THE DETECTION OF PLASMID TRANSFER GENES IN *RHIZOBIUM* SPECIES.

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ABSTRACT

In order that *Rhizobium tra* genes responsible for Sym plasmid transfer might be found, DNA probes were constructed from *Agrobacterium tra* genes. Three probes were constructed from DNA containing ;- 1) *traR,* a gene which regulates other *tra* genes on the *Agrobacterium tumefaciens* plasmid pTiC58, 2) an *OriT* site, at which nickases cleave the plasmid before conjugal transfer can take place, and 3) part of a gene required for construction of a mating bridge.

All three probes were constructed by the ligation of *tra* areas from the *A.tumefaciens* strain C58 plasmid pTiC58 into broad host range plasmid vectors and subsequent electroporation into *E.coli* cells.

The genomic DNA digests of several *Rhizobium* and *Agrobacterium* strains were blotted and probed with the three probes under various washing and hybridisation stringencies.

A.tumefaciens strain LMG64 was the only *Agrobacterium* strain aside from strain C58 to have DNA homologous to any of the probes. Neither *R.leguminosarum* bv trifolii strain ICMP2163, nor *R. leguminosarum* bv trifolii strain ICMP2163::Tn5, nor *R.leguminosarum* bv trifolii strain PN165 had any DNA homologous to any of the probes. However, *R.leguminosarum* bv trifolii strain ATCC14480 showed homology to the *tra I* probe (containing *traR*), and *R.loti* strain ATCC33669, phylogenetically the most distant relative to *A.tumefaciens* strain C58 shared homology with the tra III probe (containing DNA responsible for mating bridge assembly).

Therefore the distribution of *tra* genes from the Ti plasmid of *A.tumefaciens* strain C58 among the agrobacteria and rhizobia used in this study did not correlate to their phylogenetic relatedness to *A.tumefaciens* strain C58 or to one another.

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A : agarose gel. **B** : autoradiograph. The final wash was carried out in 0.1 x SSC at 42°C.

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1.0 Introduction

1.1 Biological Nitrogen Fixation and *Rhizobium*

Nitrogen fixation is not only vital to New Zealand agriculture, but is also crucial to food resources on a global scale as it involves the process of converting atmospheric nitrogen to ammonia. This ammonia can then be used by plants to build amino acids such as glutamine (Grant and Long,1981;Brock and Madigan,1991). Brock and Madigan describe nitrogen fixation **as "one of the most crucial biochemical processes in nature"** (Brock and Madigan,1991). Agricultural products such as wool, meat, fruit and dairy products are huge export earners in New Zealand. In 1990 over $9 billion of New Zealand's $17 billion gross export earnings came from agricultural products (NZ Yearbook, 1993). This represents about 55% of the total export earnings in New Zealand. Therefore nitrogen fixation plays a very important part in the financial welfare of our country.

In those pastures which have low levels of fixed nitrogen, there are two common ways of providing nitrogen in a form which is accessible to pasture plants.

Firstly there is the expensive and ecologically unsound use of man-made fertilizers such as urea and ammonium sulphate. These nitrogen-rich chemicals readily leech from the soil and get into rivers, lakes and underground water systems, causing eutrophication. They are lost to the pasture and require regular replenishment. However, the second method of providing accessible nitrogen to crops and pastures is a natural one. It involves the inclusion of legumes and suitable *Rhizobium* or *Bradyrhizobium* species in pastures. When white clover (*Trifolium repens)* and *Rhizobium leguminosarum* biovar trifolii are added to the soil, the plant and bacteria may form a symbiotic relationship. The *Rhizobium-legume* symbiosis is one of the most efficient nitrogen fixing relationships among the legumes (Grant and Long,1981). There are many important legume crops such as soybeans, peas and alfalfa, but of greater significance to New Zealand agriculture are the pasture legumes such as red and white clover and lucerne. Once established in a field, legumes should last long term, depending on farm management and the environmental conditions. They also retain much of their fixed nitrogen in the form of proteins. Legumes can survive in soils which are already nitrogen deficient.

Nitrogen fixing bacteria of the *Rhizobium* or *Bradyrhizobium* genera form a symbiotic relationship with their host legume. The bacteria are supplied with nutrients, energy and some physical protection against environmental hazards. In return, the bacteria convert atmospheric nitrogen to fixed nitrogen, which the plant can use to build amino acids. The enzyme complex *nitrogenase,* which is present in nitrogen fixing rhizobia, converts N_2 to NH_3 (Grant and Long,1981). This is a crucial step in the nitrogen cycle and is a reduction process which will not work under aerobic conditions. However, a small amount of oxygen is required to provide enough energy for the reaction to work. These conditions are provided within the nodules on the roots of the host legume (figure 1). This is where nitrogen fixing rhizobia are located on the host.

