represent the bands which hybridized to the 2.5kb EcoRI/HindIII probe of pPN385, as shown in figure 14.

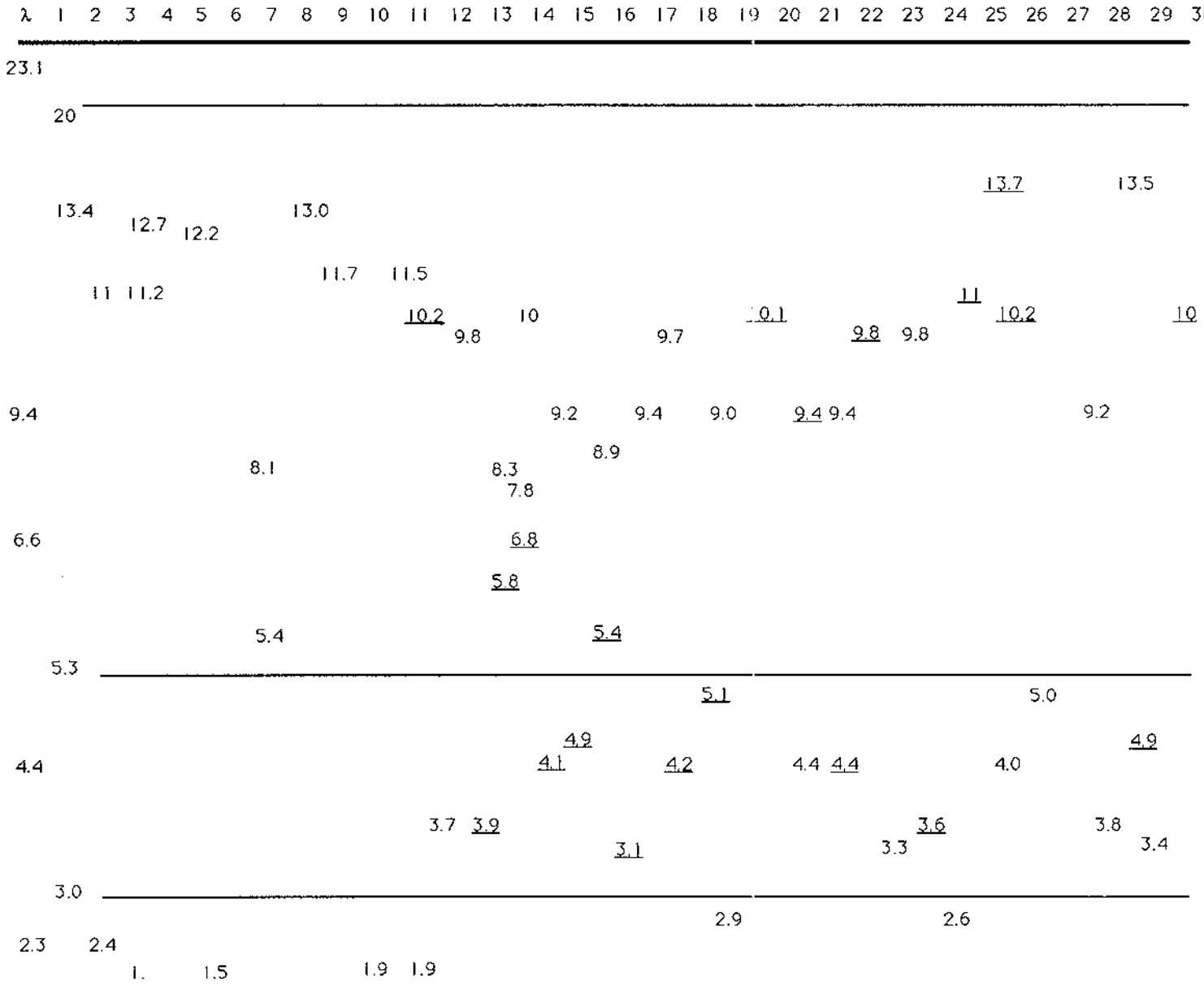
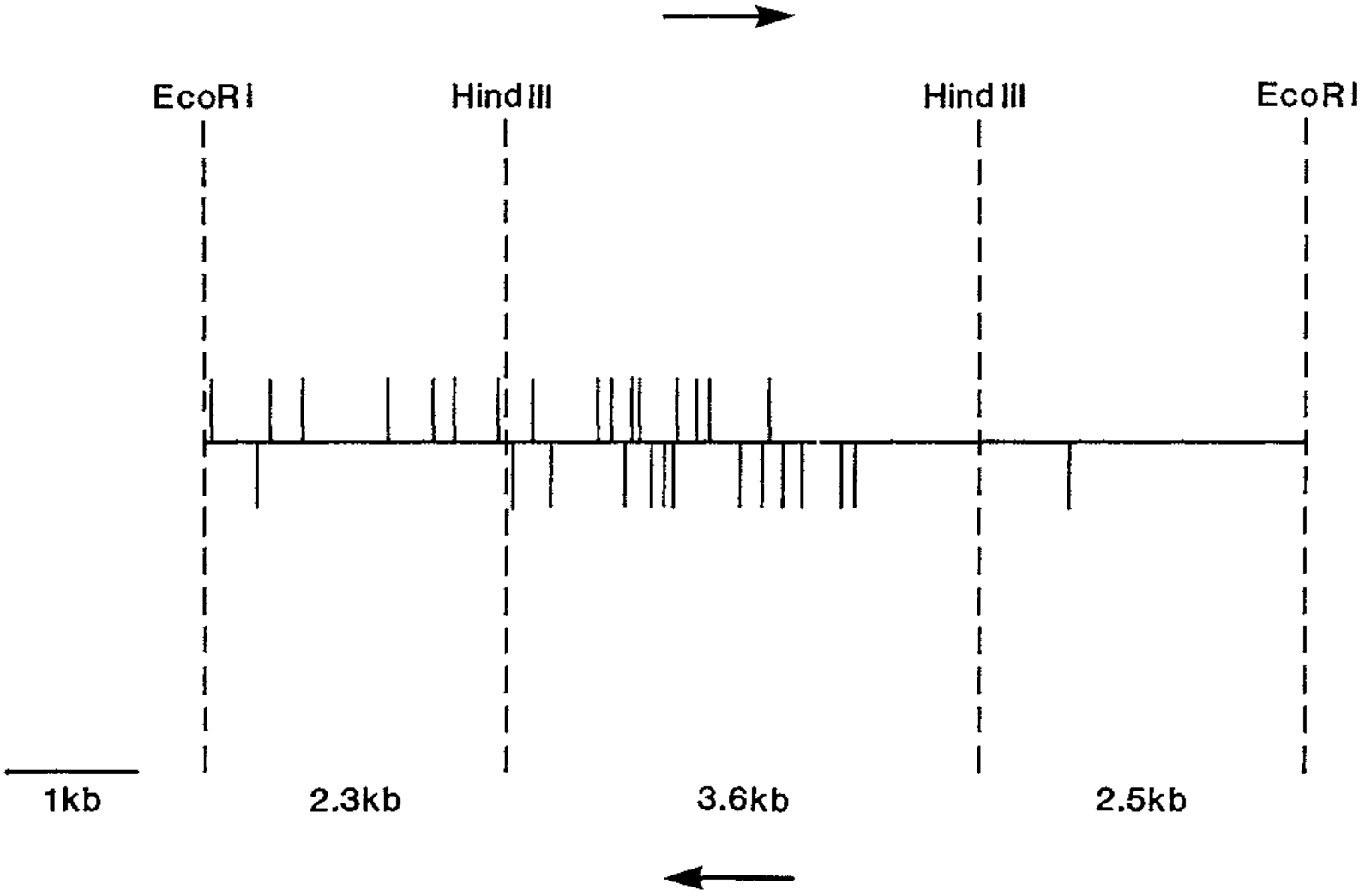

λ 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
23.1
20
13.4
12.7
12.2
13.0
11.7
11.5
11
11.2
10.2
9.8
10
9.7
10.1
9.8
9.8
11
13.7
10.2
13.5
10
9.4
9.2
9.4
9.0
9.4
9.4
9.2
8.9
8.1
8.3
7.8
6.6
6.8
5.8
5.4
5.4
5.3
5.1
5.0
4.9
4.1
4.2
4.4
4.4
4.0
4.9
4.4
3.7
3.9
3.1
3.3
3.6
3.8
3.4
3.0
2.9
2.6
2.3
2.4
1.
1.5
1.9
1.9

Figure 15. MAP OF THE Tn3-HoHo1 INSERTION SITES IN THE 8.3kb FRAGMENT OF THE PLASMID pPN385.

The orientation of the inserts in each region within the 8.3kb fragment are indicated by arrows.

Map of Tn3 - HoHo1 Inserts into pPN385



3.2) THE DIRECTED APPROACH TO CONSTRUCTING R.loti nod PROMOTER lacZ TRANSCRIPTIONAL FUSIONS.

Due to the difficulty in achieving complete saturation of the 8.3kb fragment with Tn3-HoHo1 inserts and with the availability of more sequence information on specific locations of nod genes in Rhizobium loti strains NZP2213 and NZP2037, a more directed approach to constructing lacZ transcriptional fusions was undertaken.

