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

A Thesis Presented in Partial Fulfilment
of the Requirements for the Degree
of Master of Science in Microbiology
at Massey University, New Zealand

PAUL JAMIE FISHER

1994

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.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

.

PAGE

INTRODUCTION

MATERIALS AND METHODS

RESULTS

APPENDICES

BIBLIOGRAPHY   122

List of Figures PAGE

Figure 9 : DNA concentration markers on a 0.7%
agarose gel. The wells were loaded
as follows : (1) 200ng 1kb ladder,
(2) 150ng pUC118, (4) 5ng concentration
marker pUC118, (5) 10ng concentration
marker pUC118.   64

Figure 10 : Eckhardt gel of *Rhizobium leguminosarum*
bv trifolii strain ICMP2163 and *Rhizobium*
leguminosarum bv trifolii strain PN165.
For both strains, wells were loaded with
0.7ml of cells at an optical density
(OD) of 0.600 at a 600nm wavelength.
Lane 1 contains ICMP2163 and lane 2, PN165.   67

Figure 11 : DNA from *Agrobacterium* and *Rhizobium*
strains digested with EcoR1 and separated
(1μg each) by electrophoresis in 0.7%
agarose, then blotted and probed. The
wells were loaded as follows : (1) 200ng
1kb ladder, (2) *Rhizobium leguminosarum*
bv trifolii strain ICMP2163::Tn5, (3)
Rhizobium leguminosarum bv trifolii
strain ICMP2163 ,(4) *Agrobacterium*
tumefaciens C58 strain 2-17, (5)
Rhizobium leguminosarum bv trifolii
strain PN165

A : agarose gel. B : autoradiograph.
The final wash was carried out in 0.1 x
SSC at 47°C.   68

Figure 12 : DNA from *Agrobacterium* and *Rhizobium*
strains digested with EcoR1 and separated
(1μg each) by electrophoresis in 0.7%
agarose, Southern blotted onto nitro-
cellulose and probed with the HindIII

fragment of pPF110. The wells were loaded
as follows : (1) *A.tumefaciens* C58 strain
2-17, (2) *Rhizobium leguminosarum* bv
trifolii strain PN165, (3) *Rhizobium
leguminosarum* bv trifolii strain ATCC14480,
(4) *Rhizobium leguminosarum* bv trifolii
strain ICMP2163, (5) *Rhizobium loti* strain
ATCC33669, (6) *Agrobacterium tumefaciens*
strain LMG64 (7) 200ng 1kb ladder,

follows : (1) 200ng 1kb ladder, (2) 1μg
plasmid pPF210 digested with EcoR1 and
Hind III without the 2.4kb EcoR1/Hind III
fragment containing the *oriT* site of
pTiC58.   84

Figure 22 : DNA electrophoresed on a 0.7% agarose
gel. The wells were loaded as follows :
(1) 200ng 1kb ladder, (2) 1.1kb
polymerase chain reaction product (30ng)
containing the *oriT* site of pTiC58, (4)
300bp polymerase chain reaction product
(30ng) containing *tra 3* mating bridge
assembly DNA, (6) 200ng 1kb ladder.   85

Figure 23 : DNA from *Agrobacterium* and *Rhizobium*
strains digested with EcoR1 and separated
by electrophoresis in 0.7% agarose,
Southern blotted onto nitrocellulose
and probed with the 2.2kb EcoR1/Hind III
fragment from pPF210. The wells were
loaded as follows : (1) 200ng 1kb, (2)
1μg *Agrobacterium tumefaciens* strain LMG64,
(3) 1μg *Agrobacterium rhizogenes* strain
ATCC15834, (4) 1μg *Rhizobium loti* strain
ATCC33669, (5) 1μg *Rhizobium leguminosarum*
bv trifolii strain ICMP2163::Tn5 (6) 1μg
Rhizobium leguminosarum bv trifolii strain
ICMP2163 (7) 1μg *A.tumefaciens* C58 strain
2-16

A : agarose gel. B : autoradiograph. The
final wash was carried out in 0.1 x SSC
at 37°C.   87

Figure 24 : DNA from *Agrobacterium* and *Rhizobium*
strains digested with EcoR1 and separated
by electrophoresis in 0.7% agarose,

0.7% agarose, Southern blotted onto a nylon
membrane and probed with pPF310. The wells
were loaded as follows : (1) 200ng 1kb ladder,
(2) no DNA, (3) 1μg *R.leguminosarum* bv
trifolii strain ICMP2163::Tn5, (4) 1μg *R.
leguminosarum* bv trifolii strain ICMP2163,
(5) 0.8μg *A.tumefaciens* strain C58 2-17,
(6) no DNA, (7) 150ng 1kb ladder