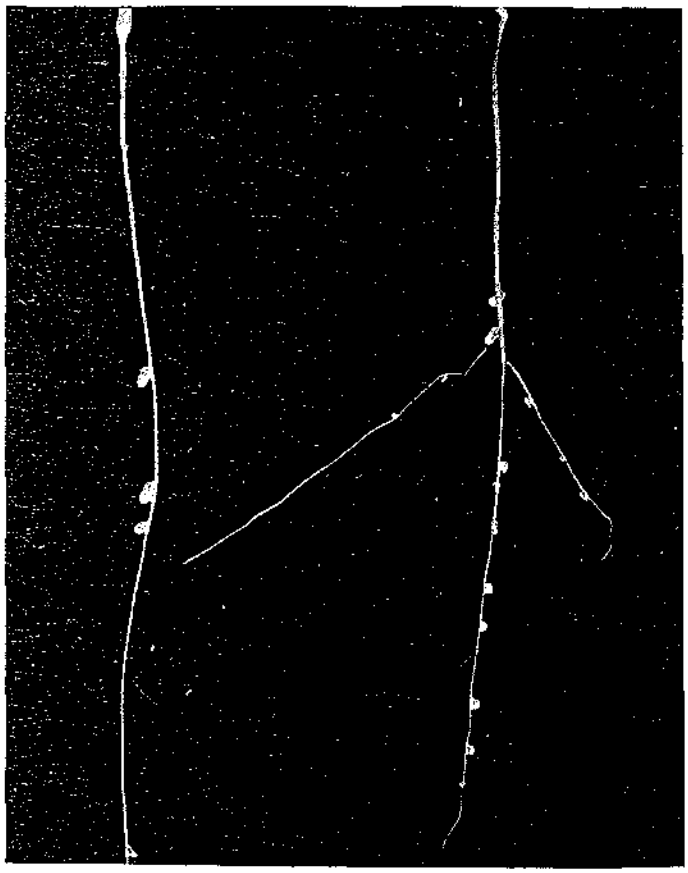
1.2 Characteristics and genetics of rhizobia

Bergey's manual of Systematic Bacteriology (Jordan,1984) indicates that a strain is a *Rhizobium* if it forms nodules on a legume. This statement must be viewed with caution as the genes responsible for nodulation are plasmid-borne (Beringer et al,1978; Brewin et al,1982;De Jong et al,1982;Hirsch et al,1980;Jarvis et al,1989;Schofield et al,1987;Zurkowski and Lorkiewick,1979). If a strain of *R.leguminosarum bv trifolii* loses it's symbiotic *(Sym)* plasmid, it becomes unclassifiable because without a *Sym* plasmid it can't nodulate. Similarly, if a strain of *Agrobacterium tumefaciens* receives a *Sym* plasmid and expresses it to nodulate a legume the *Agrobacterium* strain could be re-classified as a *Rhizobium* species!

1.3 Indigenous soil rhizobia

Many strains which are phylogenetically similar to *Rhizobium* strains are present in the soil, both within and outside of the rhizosphere, but not all are capable of a symbiotic existence (Segovia,1991,Soberon-Chavez,1989). One strain isolated from the soil belonged to the same somatic serogroup as the *R.leguminosarum* bv phaseoli type strain, but could not form nodules. When a *R.leguminosarum* bv phaseoli Sym plasmid was transferred to the soil isolate, the recipient was able to

Figure 1 : Nodules on white clover (*Trifolium repens*).



nodulate and fix nitrogen in bean roots, and could also compete effectively with other indigenous *Rhizobium* strains in the soil (Soberon-Chavez,1989). A number of nonsymbiotic soil bacteria were later shown to have identical 16S rRNA sequences to that of *R.leguminosarum* bv phaseoli, and when complemented with a *R.leguminosarum* bv phaseoli Sym plasmid were able to nodulate and fix nitrogen in bean roots (Segovia,1991). Segovia observed that the ratio of symbiotic *R.leguminosarum* strains to nonsymbiotic *R.leguminosarum* strains was less than 1 in 40, but this number was subject to variation due to changes in soil conditions and methods of sampling (Segovia,1991).

Bacterial strains were also isolated from nodules of the legume *Phaseolus vulgaris,* and they were examined by restriction fragment length polymorphism (RFLP) and 16s rRNA sequence analysis (Laguerre,1993). Two of these strains were shown to have less than 21% relatedness with recognised *Rhizobium* type strains including type strains of *R.etli* and *R.tropici* and less than 18% relatedness with one another (Laguerre,1993).

These and other results suggest that it is important to provide a meaningful biological classification of the genus *Rhizobium* based on phylogenetic, rather than phenotypic traits
(Eardly,1990;Segovia,1991).

It is concluded that perhaps not all field isolates from nodules are *Rhizobium* or *Bradyrhizobium* species, based on phylogenetic traits. If different isolation procedures were used to grow nodule isolates from the field, more species may be found.

According to *Bergey's manual of Systematic Bacteriology* (Jordan,1984) the family *Rhizobiaceae* is one of eight families of Gram negative, aerobic rods and cocci, and four genera are recognised within the *Rhizobiaceae* ;- *Rhizobium, Bradyrhizobium, Agrobacterium* and *Phyllobacterium.* Phylogenetic research, however only partially supports this classification. For instance, rRNA cistron similarities indicate that bradyrhizobia should not be in the same family as rhizobia (Jarvis et al,1986). There is also some debate, based on 23S rRNA

similarities, and from DNA:DNA and 16SrRNA:DNA binding experiments, over whether the genus *Agrobacterium* should be incorporated into the *Rhizobium* genus (De Ley et al,1973;De Ley et al,1974;De Smedt and De Ley,1977;Jarvis et al,1986;Sawada and Ieki, 1992;Willems and Collins,1993). Fig.2 shows the relationship of rhizobia and agrobacteria to one another and to other taxa of the alpha-2 subgroup of the *Proteobacteria.*