Three regions of interest were chosen. The first region, a 4.1kb SalI fragment from strain NZP2213 containing a "nod box" and a nodD gene (Scott et al., Pers. Comm.), situated slightly upstream from, and overlapping with the 8.3kb fragment (Fig. 16).

A second sub-region of this fragment was used to test the limits of the promoter activity. This was the 0.65kb EcoRI fragment from strain NZP2213 containing part of a nodD gene, and a "nod box" promoter region, situated immediately adjacent to the 8.3kb fragment (Fig. 16).

The third fragment used was a 1.4kb SalI fragment from NZP2037 known to contain (Collins-Emerson pers. comm.) a "nod box", the nodA gene (see Fig. 4) and part of the nodC gene (Fig. 17).

3.2.1 Construction of a lacZ fusion to the 4.1kb SalI Fragment from strain NZP2213.

During the course of the Tn3-HoHo1 8.3kb experiments it was found that the 0.65kb fragment, situated just upstream from the 8.3kb fragment (Fig. 16), had sequences resembling the accepted "nod box" and a nodD gene (Scott et al. unpublished). This region was also found to hybridise with two regions within the 8.3kb fragment, suggesting that there are multiple nodD genes at these positions.

Given that the 0.65kb fragment lacked sequences downstream of the "nod box" the initial approach was to clone a larger 4.1kb SalI fragment spanning this region (see Fig. 16a).

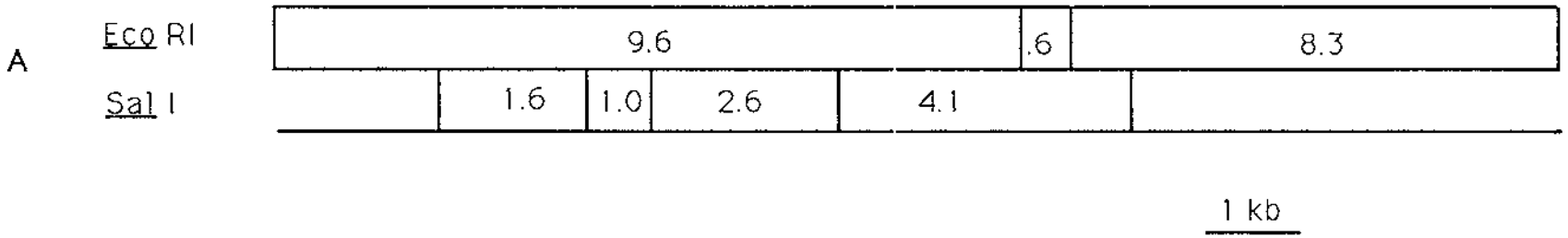
Figure 16. MAPS OF THE REGIONS WITHIN THE **R.loti** STRAIN NZP2213 USED IN CONSTRUCTING **nod-lacZ** FUSIONS.

(A) Map of the regions of interest for lacZ fusions within R.loti strain NZP2213.

The top line being the EcoRI digestion pattern showing the 8.3kb EcoRI fragment and the 0.65kb EcoRI fragment. The bottom line being the SalI digestion pattern showing the SalI fragment.

(B) A DETAILED RESTRICTION MAP OF THE 4.1kb **SalI** REGION OF **R.loti** STRAIN NZP2213.

Showing the restriction sites for SalI, EcoRI, ClaI and PstI, and the relationship between the 4.1kb SalI fragment and the 0.65kb EcoRI fragment.


A
Eco RI
Sal I
9.6
.6
8.3
1.6
1.0
2.6
4.1
1 kb

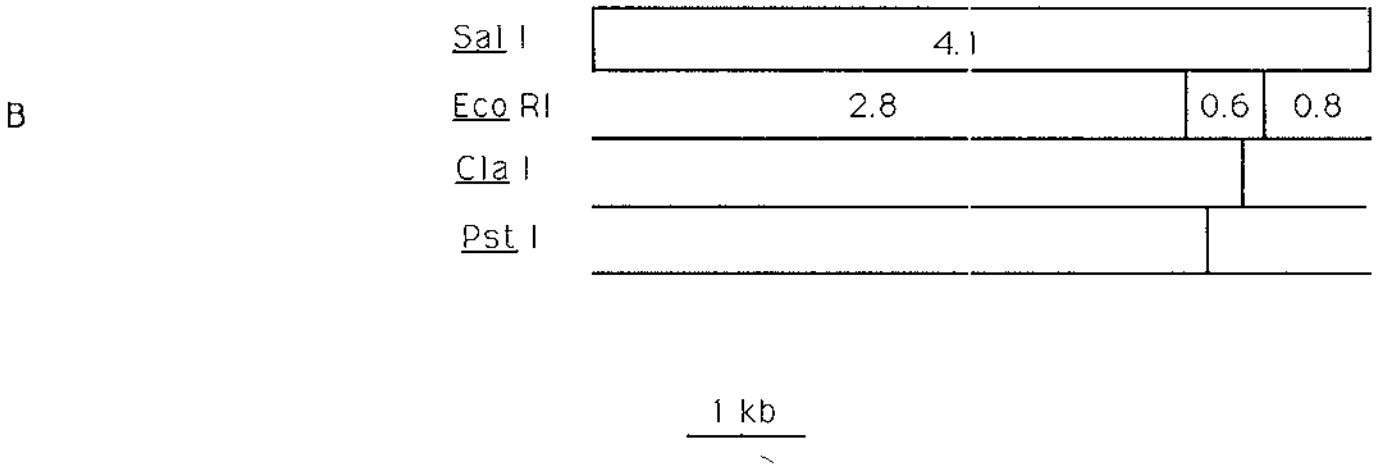
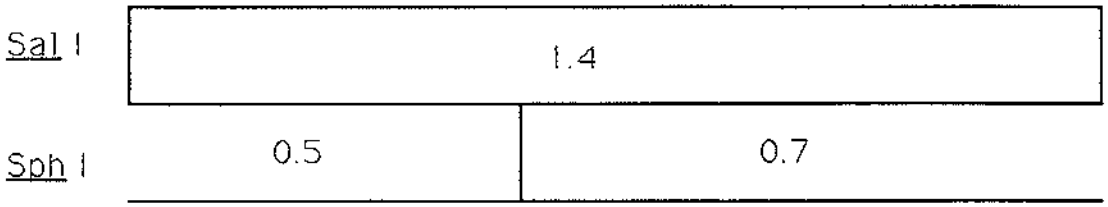

B
Sal I
Eco RI
Cla I
Pst I
4.1
2.8
0.6
0.8
1 kb

Figure 17. RESTRICTION MAP OF THE 1.4kb **SalI** FRAGMENT FROM **R.loti** STRAIN NZP2037.

The first line shows the restriction pattern with SalI, the second line demonstrates where the SphI enzyme cuts within the 1.4kb SalI fragment.
Note: The 4.1kb SalI fragment lies within the 7.1kb EcoRI fragment containing nodA as shown in Figure 4.


Sal I
1.4
Sph I
0.5
0.7
0.1 kb

To analyse any promoter activity of the 4.1kb fragment, a series of experiments were carried out in which the 4.1kb fragment was inserted into the polylinker region, of the lacZ transcriptional fusion vector pMP190 (Fig. 18b). Promoter activity resulting from the 4.1kb fragment would induce the lacZ gene to produce the β-galactosidase product, which can then be assayed.

The 4.1kb SalI fragment from the plasmid pPN366 (Fig. 4) was isolated by first colony purifying the bacterial strain (PN464), isolating plasmid DNA by the rapid boil procedure (section 2.6.2), and phenol-chloroform (section 2.7) extracting it. The purified DNA was then digested with SalI (section 2.8) and run on a minigel (section 2.9) to ensure digestion was complete. The digested DNA was then run on a Seaplaque gel (section 2.9) and the 4.1kb band extracted from the gel (section 2.11).

To obtain sufficient quantities of the 1.4kb SalI fragment it was first cloned into pUC118 (Fig. 6a). This was achieved by the following steps. The initial step was to treat the vector with calf alkaline phosphatase (section 2.14) to ensure no self ligation occurred. The vector was then ligated to the 4.1kb fragment (section 2.15) and checked on a minigel. The ligated DNA was then transformed into MC1022 (section 2.16) and white colonies selected for on LB agar containing Amp and X-gal. The transformants were then checked by the isolation of plasmid DNA (section 2.6.2), followed by SalI digestion and analysis on a minigel (results not shown) to ensure that only two bands (4.1kb and 3.2kb) were present.

The plasmid DNA from one such colony was isolated (section 2.6.2), purified by phenol-chloroform extraction (section 2.7), SalI digested, and run on a minigel to check digestion was complete. The digested DNA was then run on a Seaplaque gel and the 4.1kb fragment was isolated. The vector, pMP190, was first treated with calf alkaline phosphatase then ligated to the 4.1kb fragment (section 2.15) and checked on a minigel against un-ligated DNA. The ligated plasmid DNA was then transformed into HB101 and transformants selected on LB agar containing Cam (20μg/ml). The transformants were then checked by the isolation of plasmid DNA followed by SalI digestion and analysis on a minigel, to ensure only two bands (15kb and 4.1kb) were present.