A : agarose gel. B : autoradiograph.
The final wash was carried out in 0.1 x
SSC at 50°C.   104

Figure 33 : Genomic DNA from *Agrobacterium and
Rhizobium* strains, digested with EcoR1,
separated by electrophoresis in 0.7%
agarose, Southern blotted onto a nylon
membrane and probed with the 350bp *traIII*
polymerase chain reaction product. The
wells were loaded as follows : (1) 0.8μg
R.leguminosarum bv trifolii strain
ICMP2163, (2) 1μg *R.loti* strain ATCC33669,
(3) 0.8μg *R.leguminosarum* bv trifolii
strain ATCC14480, (4) *A.tumefaciens*
strain C58 2-17

A : agarose gel. B : autoradiograph.
The final wash was carried out in 0.1 x
SSC at 42°C.   105

Figure 34 : Genomic DNA from *Agrobacterium* strains,
digested with EcoR1, separated by
electrophoresis in 0.7% agarose,
Southern blotted onto a nylon membrane
and probed with the 350bp *traIII* polymerase
chain reaction product. The wells were
loaded as follows : (1) 1μg *A.tumefaciens*
strain LMG87, (2) 1μg *A.tumefaciens* strain

LMG196, (3) 1μg *A.tumefaciens* strain LMG219, (4) 1μg *A.tumefaciens* strain LMG26, (5) 1μg *A.rhizogenes* strain ATCC15834, (6) 1μg *A.tumefaciens* strain LMG64, (7) 1μg *A. tumefaciens* strain C58 2-17