Symbiotic (Sym) plasmids bear the genes required for nodulation. Sym plasmids range in size from 130kb (kilobases) to greater than 290kb in *R.leguminosarum* and greater than 1200kb in *Rhizobium meliloti* (Long,1989;Prakash et al,1980). These huge megaplasmids carry many genes, such as *tra, nod, fix* and *nif* genes (Brewin et al,1982;Iisma et al,1989), as well as genes whose products provide antibiotic resistance (Brewin et al,1982), hydrogenase activity (De Jong,1982), bacteriocinic properties (De Jong,1982;Hirsch,1980), affect polysaccharide production (Borthakur et al,1985;Latchford et al,1991), carbon metabolism (Djordjevic,1982) etc. *Tra* genes are responsible for the conjugative transfer of the Sym plasmid to recipient bacteria. *Nod* genes are responsible for the nodulation of the host legume. *Nif* and *fix* genes are responsible for nitrogen fixation. However it is important to note that nitrogen fixation cannot be carried out solely by the expression of the products of genes carried on the Sym plasmid. A number of important genes are present in the chromosome itself. Figure 3 (Iismaa et al,1989) shows the location of *fix, nif* and *nod* genes on the Sym plasmid of *R.leguminosarum bv trifolii.*

1.4 Nodulation

The process of nodulation can be divided into four main stages (Bauer,1981;Brock and Madigan,1991;Kondorosi,1986;Long,1989; Vance,1983) ;-

1.Recognition
2.Invasion
3.Bacteroid formation
4.Maturation

Figure 2 : Unrooted phlyogenetic tree, obtained by Fitch analysis.

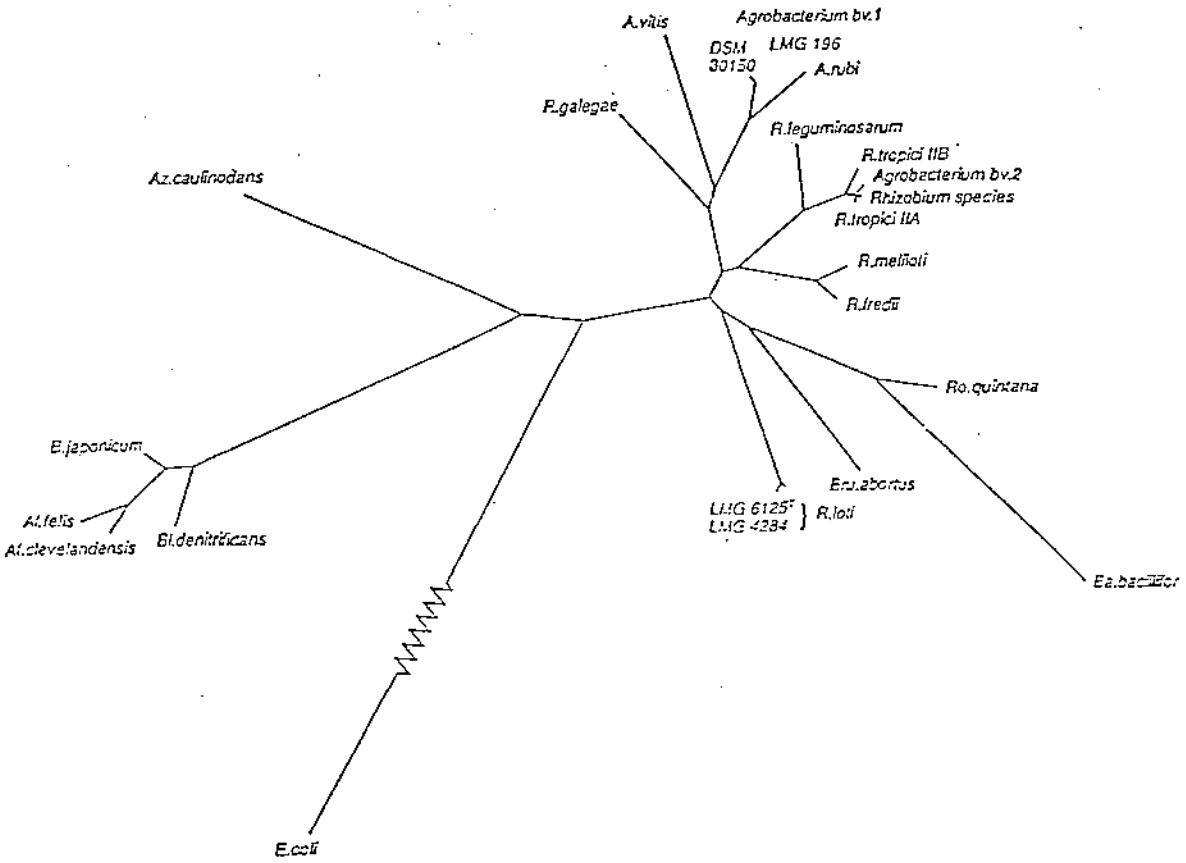
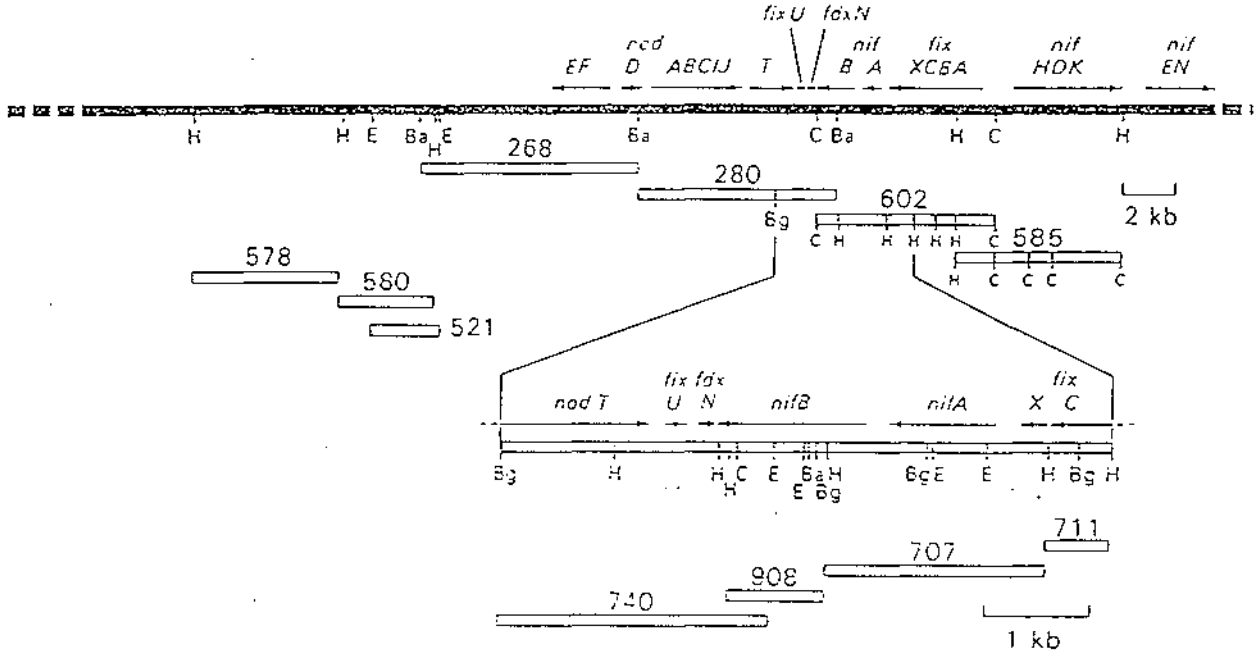