To determine the orientation of the 4.1kb fragment within the pMP190 vector (Fig. 18b) the following steps were carried out. Plasmid DNA from seven pMP190-4.1kb recombinant clones was isolated by the rapid boil method

Figure 18. PHYSICAL MAPS OF BROAD HOST RANGE EXPRESSION VECTORS CONTAINING A PROMOTERLESS **E.coli lacZ** GENE.

(A) The board host range transcription fusion vector pMP220.

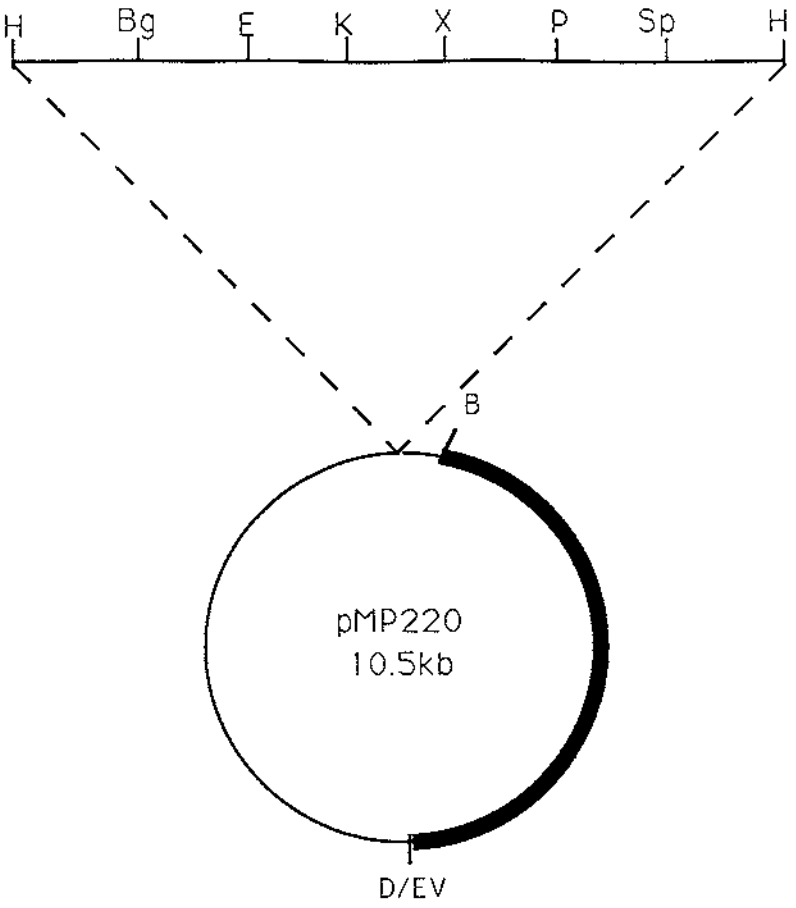
The pMP220 vector is an IncP, 10.5kb plasmid containing unique cloning sites in the polylinker region and a tetracycline resistance marker.

(B) The board host range transcription fusion vector pMP190.

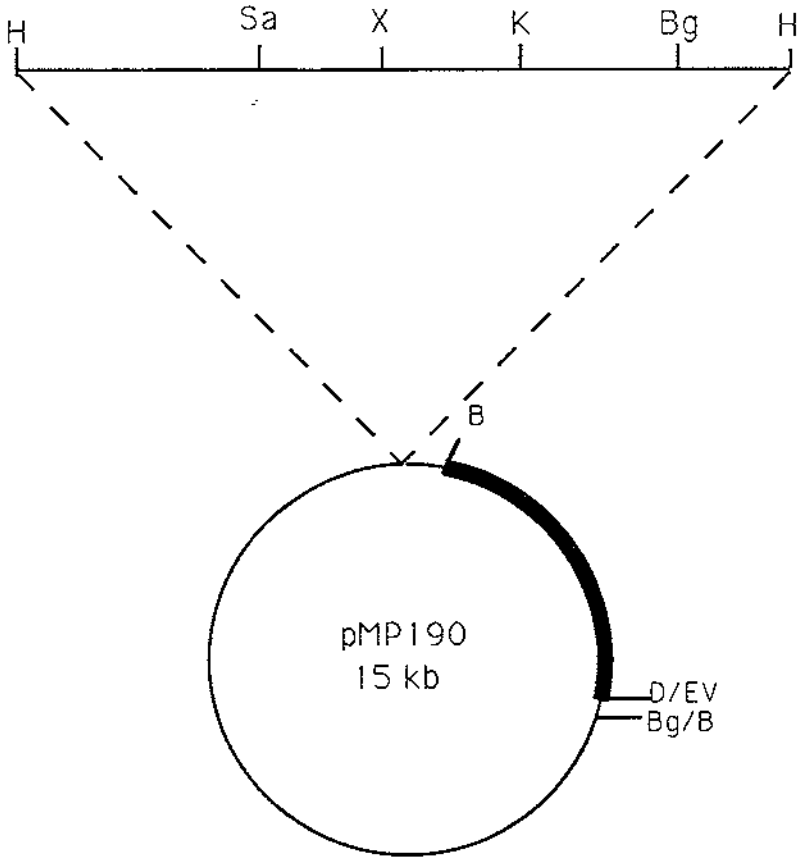
The pMP190 vector is an IncQ, 15kb plasmid containing unique cloning sites in the polylinker region, streptomycin and chloramphenicol resistance markers.

Plasmids and polylinkers have not been drawn to scale.
The black area contains the E.coli lacZ gene devoid of a lac promoter and operator.
E, EcoRI; H, HindIII; B, BamHI; D, DraI; Sp, SphI; P, PstI; X, XbaI; K, KpnI; Sa, SalI; Bg, BglII; EV, EcoRV.

A


H
Bg
E
K
X
P
Sp
H
B
pMP220
10.5kb
D/EV

B


H
Sa
X
K
Bg
H
B
pMP190
15 kb
D/EV
Bg/B

(section 2.6.2), double digested with ClaI then KpnI (section 2.8), RNAased with a 1/50 dilution of RNAase, run on a minigel to check for complete digestion, then on a 1% overnight gel as shown in Figure 19. The ClaI/KpnI combination of enzymes was chosen as there are no KpnI sites in the insert but one in the vector and one ClaI site had been identified within the 0.65kb fragment (see Fig. 16). No KpnI/ClaI map of the 4.1kb SalI fragment was avaliable but the orientation of the fragment could be deduced.

The orientation of the 4.1kb fragment within the pMP190 vector was determined from the pattern of fragments observed in Figure 19.

The two unique patterns observed, corresponding to the two orientations are shown in lanes 4 to 9. The pattern in lanes 4 (pPN38), 5, 6, 7, and 9 consisted of three bands of; 15kb, 3.1kb and 1.05kb. The pattern observed in lane 8 (pPN37) consisted of three bands of; 17kb, 1.05kb and 0.9kb.

From these results the following deductions could be made;

1) The 1.05kb band (which was also observed in vector only, Lane 3) is vector DNA,
2) That the differences in the two patterns are a result of two different orientations.

Therefore the orientations could be resolved:

Orientation 1.

(left to right with respect to Figure 20) produces fragments of 17kb, 1.05kb and 0.9kb when double digested with KpnI and ClaI (Fig. 19 lane 8 corresponds to this, pPN37).

Orientation 2.

(right to left with respect to Figure 20) produces fragments of 15kb, 3.1kb and 1.05kb when double digested with KpnI and ClaI (Fig. 19 lanes 4, 5, 6, 7, and 8 correspond to this). One such clone (Fig. 19 lane 4) was kept and designated as pPN38.

3.2.2 Construction of a lacZ fusion to the 0.65kb EcoRI Fragment from strain NZP2213.

In order to determine whether the 0.65kb EcoRI fragment (Fig. 16) from strain NZP2213 contains a functional promoter, a set of experiments were carried out in which the 0.65kb fragment was inserted into the polylinker region, of the

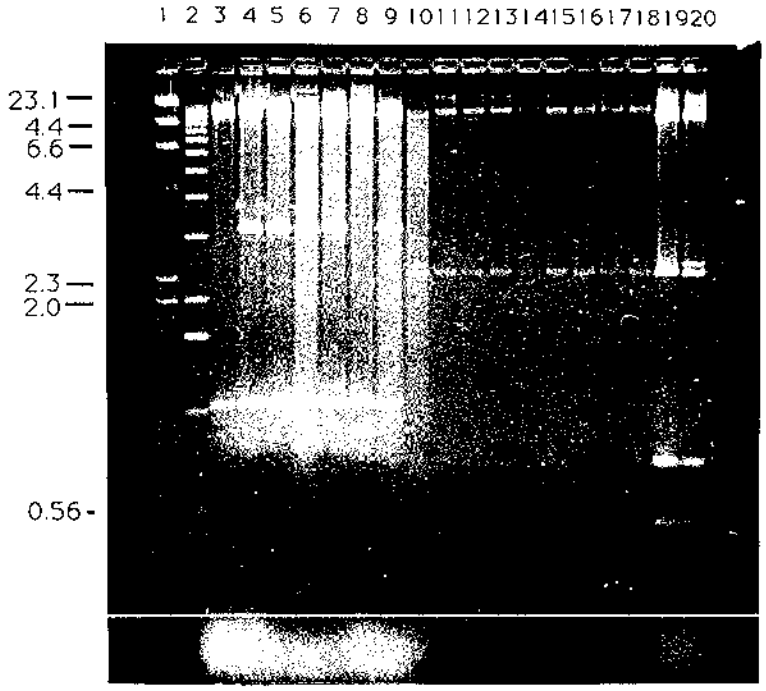
Figure 19. AGAROSE GEL OF DNA DIGESTS USED TO DETERMINE THE ORIENTATION OF THE 4.1kb FRAGMENT WITHIN THE pMP190 VECTOR.