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

106

LIST OF TABLES

TABLE

PAGE

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*).
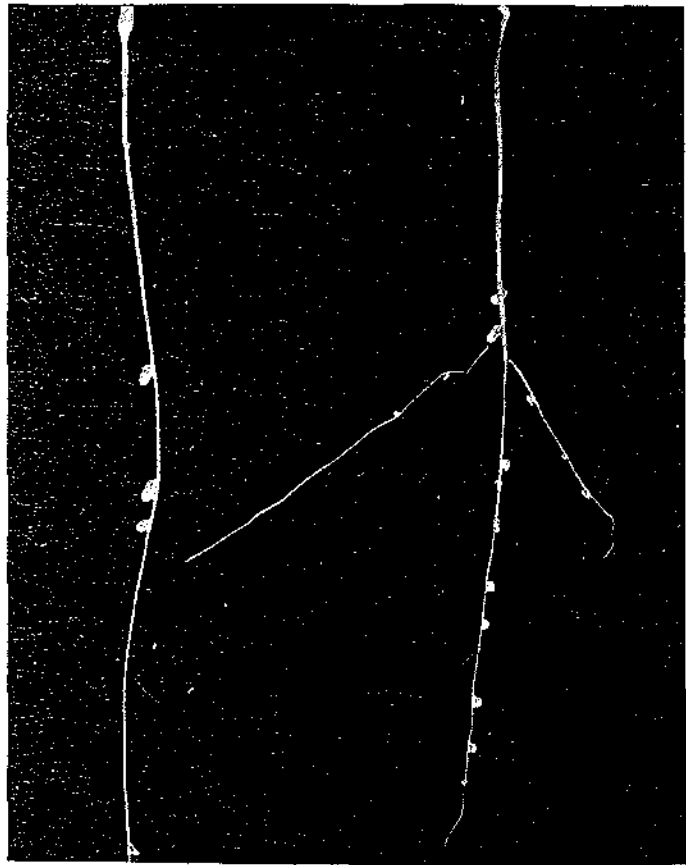
nodulate and fix nitrogen in bean roots, and could also compete effectively with other indigenous *Rhizobium* strains in the soil (Soberon-Chavez,1989). A number of nonsymbiotic soil bacteria were later shown to have identical 16S rRNA sequences to that of *R.leguminosarum* bv phaseoli, and when complemented 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.
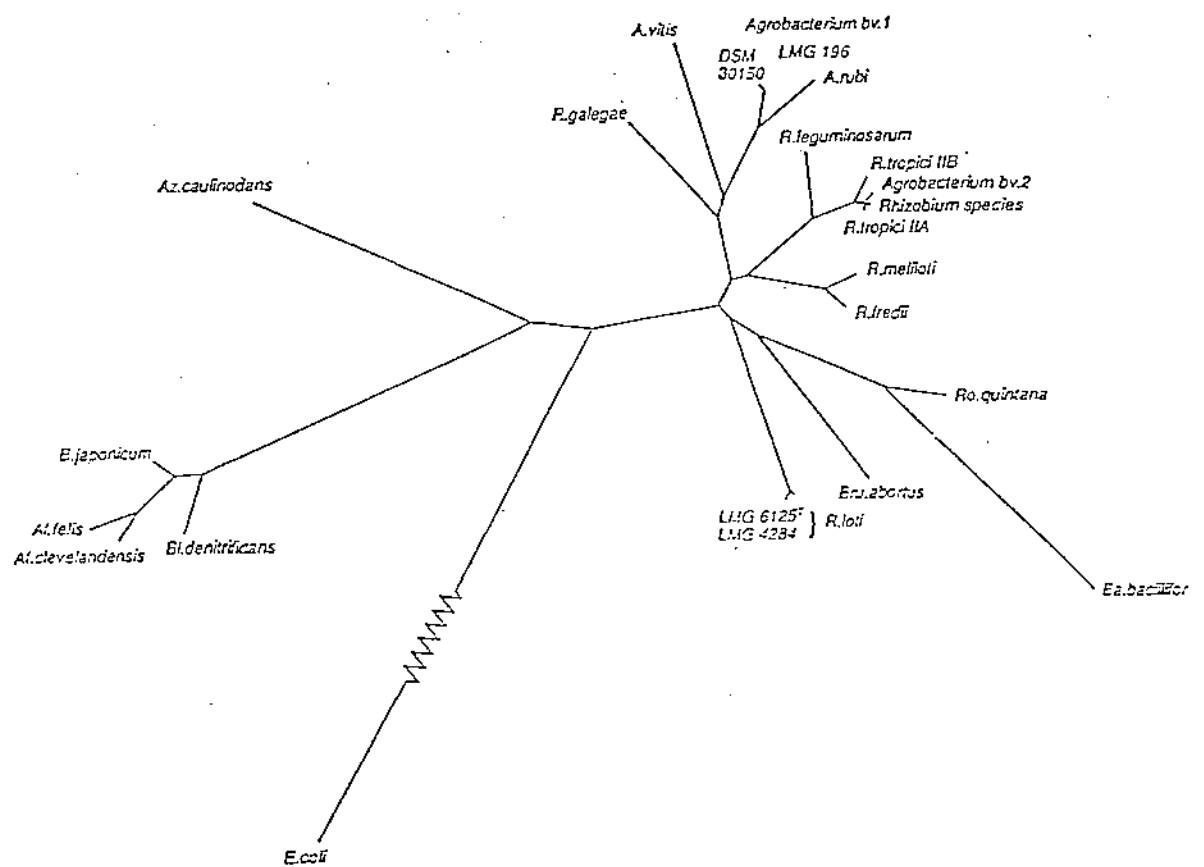
Figure 3 : A 45kb region of the Sym plasmid of
R.leguminosarum bv trifolii strain
ANU843, showing some *nif, fix, fdx*
and *nod* genes. Many other genes are
also present on the Sym plasmid,
but are not shown here.
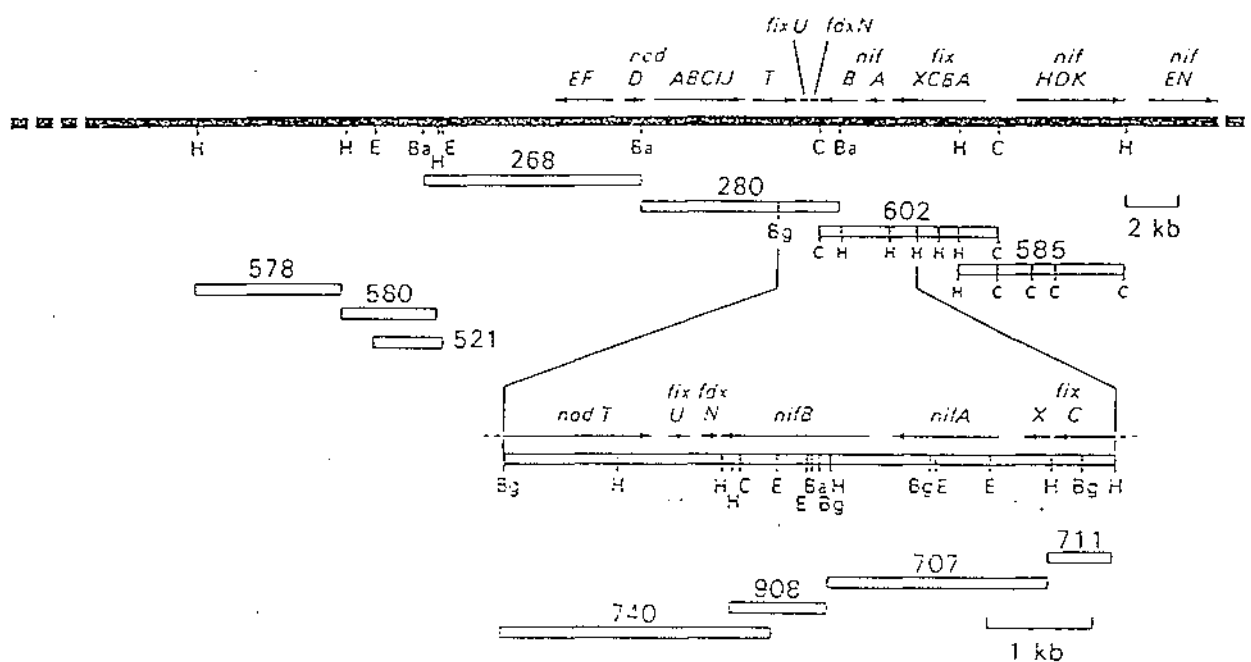
1.4.1 Recognition