A.vitis
Agrobacterium bv.1
DSM 30150
LMG 196
A.rubi
P.galegae
R.leguminosarum
R.tropici IIB
Agrobacterium bv.2
Rhizobium species
R.tropici IIA
Az.caulinodans
R.meliloti
R.trefii
Ro.quintana
B.japonicum
Ochrobactrum
LMG 6125T
LMG 4284
R.loti
Af.felis
Af.clevelandensis
Bl.denitrificans
Ea.bacilliformis
E.coli

Figure 3 : A 45kb region of the Sym plasmid of *R.leguminosarum* bv trifolii strain ANU843, showing some *nif, fix, fdx* and *nod* genes. Many other genes are also present on the Sym plasmid, but are not shown here.


fix U fdx N
EF
ccd D ABCIJ
T
nif B A
fix XCBA
nif HDK
nif EN
268
280
602
585
578
580
521
2 kb
nod T
fix fdx U N
nifB
nifA
fix X C
711
707
908
740
1 kb

1.4.1 Recognition

Legume roots secrete a range of organic compounds which aid growth of micro-organisms in the rhizosphere (Long,1989). These are not specific to rhizobia, but they assist the growth of any and all bacteria within the rhizosphere. Among these are flavanoids. Flavanoids induce *nod* gene transcription in certain species of rhizobia (Djordjevic et al,1987;Downie and Johnston,1986;Long, 1989). In particular, *nodD* gene transcription is induced by flavones (Long,1989). The *nodD* gene itself is a regulatory gene, and its protein product induces other *nod* genes (Long,1989;Downie and Johnston,1986). These *nod* genes are responsible for nodulation of a host legume, and their products are only present in high enough quantities to cause nodulation after the genes have been induced by *nodD*. Chemicals called isoflavones can also inhibit *nodD* gene transcription in different species, and so we have a form of plant-bacterium specificity (Djordjevic et al 1987;Downie and Johnston,1986;Long, 1989) as there is no longer any positive gene induction of the *nod* genes by the *nodD* gene product.

The root hairs of legumes such as white clover also contain lectins on their surface (Bauer,1981;Djordjevic et al,1987). These lectins are proteins, produced by the legume, and they are present before, during and after nodulation (Djordjevic et al,1987). These lectins bind specifically to exo- and capsular polysaccharides (Vance,1983; Latchford et al,1991) and also to glucans and lipopolysaccharides produced by rhizobia (Bauer,1981;Djordjevic et al,1987;Dowling and Broughton,1986;Downie and Johnston,1986;Long,1989). This lectin-polysaccharide binding is another source of legume-*Rhizobium* specificity (Long,1989), and is involved in the binding of bacterial cells to the root hair (Djordjevic,1987). In many cases only a specific strain of *Rhizobium* can bind to a specific host legume, however there are exceptions to the rule (Vance,1983). Dazzo and Hubbell (1975) proposed that polyvalent plant lectins cross-bridge common antigens on the host root hair and on the bacterial cell surface. Capsular polysaccharides of *R.leguminosarum bv trifolii* bind specifically to the lectins on white clover, but capsular polysaccharides from *R.meliloti*

cannot (Dazzo and Brill,1977). More important in the nodulation process are the exopolysaccharides (Reuber et al,1991). The amount of exopolysaccharide produced is directly proportional to the frequency of nodulation (Vance,1983). Once *Rhizobium* has bound to the root hair, infection can begin.