Lane 1 contains the lambda HindIII size markers.

Lane 2 contains the BRL 1kb size markers.

Lanes 3 to 9 are ClaI/KpnI double digests of 7 pMP190-4.1kb SalI recombinant plasmid DNA preparations.

Lanes 10 to 20 are SphI digests of pMP190-1.4kb SalI recombinant plasmid DNA preparations.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
23.1
4.4
6.6
4.4
2.3
2.0
0.56

plasmid pMP220 (Fig. 18a), upstream of a promoterless lacZ gene. Therefore any promoter activity within the 0.65kb fragment induces lacZ to produce the β-galactosidase product which, can then be assayed.

The initial step in this process was the insertion of the 0.65kb fragment into the vector pMP220. This was achieved by using the bacterial strains PN1165 (containing pPN30) and PN1175 (containing pMP220). These strains were first colony purified, to ensure pure cultures, then the plasmid DNA was isolated from both strains by the rapid boil method (section 2.6.2), purified by phenol-chloroform extraction (section 2.7) and digested with EcoRI (section 2.8). The samples were then run on a minigel (section 2.9) to ensure digestion was complete. The digested DNA from each strain was then run on separate Seaplaque gels (section 2.11) and the required band (0.65kb from PN1165 and 10.5kb from PN1175) from each gel extracted. To clone the 0.65kb fragment into the polylinker region of the pMP220 vector, a ligation (section 2.15) of the EcoRI digested DNA was carried out. The first step in this process was to treat the pMP220 vector with calf alkaline phosphatase (section 2.14), to stop self ligation. This treated DNA was ligated to the 0.65kb fragment (section 2.15) and the ligation checked on a minigel. The ligated DNA was then transformed (section 2.16) into HB101 and transformants selected by plating on LB agar containing Amp. The transformants were then checked by the isolation of the plasmid DNA (section 2.6.2), digestion with EcoRI and analysis on minigels to ensure that only two bands (10.5kb and 0.65kb) were present (results not shown).

To determine the orientation of the 0.65kb fragment within the pMP220 vector the following steps were carried out. Plasmid DNA from each of the sixteen 0.65kb recombinants was isolated by the rapid boil method (section 2.6.2), digested with PstI (section 2.8) to completion, RNAased with a 1/50 dilution of RNAase, then run on a 0.4% overnight gel. The separation of these samples is shown in Figure 21.

The orientation of the 0.65kb fragment within the pMP220 vector was determined from the size of the fragments observed on the gel (Fig. 21).

The two unique patterns observed are shown in lanes 2 to 17. The observed pattern for lanes 2, 4, 9, 11, 12, 14, 15, and 16 consisted of one band of 10.5kb. The observed pattern for lanes 3, 5, 6, 7, 8, 10, 13, and 17 consisted of one

Figure 20 MAP OF pPN37.

Map of pPN37 showing the orientation of the 4.1kb SalI nod fragment cloned into pMP190 (see figure 18). Insertion of the 4.1kb SalI fragment in the opposite orientation (orientation 2) gives rise to pPN38.
The scale for the insert is 1cm = 1kb.
The plasmid and the polylinker region are not drawn to scale. Sa, SalI; E, EcoRI; H, HindIII; X, XhoI; K, KpnI; Bg, BglII; B, BamHI; D, DraI; EV, EcoRV.

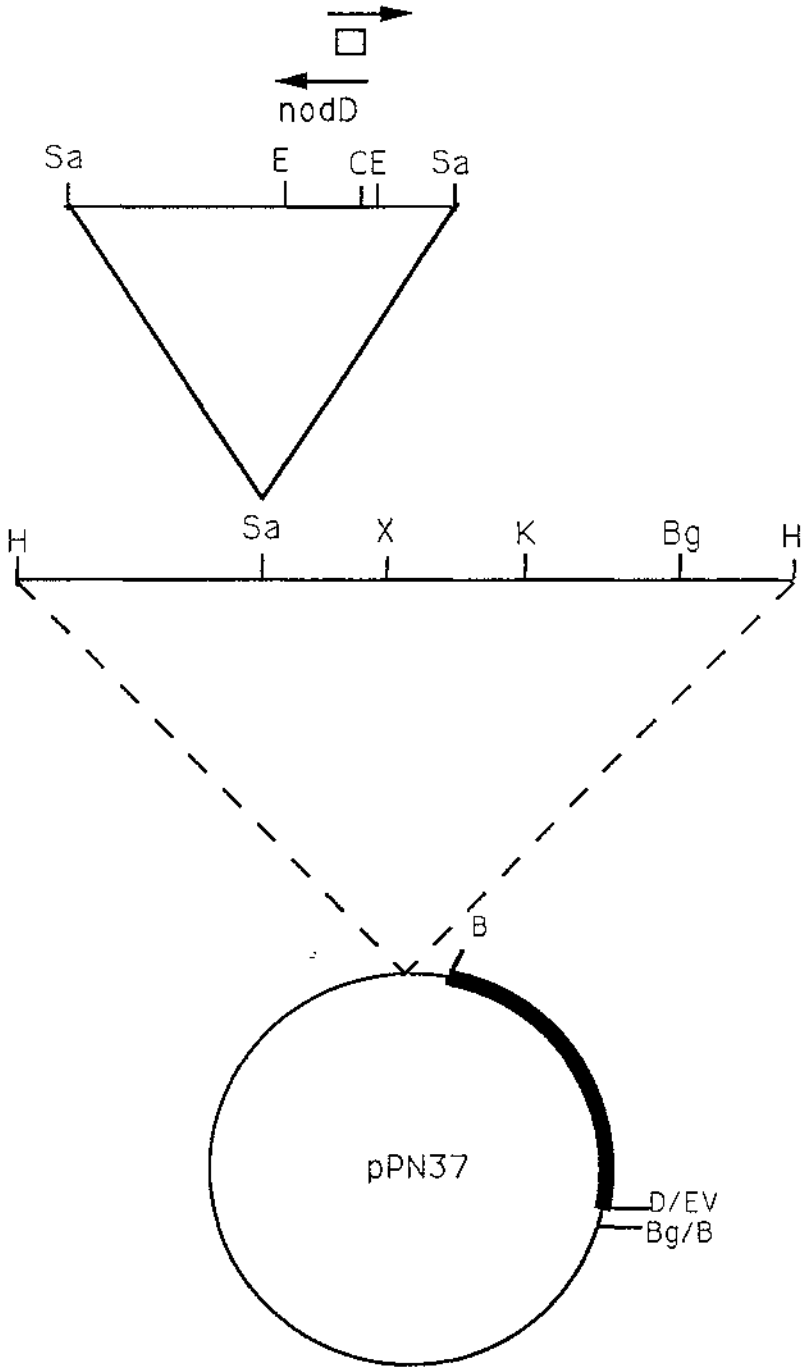
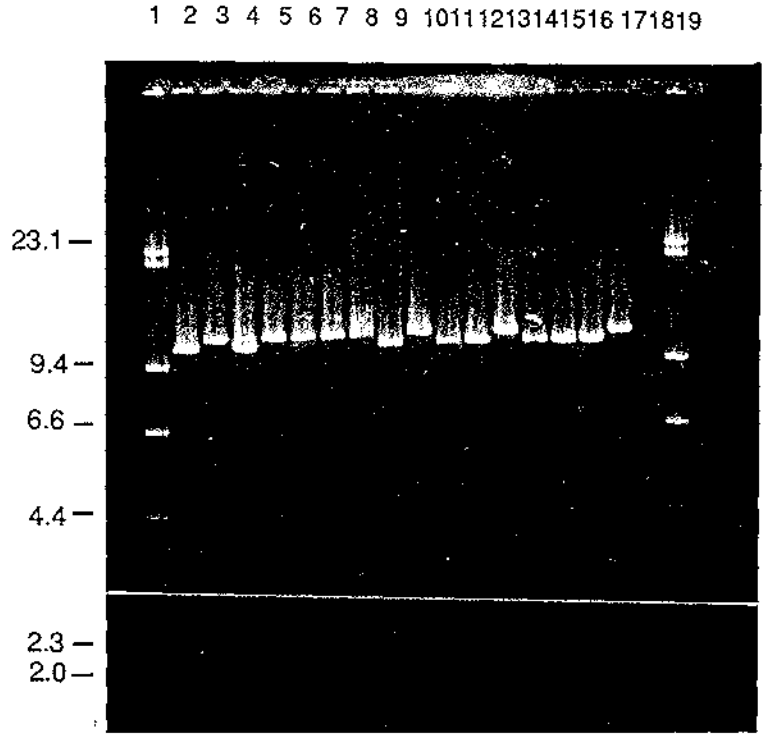

nodD
Sa
E
CE
Sa
H
Sa
X
K
Bg
H
B
pPN37
D/EV
Bg/B

Figure 21. AGAROSE GEL OF **PstI** DNA DIGESTS USED TO DETERMINE THE ORIENTATION OF THE 0.65kb FRAGMENT INSERTS INTO THE pMP220 VECTOR.

Lanes 1 and 19 contain the lambda HindIII size markers.
Lanes 2 to 18 contain PstI digests of 16 pMP220-0.65kb recombinant plasmid DNA preparations.
Note: the gel only shows the larger of the two bands because of the percentage gel and the time required to separate the larger bands is sufficient for the smaller bands to have disappeared off the end of the gel.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
23.1
9.4
6.6
4.4
2.3
2.0

band of 11kb. The predicted PstI digestion pattern (see Figure 22) for the two orientations was not observed because the running time of the gel was such that the smaller fragments had disappeared off the end of the gel.

From the observed and predicted results the orientations could be resolved:
Orientation 1. (left to right with respect to Figure 22) produces fragments of 11kb and 0.1kb when digested with PstI. Figure 21 lanes 3, 5, 7, 10, 13, and 17 correspond to this orientation, one such clone (lane 7) was kept and designated as pPN41.
Orientation 2. (right to left with respect to Figure 22) produces fragments of 10.5kb and 0.5kb when digested with PstI. Figure 21 lanes 2, 4, 9, 12, 14, 15, and 16 correspond to this orientation, one such clone was kept (lane 9) and designated as pPN42.

3.2.3 Construction of lacZ fusions to the 1.4kb SalI Fragment from strain NZP2037.

The last region cloned into the promoterless lacZ vector was a 1.4kb SalI fragment (Fig. 17) from strain NZP2037. This fragment contained a "nod box", a nodA gene, and the initial part of a nodC gene (Collins-Emerson pers. comm.). The significance of including this region was that sequence data was available (Collins-Emerson pers.comm.) that strongly implicated the presence of a plant inducible nodA promoter within this region.

To analyse the promoter activity of the "nod box" within the 1.4kb SalI fragment, a series of experiments were carried out in which the 1.4kb SalI fragment was inserted into the polylinker region, of the plasmid pMP190, upstream of a promoterless lacZ gene.

The initial step in this process was to isolate the DNA from the vector, pMP190, (Fig. 18b) this was achieved by using the rapid boil method (section 2.6.2), followed by purification by phenol-chloroform extraction (section 2.7). Once the vector DNA was purified, it was cut out with SalI, then treated with calf alkaline phosphatase (section 2.14) to stop self ligation. This vector was then ligated (section 2.15) to a previously purified sample of the 1.4kb SalI fragment (this fragment was isolated and purified from the pPN25 cosmid (Fig. 4) by Robert Cleaver, Molecular Genetics Unit, Massey University). The ligated DNA was checked on a minigel against un-ligated DNA to ensure ligation was

achieved. This DNA was transformed (section 2.16) into HB101 and transformants selected on LB agar containing Cam (20µg/ml). The transformants were then checked by the isolation of plasmid DNA followed by SalI digestion and analysis on a minigel, to ensure only two bands (15kb and 1.4kb) were present.

To determine the orientation of the 1.4kb fragment within the pMP190 vector a series of experiments were carried out. The first of these involved the isolation of plasmid DNA, by the rapid boil method (section 2.6.2), from six transformants. The plasmid DNA was then digested with the enzyme SphI (as the 1.4kb SalI fragment was known to contain a single SphI site asymmetrically positioned from the SalI ends), run on a minigel to ensure digestion was complete, RNAase treated with a 1/50 dilution of RNAase, then run on a 1% overnight gel. The results are shown in Figure 23.

The orientation of the 1.4kb fragment within the pMP190 vector was determined from the digestion patterns observed on the gel (Fig. 23).

Two unique patterns, representing the two different orientations, were observed in Figure 23 (lanes 3 to 8).

The first banding pattern observed (lanes 3, 4, 6, and 8) consisted of the following fragments; 12.3kb, 2.3kb, 0.7kb, 0.45kb and 0.35kb. The second banding pattern observed (lanes 5 and 7) consisted of the following fragments; 12.5kb, 2.3kb, 0.5kb, 0.45kb and 0.35kb. It was observed that lighter bands were present, these were assumed to be partial digests of the DNA.

From these digestion patterns and those observed for SphI digested pMP190 vector DNA of 12kb, 2.3kb, 0.45kb and 0.35kb (Fig. 24b) and SphI digested 1.4kb SalI fragment DNA of 0.7kb and 0.5kb (Fig. 24a) the orientation of the 1.4kb SalI fragment could be determined.

Orientation 1. (left to right as in Fig. 25) produces fragments of 12.5kb, 2.3kb, 0.5kb, 0.45kb and 0.35kb when digested with SphI. Figure 23 lanes 5 and 7 corresponding to this orientation, one such clone (lane 5) was kept and designated as pPN39.

Figure 22. MAP OF THE pMP190 VECTOR CONTAINING THE 0.65kb EcoRI FRAGMENT.

The orientation shown corresponds to pPN41 and the opposite orientation corresponds to that of pPN42.

The plasmid, polylinker and insert are not drawn to scale. E, EcoRI; P, PstI; H, HindIII; Bg, BglII; K, KpnI; X, XbaI; Sp, SphI.

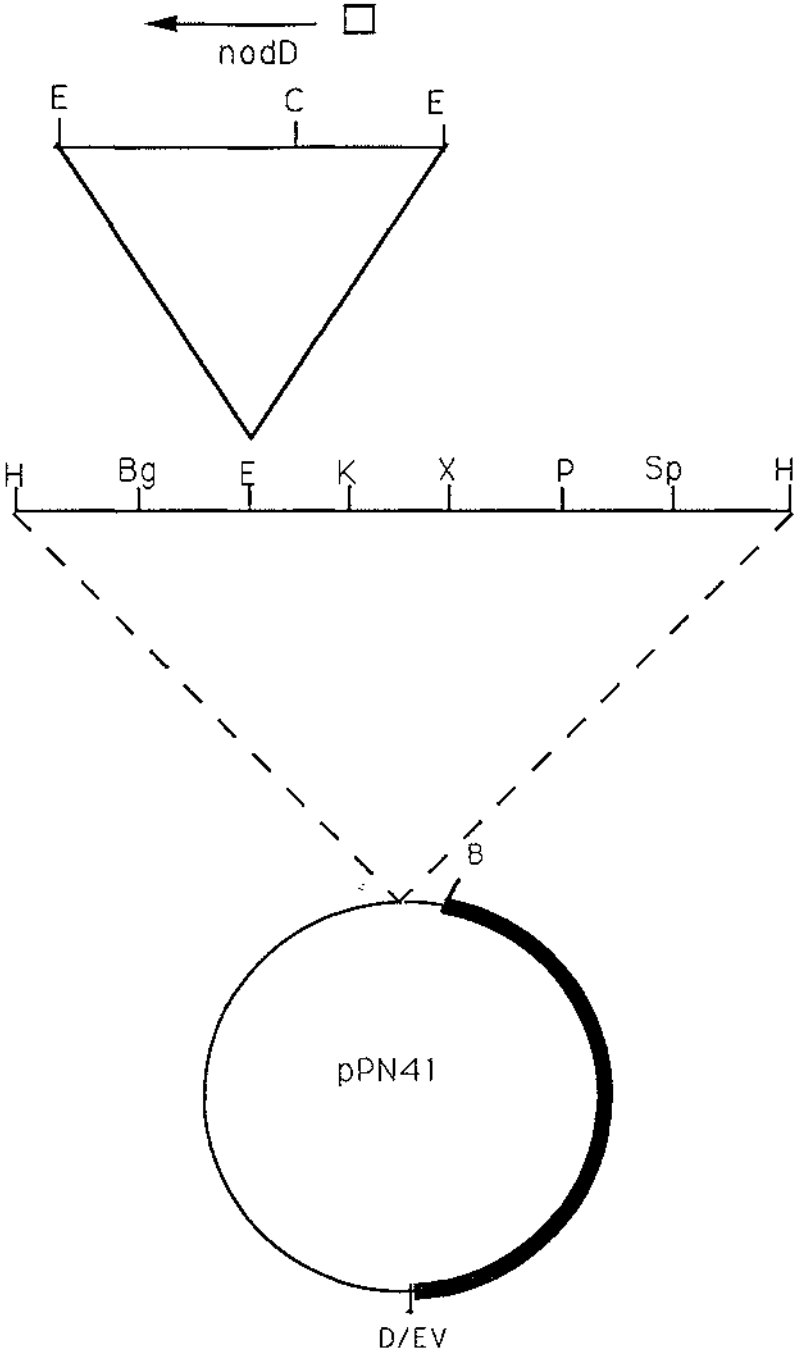

nodD
E
C
E
H
Bg
E
K
X
P
Sp
H
B
pPN41
D/EV

Figure 23. AGAROSE GEL TO DETERMINE THE ORIENTATION OF THE 1.4KB FRAGMENT WITHIN THE pMP190 VECTOR.

Lane 1 contains the lambda HindIII size markers.

Lane 2 contains the BRL 1kb size markers.

Lanes 3 to 8 contain the SphI digests of pMP190-1.4kb recombinant plasmid DNA preparations.