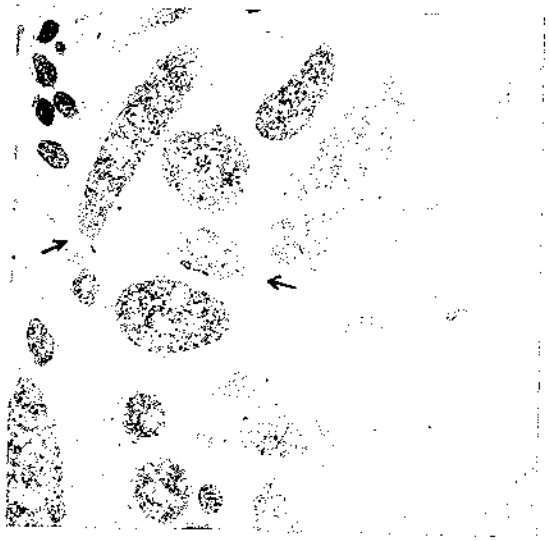
1.4.2 Invasion

After binding occurs, the root hair curls and the bacteria enter the tip of the root hair (Bauer,1981). This induces the formation of an infection thread (Long,1989;Vance,1983) which grows down the root hair and into the cortex of the root, allowing the bacteria to infect adjacent plant cells. The infection thread appears to result from invagination of the root hair's cell wall. As yet the bacteria are still considered to be outside the cell and there are no pores by which the bacteria could infect the plant itself (Napoli and Hubbell,1975;Reuber et al,1991;Vance et al,1980). For infection to occur the plant cell wall must be partially degraded. Genes responsible for pectolytic and cellulolytic enzymes, present in the plant itself are induced by the *Rhizobium* exopolysaccharides (Bauer,1981) which pass through the cell wall and into the plant cell nucleus. The pectolytic and cellulolytic enzymes loosen the cell wall, thus allowing the release of the rhizobia into the cells of the plant cortex (Ljunggren and Fahraeus,1959; Ljunggren and Fahraeus,1961). The majority of these plant cells are diploid and die when invaded by bacteria. However, the small number of tetraploid cells present in the neighbouring root area grow rapidly, forming a tumour-like nodule. Cytokinins produced by rhizobia seem to be at least partially responsible for this nodule formation (Vance,1983).

1.4.3 Bacteroid Formation

Bacteroids (bacterial cells surrounded by plant membrane) now develop within the tetraploid cells (Fig. 4). The plant membrane is referred to now as the peribacteroid membrane (Long,1989). There are a number of other differences between pre- and post-bacteroid rhizobia. Verma and Long (1983) liken bacteroids to chloroplasts and mitochondria. They suggest that bacteroids may be in an early stage,

Figure 4 : Electron micrograph 11,200 x magnification) showing bacteroids inside a nodule which was isolated from a white clover plant. The peri-bacteroid membrane is clearly visible, as indicated by the arrows.



evolutionarily, of becoming organelles. However, bacteroids divide at a different rate from the host cells and are capable of extra-cellular existence, unlike chloroplasts and mitochondria. There is some free exchange of cell constituents, such as growth hormones and flavanoids, across both the bacterial and the peribacteroid membrane, allowing legume-*Rhizobium* communication. For example, naphthylphthalamic acid (NPA), an "anti-auxin", can induce alfalfa nodulation and nodulin gene expression (Long,1989). Flavanoids can bind to legume NPA receptors, also acting as "anti-auxins", and so may have a role in nodulin induction (Jacobs and Rubery,1988). Nodulins are plant gene products which are expressed only in nodules, and their expression is regulated either directly or indirectly by *Rhizobium* inducers. A number of molecular changes follow this gene induction (Verma and Long,1983). Such nodulins are found both within the bacteroid membrane and inside the bacteroid itself (Verma and Long,1983).

1.4.4 Maturation

During nodule maturation the bacteroid experiences a different environment from that outside the peribacteroid membrane. Irigoyen et al (1990) examined the activity of a number of *R.meliloti* enzymes in bacteroids and free-living cells. Activities in the bacteroid state which were significantly reduced included : aldolase, alcohol dehydrogenase, pyruvate kinase, citric acid cycle, pentose phosphate pathway and Entner-Doudoroff pathway enzymes. It is essential that these changes take place before nitrogen fixation can work efficiently (Irigoyen et al,1990), but why this should be so is unknown.

Because the nitrogenase complex requires some energy to work, yet is inhibited by oxygen, the role of another protein, leghaemoglobin is significant (Brock and Madigan.Ed.,1991;Downie and Johnston, 1986;Long,1989;Verma and Long,1983). Leghaemoglobin is a nodulin, present only in the nodule itself (Long,1989).
Leghaemoglobin binds to oxygen thus reducing considerably the amount of free oxygen available to inhibit nitrogenase activity (Brock and Madigan,1991;Long,1989). The interesting thing about leghaemoglobin is that it has two sub-units. The haem sub-unit is transcribed from *Rhizobium* DNA, whereas the globin sub-unit is

transcribed from legume DNA (Brock and Madigan,1991; Verma and Long,1983). The leghaemoglobin situation is another another example of the symbiotic nature of the legume-*Rhizobium* system, and a target for legume-*Rhizobium* specificity as both *Rhizobium* and the host legume must produce the correct sub-unit to get a complete and functional molecule (Downie and Johnston,1986;Long,1989;Verma and Long,1983).

1.5 Sym Plasmid Transfer and Nodulation

If a Sym megaplasmid is transferred conjugatively from one *Rhizobium* strain to another *Rhizobium* strain, or even to a different species such as *Ag.tumefaciens*, the recipient may gain the ability to nodulate the donor's host legume. This is because the *nod* genes on the Sym plasmid are sufficient to cause nodulation in some but not all legume-bacteria associations. Consequently host-bacteria specificity can be affected by Sym plasmid transfer. This has been shown to occur with strains from a number of different genera, including *Rhizobium* (Beringer et al,1978;Beynon et al,1980;Brewin et al,1983;Broughton et al,1987;Djordjevic et al,1982;Dowling and Broughton,1986;Espuny et al,1987;Hooykaas et al,1981;Jarvis et al,1989;Rolfe et al 1980; Schofield et al,1987), *Agrobacterium* (Hooykaas et al,1981;Verma and Brisson,1987) and even *Lignobacter* and *Psuedomonas* strains (Plazinski and Rolfe,1985). This situation may or may not be beneficial to the host as not all Sym plasmid recipients carry the chromosomal genes required to fix nitrogen effectively in nodules. Therefore the plant would be harbouring bacteria which don't supply it with accessible forms of nitrogen, and to do this there is some energy and carbon cost (Skot et al,1986). Strains which can nodulate a legume, but do not efficiently fix nitrogen are called **ineffective** nodulaters (Vance,1983). Pastures with clover in them require the presence of nitrogen-fixing bacteria in nodules on their roots, thus providing adequate levels of protein in the diet of herbivores for healthy growth.