Note: the other bands present at the 2.3kb level are partial digests.

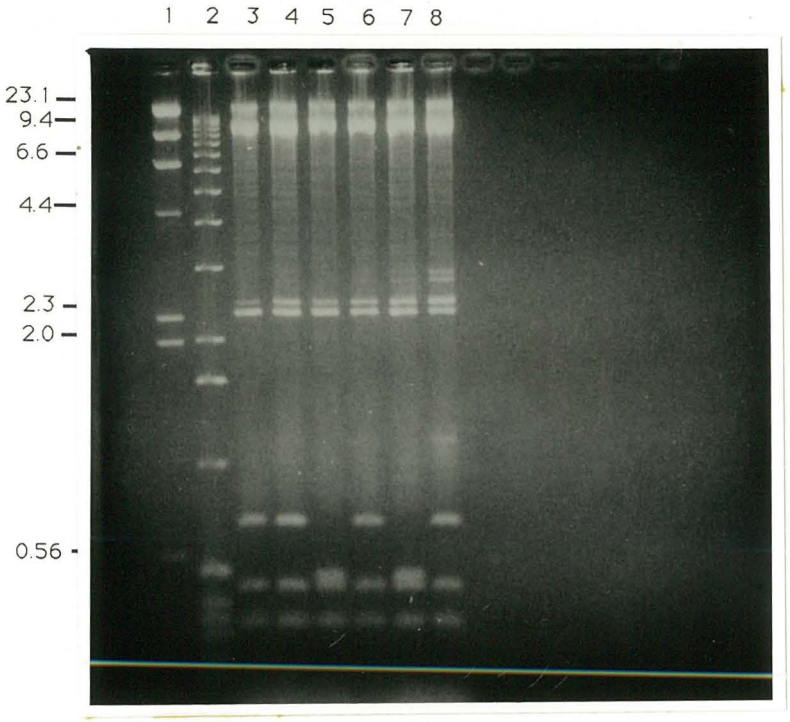

1 2 3 4 5 6 7 8
23.1
9.4
6.6
4.4
2.3
2.0
0.56

Figure 24. AGAROSE GEL TO DETERMINE THE CORRECT DIGESTION PATTERNS FOR THE CONTROLS USED IN THE 1.4kb DIRECTED **lacZ** FUSIONS.

(A) **SphI** DIGESTION PATTERN OF THE 1.4kb **SalI** FRAGMENT ALONE.

Lane 1 contains the lambda HindIII size markers.
Lane 2 contains the BRL 1kb size markers.
Lane 3 contains the SphI digest of the 1.4kb SalI fragment.

(B) THE **SphI** DIGESTION PATTERN OF THE pMP190 VECTOR ALONE.

Lane 1 contains the lambda HindIII size markers.
Lane 2 contains the BRL 1kb size markers.
Lane 3 contains the SphI digest of the pMP190 vector.

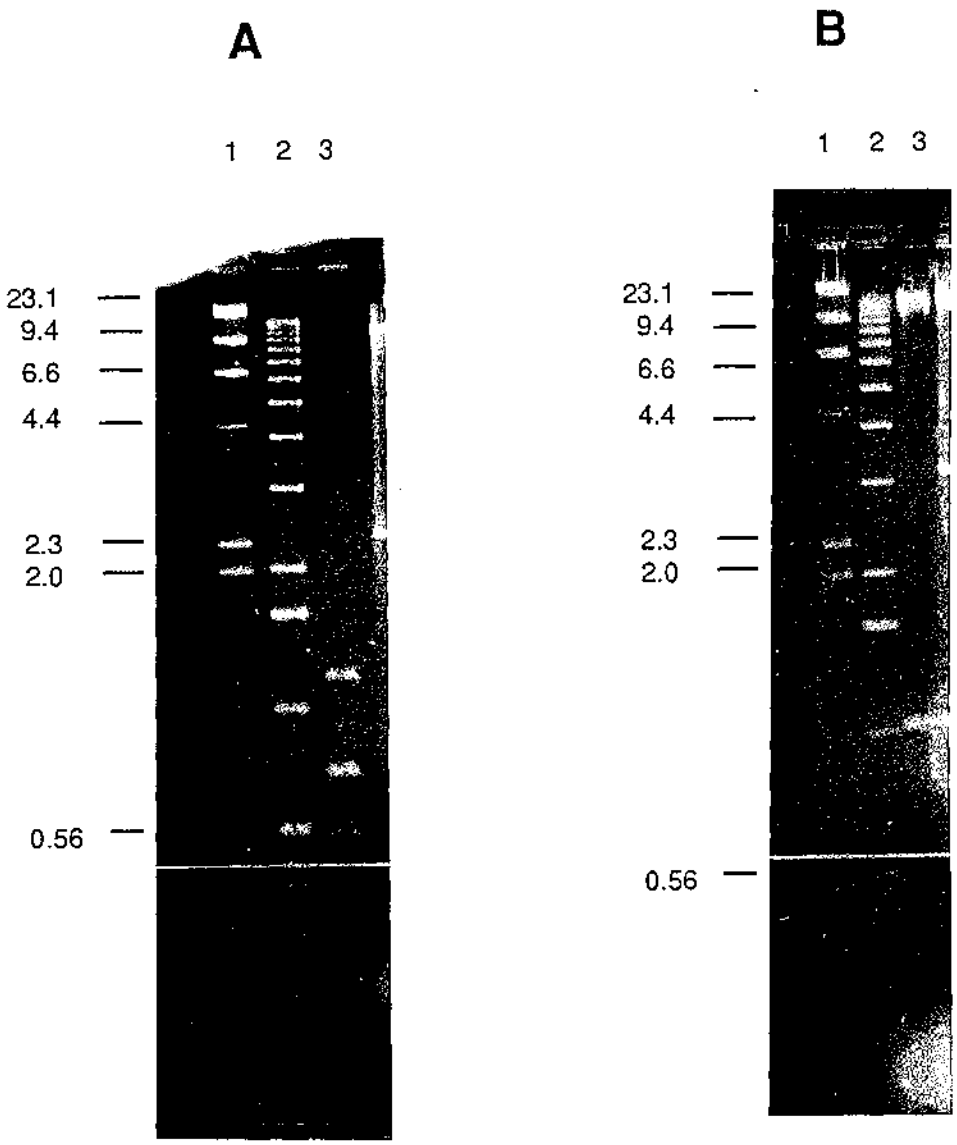
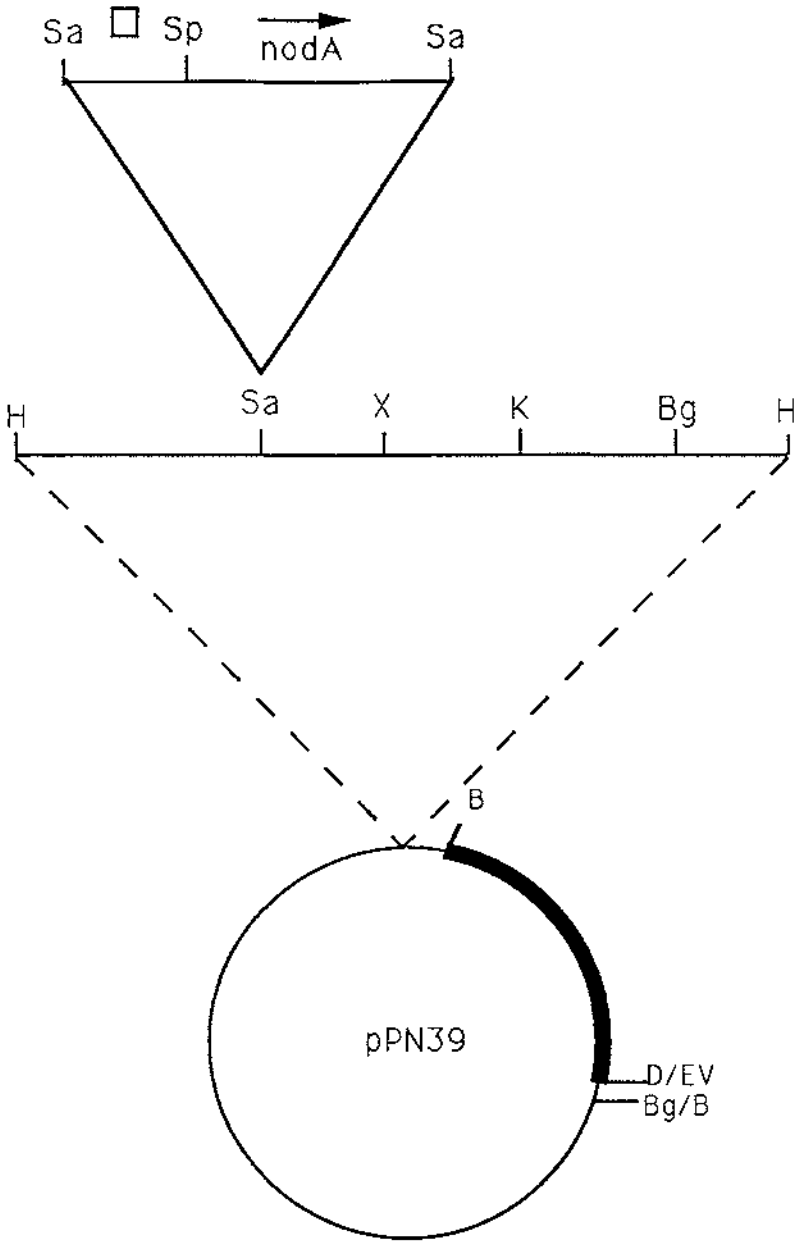

A
1 2 3
23.1
9.4
6.6
4.4
2.3
2.0
0.56
B
1 2 3
23.1
9.4
6.6
4.4
2.3
2.0
0.56

Figure 25. MAP OF pPN39 SHOWING THE 1.4kb **SalI** FRAGMENT OF **R.loti** STRAIN NZP2037 FRAGMENT JOINED TO THE POLYLINKER REGION AT THE **SalI** SITE.