1.6 Nodulation of White Clover by *R.leguminosarum* bv trifolii in a Laboratory, and in the Field.

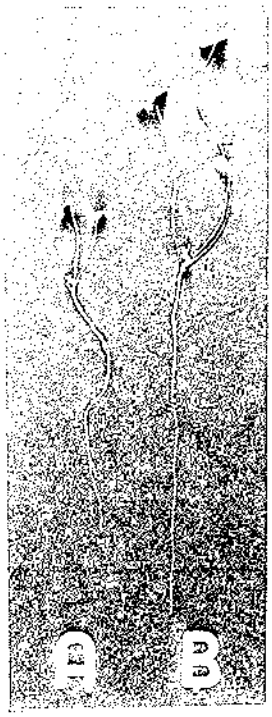
Fig.5 shows a comparison of two white clover plants. One plant (B) had nodules inhabited by *R.leguminosarum* bv trifolii strain ICMP2163. The other plant was grown in sterile, nitrogen-free media. The two plants show a marked difference in size. This size difference correlates with a difference in dry weight, and is due to nitrogen fixation by strain ICMP2163. The temperature and pH were optimised, and both plants were grown in sterile conditions. Consequently nodulation and nitrogen fixation were easily achieved.

Seed-producing companies ensure the presence of nitrogen-fixing bacteria near clover seeds by inoculating the seeds with commercial strains of *R.leguminosarum* bv trifolii such as ICMP strains 2163,2663 and 2668. Inocula of the bacteria are added to the seeds in a broth which is rolled onto the seed surface. However this may not guarantee nodulation or long term nitrogen fixation (Roughley et al,1976). Even if nitrogen fixation does occur in clover in the field, some pastures show a marked decrease in clover growth and size over a period of months or weeks due to the loss of root nodules containing nitrogen-fixing bacteria (Roughley et al,1976). This phenomenon may be due to a number of factors which affect the viability of the rhizobia located within the clover roots (Dowling and Broughton, 1986). The rhizobia may still be present in the rhizosphere, but if they have lost the Sym plasmid they can no longer form nodules (Brewin et al,1983).

1.7 Factors Affecting *Rhizobium* Survival in Soil.

An example of an abiotic factor which affects the survival of rhizobia both in the rhizosphere and in the comparative safety of the nodule is the pH of the soil (Dowling and Broughton,1986). The number of rhizobia in the rhizosphere decrease markedly when soil pH drops below 6.0 (Dowling and Broughton, 1986). This is particularly important in New Zealand as farmers may be advised to keep their soil below pH 6.0 to allow rock phosphate to dissolve. Other abiotic factors affecting *Rhizobium* survival include soil type, salinity of the soil, temperature, pesticides, moisture and even the size of the pores within the soil (Brewin et al,1983;Dowling and Broughton,1986; Postma and van Veen,1990;Rao et al). As well as abiotic factors, biotic factors affect survival in the rhizosphere. These include the

Figure 5 : White clover plants after growth in nitrogen-deficient media. Plant A was added aseptically to the medium as a seedling, whereas plant B was added with an inoculum of *R.leguminosarum* bv trifolii strain ICMP2163.


B
C

effect of bacteriophage, epiphytic bacteria, protozoa, mycorrhiza *Bdellovibrio,* bacteriocins and competition with neighbouring bacteria within the rhizosphere (Bauer,1981;Brewin et al,1983;Broughton et al,1987;Djordjevic et al,1982;Dowling and Broughton,1986;Rao et al;Schofield et al,1987). Some of these interactions may suppress nodulation, as is the case with the epiphytic bacterium (epiphytic meaning that they grow on, but are not parasitic to the host plant) *E.herbicola,* which blocks *Rhizobium* attachment sites on root hairs (Dowling and Broughton,1986). Other interactions may aid both nodulation and nitrogen fixation, as is the case with some mycorrhiza, which provide usable phosphates to the plant (Barea et al,1983), but their affect on *Rhizobium* survival in the soil is still unknown (Dowling and Broughton, 1986). The involvement of Sym plasmid transfer to recipients which are unable to fix nitrogen may also affect the survival of nitrogen-fixing bacteria in soil. Dowling and Broughton (1986) stated that "*it must be assumed that genetic exchange occurs among rhizobia in the field, and that this exchange can lead to altered competitiveness and nodulation properties of the recipient bacteria.*" Plasmid transfer can be widespread among rhizobia (Eardly,1990), and chromosomally related *R.leguminosarum* bv trifolii isolates have been shown to have unrelated plasmid profiles, whereas less chromosomally related *R.leguminosarum* bv trifolii isolates may have very similar plasmid contents (Schofield,1987). Also some strains which show very little chromosomal homology to any *Rhizobium* type strain have been isolated from nodules taken directly from a field (Laguerre,1993). A nonsymbiotic soil isolate which was shown to be a *R.leguminosarum* biovar phaseoli strain without a Sym plasmid was complemented with a Sym plasmid from another *R.leguminosarum* bv phaseoli strain, then placed back in the soil. The Sym plasmid recipient was able to compete with the indigenous rhizobia, could form nodules and even fix nitrogen (Soberon-Chavez,1989).