The orientation of the 1.4kb SalI fragment shown corresponds to pPN39 the opposite orientation corresponds to pPN40.
The plasmid, polylinker and insert are not drawn to scale.
Sa, SalI; Sp, SphI; H, HindIII; X, XhoI; K, KpnI; Bg, BglII.


Sa
Sp
nodA
Sa
H
Sa
X
K
Bg
H
B
pPN39
D/EV
Bg/B

Orientation 2. (right to left as in Fig. 25) produces fragments of 12.3kb, 2.3kb, 0.7kb, 0.45kb and 0.35kb when digested with SphI. Figure 23 lanes 3, 4, 6, and 8 corresponding to this orientation, one such clone (lane 4) was kept and designated as pPN40.

3.2.4 Transfer of the three promoterless lacZ fusions into Rhizobium loti.

Representative strains of each orientation were crossed with Rhizobium loti in order to transfer the recombinant plasmid into a wild type background, to enable the Rhizobium promoters within the different regions to be expressed. Transconjugants were selected for on S20 media containing Str and Cam.

This was achieved by a triparental cross (section 2.17) with R.loti strain NZP2213 for recombinants containing the 0.65kb fragment and 4.1kb fragment and with R.loti strain NZP2037 for the 1.4kb recombinant plasmids. In addition the plasmids pMP190, pMP220, and pLAFR3 were each crossed into both NZP2213 and NZP2037 as controls for the β-galactosidase assays. Along with two R.loti NZP2037 strains containing Tn3-HoHo1 inserts, D71 and D95, previously constructed by M^{c}Sweeney (Honours project, 1987), which were used as negative and positive controls respectively.

To confirm that the respective plasmids were transferred to the wild type Rhizobium strains, plasmids present in the transconjugants were analysed by the Eckhardt method (section 2.20). Results are shown in Figure 26.

The first relevant lanes (lanes 3 to 5) showed two different banding patterns. The first pattern, present in lane 3, represents R.loti strain NZP2037 DNA plus pMP190 vector DNA (15kb band) (MG8). The second pattern, present in lanes 4 and 5, represents R.loti strain NZP2037 DNA containing pPN39 (as per Fig. 25) (MG1) and pPN40 (MG2) respectively.

The next relevant lanes (lanes 7 to 9) also showed two different patterns. The first pattern, present in lane 7, represents R.loti strain NZP2213 DNA plus pMP190 vector DNA (15kb) (MG10). The second pattern, present in lanes 8 and 9, represents R.loti strain NZP2213 DNA containing pPN37 (as per Fig. 20) (MG4) and pPN38 (MG5) respectively. The other low molecular weight

band present in lanes 3, 4, 5, 7, 8, and 9 is assumed to be the helper plasmid RK2013. This is shown by comparison to lane 1 (control) which contains R68.45 (60kb) which is similar in size to RK2013.

This shows that the addition of the 1.4kb or 4.1kb fragment increases the size of the lowest band (vector DNA, pMP190) on the gel thus confirming that the plasmids were transferred.

Figure 26. ECKHARDT GEL ANALYSIS OF **Rhizobium** TRANSCONJUGANTS CONTAINING pMP190 RECOMBINANT PLASMIDS.

Lane 1 contains a control sample; E.coli PN480 containing R68.45 (60kb).

Lane 2 contains Rhizobium loti strain NZP2037.

Lane 3 contains Rhizobium loti strain NZP2037 plus pMP190 (15kb) (=MG8).

Lanes 4 and 5 contain Rhizobium loti strain NZP2037 plus pPN39 (MG1) or pPN40 (MG2) respectively (= pMP190 plus the 1.4kb fragment).

Lane 6 contains Rhizobium loti strain NZP2213.

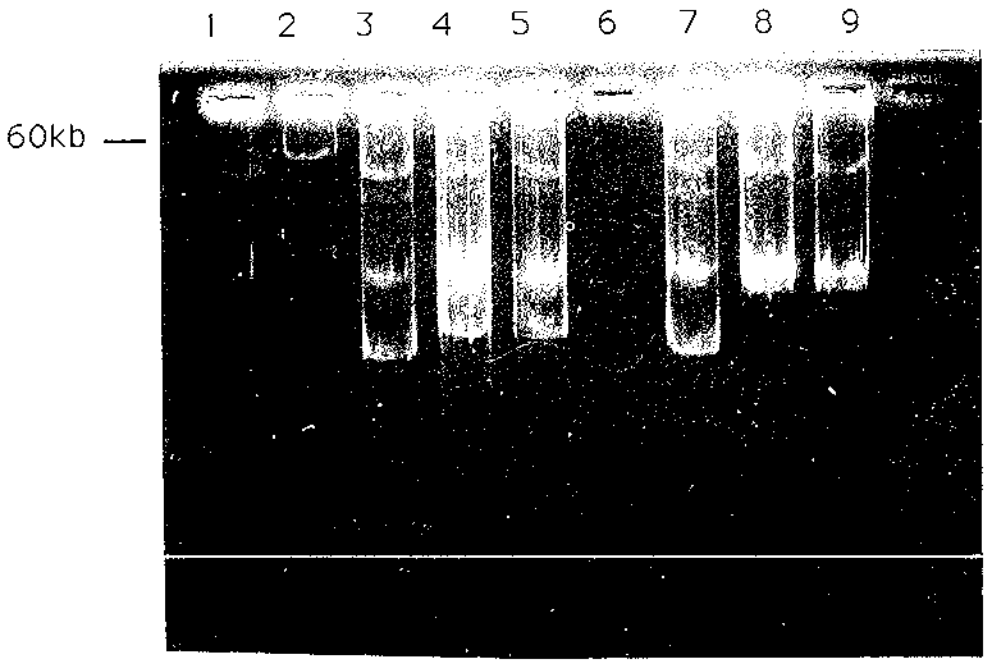
Lane 7 contains (MG10) Rhizobium loti strain NZP2213 plus pMP190 (15kb).

Lanes 8 and 9 contain Rhizobium loti strain NZP2213 plus pPN37 (MG4) or pPN38 (MG5) respectively (= pMP190 plus the 4.1kb fragment).

Note: i) The single large indigenous plasmids of NZP2037 and NZP2213 were presumably not visible in these transconjugants.

ii) The high molecular weight (60kb) band present in lanes 3, 4, 5, 7, 8, and 9 corresponds to the helper plasmid RK2013 (60kb).

iii) The lower bands represent recombinant plasmid DNA and the remaining faint bands could be open circular forms.


1 2 3 4 5 6 7 8 9
60kb

3.3) The β-Galactosidase assays.

Once all the lac constructs, that is random and directed constructs, were crossed into the appropriate R.loti strains, β-galactosidase assays were performed.

To assay for β-galactosidase activity, cells were inoculated in TY media and grown for 2 days in a 30°C shaking incubator (250rpm). A 1/50 dilution of this culture was then transferred into 10ml of M^- media and grown at 30°C for a further 2 days.

β-galactosidase assays were then performed immediately on a sample of these cultures (section 2.19). Further samples were then incubated with and without L. tenuis seed exudate, as described in section 2.19, for a further sixteen hours before assaying for β-galactosidase activity.

The results for the strains analysed are shown in Table 3.

In summary these results show that;

1) A five fold increase in activity for MG1 (pPN39), which contains a nodA lacZ fusion, was observed, whereas no induction was observed for the opposite orientation, MG2 (pPN40) with respect to Figure 17.

2) Very low activity was observed in controls of NZP2037 alone (line 12, table 3), MG8 (line 13) and MG9 (line 14).

3) High β-galactosidase activity in MG4 (pPN37) and MG5 (pPN38) identifies constitutive promoters in both orientations. The higher activity (2x) in MG5 would suggest this is a more active promoter.

4) Only background activity was observed for the small 0.65kb EcoRI fragment cloned in both orientations, MG6 (pPN41) and MG7 (pPN42).

5) Very low activity was also observed in controls of NZP2213 alone (line 1, table 3), MG10 (line 2) and MG11 (line 3).

6) All Tn3-HoHo1 inserts analysed from the 3.6kb HindIII/HindIII (line 9), 2.5kb HindIII/EcoRI (line 10) and 2.3kb HindIII/EcoRI (line 11) were negative suggesting that there are no active promoters (constitutive or inducible) situated upstream of these insertion sites.

7) Two previously isolated (M^{c}Sweeney 1987, Honours Thesis) Tn3-HoHo1 fusions one negative (D71) and one positive (D95) were also included for comparison.