Because a relatively low number of soil isolates containing chromosomal DNA identical to *R.leguminosarum* bv phaseoli contain the Sym plasmid required for the nodulation of beans (Segovia,1991), it could be postulated that there is a tendancy for rhizobia to get rid of their large, cumbersome Sym plasmids when the plasmids are not being used. Such a theory requires further investigation, and the

production of a non-transferrable Sym plasmid (section 1.7) may theoretically result in reduced survivability of rhizobia in the soil under certain conditions.

Fig.6 summarizes some of the interactions which affect *Rhizobium* survival.

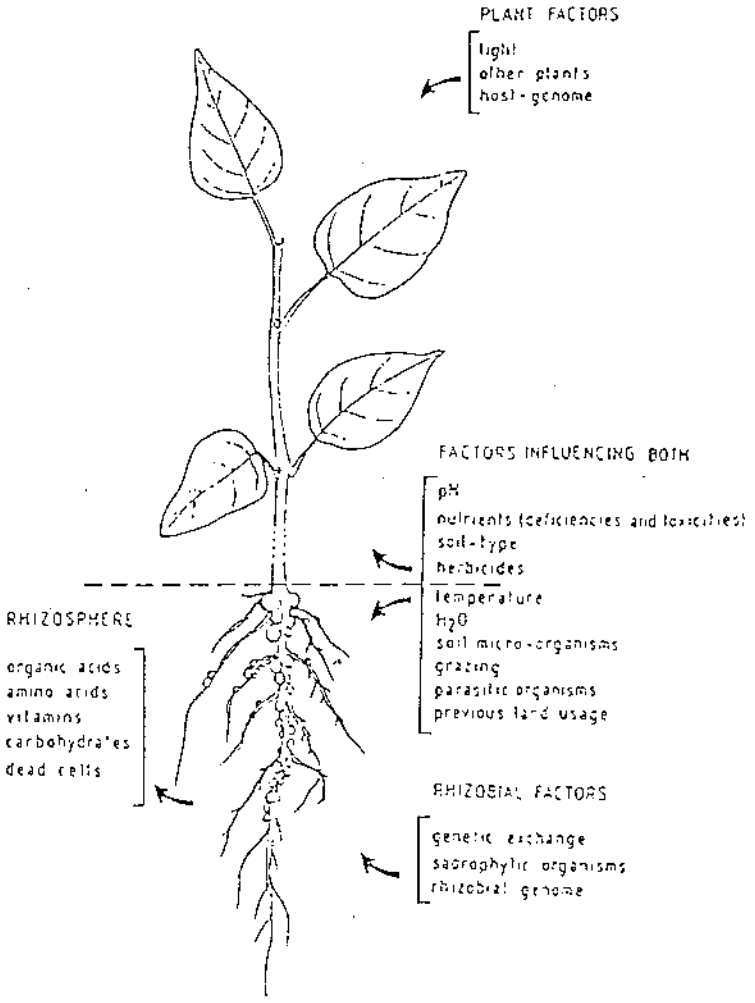
1.8 Hypothesis

If a nod$^-$ strain (i.e. a strain unable to form nodules), such as *Agrobacterium tumefaciens* strain C58, receives the Sym plasmid of *R.leguminosarum bv trifolii* strain 2163, the ability to nodulate the donor's host plant *(Trifolium repens)* may also be transferred. This has been shown to occur in nod$^-$ rhizobia as well as non-*Rhizobium* genera (Beynon et al,1980;Verma and Brisson,1987; Plazinski and Rolfe,1985). Theoretically this could happen in the rhizosphere by means of transconjugation. The rhizosphere has a dense bacterial population, so transconjugation would be expected to occur at a higher frequency here than it would elsewhere in the soil.

By crossing soil bacteria with *R.leguminosarum* bv trifolii strain ICMP2163 under the appropriate antibiotic selection pressure the soil bacteria not only received the Sym plasmid, but gained the ability to nodulate white clover (Jarvis et al,1989). The antibiotics selected against the Sym plasmid donor (*R.leguminosarum* bv trifolii), and by inoculating white clover plants with the antibiotic resistant recipient strain, only those bacteria which had received the Sym plasmid would be able to form nodules. The nitrogen fixing ability of Sym plasmid recipients varies from being at least as efficient as the donor to being very poor (personal communications, Fenton).

It is not known how plasmid transfer affects nodulation, nitrogen fixation, or even *Rhizobium* survival in the field, but very few *R.leguminosarum* isolates contain a Sym plasmid in fields unless recent inoculations have been made (Segovia, 1991), indicating that there is a tendancy of rhizobia to get rid of their large Sym plasmids when the plasmids are not being used. It may be possible that rhizobia which have got rid of these large plasmids are more likely to survive because

Figure 6 : Factors which affect the survival of *Rhizobium* species in the soil environment.


PLANT FACTORS
light
other plants
host-genome
FACTORS INFLUENCING BOTH
pH
nutrients (deficiencies and toxicities)
soil-type
herbicides
temperature
H_2O
soil micro-organisms
grazing
parasitic organisms
previous land usage
RHIZOSPHERE
organic acids
amino acids
vitamins
carbohydrates
dead cells
RHIZOBIAL FACTORS
genetic exchange
saprophytic organisms
rhizobial genome

they are not expending precious energy reserves on the maintenance of the Sym plasmid and the production of unnecessary genes.

Potentially, Sym plasmid transfer could result in less efficient nodulation and/or nitrogen fixation, or more efficient nodulation and/or nitrogen fixation. Alternatively, expression of nodulation and nitrogen fixing genes may be totally suppressed, but the Sym plasmid may be maintained in a bacterium which survives better than the donor in the soil environment. An ideal situation would occur if a Sym plasmid recipient fixed nitrogen efficiently and survived and competed more efficiently in the soil than the donor.