Table 3. INDUCTION OF *Rhizobium loti nod lacZ* FUSIONS BY *Lotus* SEED EXUDATE.

The data represented is the means of four independent experiments and standard errors are indicated.

UNITS OF β-GALACTOSIDASE

STRAIN	0hr MEDIA ALONE	16 hrs MEDIA PLUS WATER	16 hrs MEDIA PLUS EXUDATE
NZP2213 derivatives:			
Wild type	1.3±0	4.4±1.9	4.1±0
MG10	31.1±9.2	46.5±9.3	49.4±14.0
MG11	24.8±2.9	14.5±0.4	26.8±4.4
MG4	144.3±71.4	126.3±27.3	145.9±13.4
MG5	212.6±75.1	312.1±32.2	306.6±103.1
MG6	3.4±1.5	11.9±2.1	16.1±1.5
MG7	9.9±0.5	13.4±5.7	15.4±3.0
MG12	6.5±2.5	2.3±2.9	11.1±4.4
3.6kb	4.9±9.3	7.1±3.5	34.0±13.0
2.5kb	3.5±4.3	7.7±1.4	30.0±8.8
2.3kb	9.7±5.7	13.6±0.3	27.8±2.4
NZP2037 derivatives:			
Wild type	3.3±1.6	2.0±2.4	2.8±0
MG8	23.5±10.5	33.0±4.0	38.8±9.2
MG9	45.8±3.5	32.0±1.3	37.1±3.5
MG1	66.9±7.6	43.8±2.0	263.0±16.9
MG2	17.0±3.7	5.1±0.3	18.4±18.7
MG3	94.2±8.3	83.8±16.3	380.3±11.7
D71	15.4±3.4	13.1±0.3	18.4±2.8
D95	160.3±2.6	116.5±4.6	142.3±5.0

Chapter 4 - DISCUSSION

The aim of this work was to analysis the regulation of nod gene expression in Rhizobium loti strain NZP2213 and NZP20237 by constructing lacZ transcription/translation fusions in nod regions of these strains. Two approaches were used to make these fusions:

i) a random Tn3-HoHo1 method; and

ii) a direct cloning method using the vectors pMP190 and pMP220.

The first approach makes use of a transposon, Tn3-HoHo1, to generate lacZ fusions. This system was used successfully in Agrobacterium tumefaciens for identifying the transcriptional units associated with the vir region (Stachel et al., 1985), and has been used by a number of other groups. Using this method 290 inserts were produced in the 8.3kb nod fragment of R.loti NZP2213. Analysis of the Tn3-HoHo1 inserts into the 8.3kb EcoRI region was achieved by three methods: i) restriction enzyme digestion of the fragments by HindIII/EcoRI double digestion (Fig. 13), ii) single restriction enzyme digestion of the fragments by EcoRI (Fig. 14a), and iii) probing, using a ^{32}P-labelled 2.5kb EcoRI/HindIII fragment, of an autoradiograph containing Tn3-HoHo1 inserts within the 3.6kb HindIII/HindIII region (Fig 14b). The completed analysis of the Tn3-HoHo1 inserts was shown to be clustered into certain regions (Fig. 15).

Through recent work by Young and Scott (In prep) it is now known that a nodB gene is present in the 2.3kb EcoRI/HindIII region of the 8.3kb EcoRI fragment (Fig. 4), however, the "nod box" is in the adjacent 0.65kb EcoRI fragment (Fig. 4), thus any insertions within this region would lack a promoter.

Young and Scott (In prep) also found by hybridisation the presence of a nodD gene, nodD1, in the 3.6kb HindIII/HindIII fragment (Fig. 4), but no information on its direction of transcription is available. If there is a promoter within this region it must be outside the area of the Tn3-HoHo1 insertions. This is possible, as there were regions of the 3.6kb HindIII/HindIII fragment devoid of insertions (Fig. 15).

Another nodD gene (nodD3) was also found within this 8.3kb EcoRI fragment (Young and Scott, In prep.). This copy of nodD was located in the 2.5kb EcoRI/HindIII fragment. However, only one insertion was isolated in this region.

As illustrated above the problem with this system is its randomness ie the position of the insertions cannot be predetermined, however, a full spread of insertions along the fragment should have been achieved. This lack of complete randomness could be attributed to the following factors:

i) insufficient independent crosses carried out,

ii) insufficient Tn3-HoHo1 inserts analysed, and

iii) Tn3 preferentially inserts into AT rich sequences (Heffron, 1983).

With the availability of nod gene sequence data during the course of this work a second approach was undertaken. The second approach to construct lacZ transcriptional fusions was less random and involved the cloning of three separate nod gene fragments containing putative nod promoter elements:

i) a 4.1kb SalI fragment isolated from the nod region of the 8.3kb EcoRI fragment of R.loti strain NZP2213 (Fig. 4),

ii) a 0.65kb EcoRI fragment isolated from the 4.1kb SalI fragment of R.loti strain NZP2213 (Fig. 4), and

iii) a 1.4kb SalI fragment isolated from the 7.1kb EcoRI region of R.loti strain NZP2037 (Fig. 4).

These fragments (4.1kb, 0.65kb and 1.4kb) were isolated and cloned into vectors pMP190, pMP220 and pMP190 respectively, in both orientations. Each lacZ fusion was transferred into R.loti strains from which the fragment had originated ie either NZP2213 or NZP2037.

The 4.1kb SalI construct from R.loti strain NZP2213 was found to have β-galactosidase activity in both orientations (Table 3), indicating that at least two constitutive 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 result for orientation pPN37 was unexpected as we now know, from sequencing studies (Young and Scott, In prep.), that a "nod box" and an open reading frame, homologous to nodB, read into the lacZ present in this orientation (Fig. 4) and therefore we would expect this putative promoter to be inducible by seed exudate, as has been found for other nod promoters identified to date (Mulligan and Long, 1985; Firmin et al., 1987; Fisher et al., 1987a; Redmond et al., 1986; Spaink et al., 1987b). One possible explaination is the presence of a constitutive promoter to the left of this region that is reading through the nod promoter. Further work will be needed to clarify the nature of this promoter.

The smaller 0.65kb EcoRI fragment, that lies within the larger 4.1kb SalI fragment (Fig. 16b), contains a "nod box" and part of a nodD-like gene (Scott et al., In prep.). The nodD-like gene reads into the lacZ in pPN42 and the "nod box" reads into the lacZ in pPN41. No significant β-galactosidase activity was observed in either orientation with or without seed exudate. This result shows that the "nod box" alone is insufficient to act as a promoter. Recent work has shown that the "nod box" is the site of NodD binding for activation of the nod operons (Fisher et al., 1987a; Fisher et al., 1987b; Fisher et al., 1988; Hong et al., 1987 and Spaink et al., 1987a). Fisher et al. (1987b) mapped the transcriptional start sites for R.meliloti nodD, A, F, and H operons by primer extension analysis and showed that the four transcriptional initiation sites are located between the "nod box" and the ATG translational start site. They also showed that that the nodD1 gene, which is divergently transcribed and regulates the other nod operons, is located in the same region. This demonstrates that the "nod box" is required solely as a site for positive regulation, ie the binding of the activated form of NodD. Therefore it is not surprising in retrospect that the 0.65kb EcoRI fragment will not serve as a promoter sequence, as it only contains the "nod box" and not a full promoter.

The 1.4kb SalI construct from R.loti strain NZP2037, that was known to contain a nodA promoter and a "nod box" upstream of this gene (Emerson-Colins pers. comm.), showed inducible expression in pPN39 (Fig. 25), corresponding to a fusion between nodA and lacZ. No significant constitutive or inducible activity was detected in the reverse orientation, corresponding to pPN40. This result is in agreement with work by other groups (Mulligan and Long, 1985; Firmin et al., 1987; Fisher et al., 1987a & b; Redmond et al., 1986; Spaink et al., 1987b). However, in comparison to the β-galactosidase activity observed by these groups, the activities reported here are rather low. This could be a result of the crude exudate used in these β-galactosidase assays compared to the purified chemicals used by these other groups. This low activity could be a result of the presence of inhibitors. Therefore, before any further analysis of this region can begin, the nature of the inducer(s) for R.loti need to be characterised.

In summary, further research needs to be performed on the 8.3kb EcoRI region to show the presence of a strong constitutive promoter which seems to be reading though the nod promoter and characterisation of the inducer is essential to determine if the β-galactosidase results, of the 1.4kb SalI region, are low or reduced by inhibitors in the crude extracts.

CONCLUSIONS

The regulation of nod genes expression in Rhizobium loti was analysed by constructing lacZ transcriptional fusions. Two different approaches were used to make these fusions: i) a random Tn3-HoHo1 method and ii) a direct cloning method using the vectors pMP190 and pMP220. Using Tn3-HoHo1 290 independent lacZ insertions were generated in the 8.3kb EcoRI nod fragment of NZP2213. No significant β-galactosidase activity was detected for any of these insertions. Direct cloning of DNA fragments containing putative nod promoter elements into promoterless lacZ vectors confirmed that the nodA promoter of Rhizobium loti NZP2037 is plant inducible and identified two constitutive promoters on a second nod fragment from Rhizobium loti NZP2213. Presently further investigation is being carried out on both of these nod regions as to identify the sequences behind these mechanisms.

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