Therefore, is it better to fix the Sym plasmid inside a *Rhizobium* inoculant which nodulates and fixes nitrogen efficiently, but may not survive very well in adverse conditions, or to allow the Sym plasmid to transfer freely among the soil bacteria in order that it has a greater chance of being maintained in the soil population, even though it may not be efficiently expressed? And does the expulsion of a Sym plasmid in adverse conditions aid in the survival of its donor so that it may receive the plasmid again at a later date when conditions have become more favourable?

In order that these questions may be resolved a *tra*$^-$ mutant needs to be produced, so that it can be compared to its wild type counter-part. Sym plasmid transfer through the soil population can be followed, and fixed nitrogen and the frequency of nodulation measured after inoculations of legume seeds with the wild type *Rhizobium*, or the *tra*$^-$ mutant have been carried out, and comparisons can be made in order that the affect of Sym plasmid transfer on long term nitrogen fixation can be estimated.

1.9 The Role of *Agrobacterium* in Locating Sym Plasmid *tra* Genes

Before *tra* genes can be targeted they must be located and isolated. *Agrobacterium tra* genes have been located and inactivated by von Bodman et al (1989). A number of Tn5 insertions in *Agrobacterium tumefaciens strain C58* were made by this group. Some insertions inactivated *tra* genes, thus preventing Ti plasmid transfer. Three *tra*

regions were found in *A.tumefaciens* strain C58, and these regions will be referred to as *tra 1, tra 2,* and *tra 3*. One clone from each tra region was obtained. The strains *Tra⁻2-16, Tra⁻2-17* and *Tra⁻15-26* have one Tn5 insertion each and the location of each Tn5 insertion has been mapped (figures 13 and 25). A sub-clone of the Hind III fragment 3 was prepared in the broad host range vector pSa152 (Tait et al,1983). This was cloned into *E.coli* strain DH5-alpha.

Agrobacterium and the *Rhizobium* are closely related genera, as can be shown by DNA:DNA and rRNA:DNA homologies, and rRNA sequencing (De Ley et al,1973;De Ley,1974;De Smedt and De Ley, 1977;Ruiz-Sainz,1984;Willems and Collins,1993). Some researchers think that they should be classified as members of the same genus (Willems and Collins, 1993). Not only are the ribosomal RNA gene sequences of *Agrobacterium* similar to those of *Rhizobium* ribosomal RNA gene sequences, indicating a close phylogenetic link between the two genera, but *Agrobacterium* Ti plasmids can be expressed in *Rhizobium* strains and *Rhizobium* Sym plasmids can be expressed in *Agrobacterium* strains (Hooykaas,1981; Verma and Brisson,1987). Therefore an *Agrobacterium tra* DNA probe may identify *tra* sequences in *Rhizobium* DNA.

With Tn5 inserted in *tra* gene areas, it is possible to locate the *Agrobacterium tra* genes within a genomic digest by labelling Tn5 and probing a Southern blot. A plasmid preparation is not used to locate the *tra* genes as the Ti plasmid, is too large to manipulate easily. The Tn5 probe identifies the band on a gel with respect to a size ladder and this can be compared with the band shown on a restriction map of all three *Tra* gene areas (von Bodman et al, 1989). The bands contain Tn5 DNA and flanking *Tra* regions from each gene. These flanking regions can be isolated and used as probes to examine strains of *Agrobacterium* and *Rhizobium* for homologous sequences.

To determine whether *tra* genes from *A.tumefaciens* strain C58 can be used to identify *tra* genes in other bacteria, a number of strains are to be selected from *Agrobacterium* clusters 1 and 2 (De Ley et al,1973), *Rhizobium loti* and a *Rhizobium leguminosarum* bv trifolii.

R.leguminosarum bv trifolii seems to be more closely related to *Agrobacterium* cluster 2, yet *Ag.tumefaciens* strain C58 (which has the *tra* mutants) is in cluster 1 (De Ley,1974;Jarvis et al,1986), so it is more likely that C58 DNA would hybridise to *Rhizobium galegae,* but it is the commercially useful inoculant strain, *R.leguminosarum* bv trifolii strain ICMP2163 (Rao et al) which was of interest in this study.

Because the genes in question are located on plasmids, it was of interest to see which groups within and among different clusters of rhizobia and agrobacteria shared homologous sequences to the three *Tra* areas of the Ti plasmid of *Ag.tumefaciens* strain C58.

1.10 Aims of This Investigation

1. To detect *tra 2* and *tra 3* in mutants of *A.tumefaciens* strain C58

2. To clone *tra 2* and *tra 3* DNA from *A.tumefaciens* strain C58.

3. To probe genomic digests of *Agrobacterium* and *Rhizobium* strains to determine whether *tra 1, 2* or *3* could be used to detect *tra* sequences in *Rhizobium* species.

1.11 Re-classification of *Agrobacterium* Strains

A proposal for the rejection of *A.tumefaciens,* and revised descriptions for the genus *Agrobacterium* and for *A.radiobacter* and *A.rhizogenes* was published in October,1993 (Sawada et al,1993). According to this proposal, the cluster 1 agrobacteria mentioned in this thesis should be referred to as strains of *Agrobacterium radiobacter,* and cluster 2 agrobacteria as *Agrobacterium rhizogenes.* Sawada et al (1993) also rejected the name *Agrobacterium tumefaciens* because the type strain of this species was assigned to *Agrobacterium radiobacter. Agrobacterium vitis* and *Agrobacterium rubi* remain unchanged. In this thesis the original names for all agrobacteria are used, regardless of which cluster they belong in.