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.
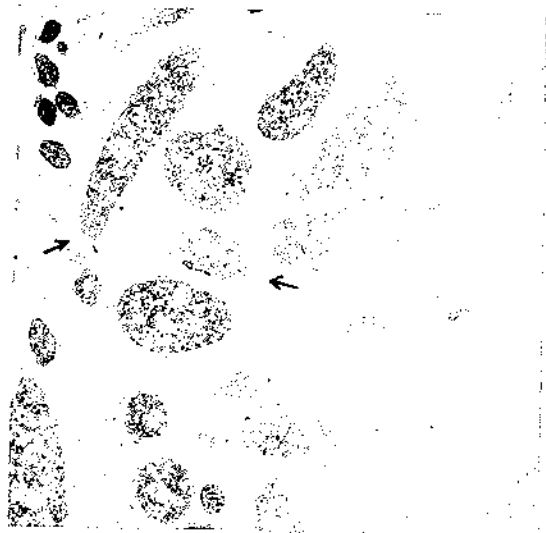
evolutionarily, of becoming organelles. However, bacteroids divide at a different rate from the host cells and are capable of extra-cellular existence, unlike chloroplasts and mitochondria. There is some free exchange of cell constituents, such as growth hormones and flavanoids, across both the bacterial 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* by 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.
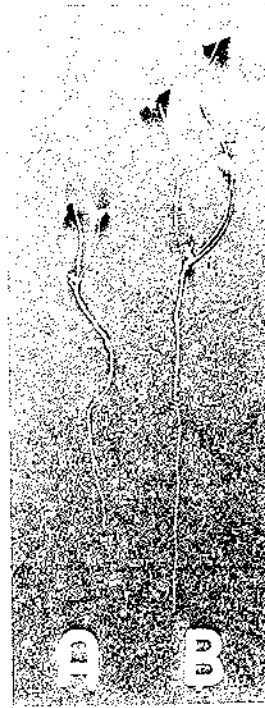
effect of bacteriophage, epiphytic bacteria, protozoa, mycorrhiza *Bdellovibrio*, bacteriocins and competition with neighbouring bacteria within the rhizosphere (Bauer,1981;Brewin et al,1983;Broughton et al,1987;Djordjevic et al,1982;Dowling and Broughton,1986;Rao et al;Schofield et al,1987). Some of 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.

Section 2   Materials and Methods

2.1   Media

All media, axcept for SOC medium were sterilized by autoclaving at 121°C for 15 min. SOC medium was filter sterilised (section 2.19)

Luria Broth (LB) (Millar,1972) (g/L) 5.0, NaCl; 10.0, tryptone (Difco);5.0, yeast extract (Difco). LB agar was obtained by adding 15.0g of agar (Davis) to each litre of LB. LB top agar was obtained by adding 7.5g of agar to each litre of LB.

Tryptone Yeast Extract Broth (TY) (Beringer,1974) (g/L) 5.0, tryptone (Difco);3.0, yeast extract (Difco);1.3, $CaCl_2.6H_2O$. The calcium chloride was filter sterilised, and aseptically added after autoclaving to prevent precipitation. TY agar was obtained by adding 15.0g of agar (Davis) to each litre of TY broth before autoclaving.

Thornton's Seedling agar (Thornton,1930) (g/L) 2.0, $Ca_3(PO_4)_2$;0.5, K_2HPO_4;0.2, $MgSO_4.7H_2O$;0.1, NaCl;1.0, $FePO_4$;0.017, $FeCl_3.6H_2O$;15.0, agar (Davis);1ml Hogland's Trace Element Solution. 10ml aliquots were added to test tubes (15 x 150mm), autoclaved, then set on a slope.

Hogland's Trace Element Solution (g/L) 2.9, H_3BO_3;1.8, $MgCl_2.4H_2O$;0.22, $ZnSO_4.H_2O$;0.095, $CuSO_4.7H_2O$;0.08, $CuSO_4.H_2O$;0.054, $Na_2MoO_4.2H_2O$;

SOC medium (Ausabel,1991) (g/L) 20, tryptone (Difco);5, yeast extract (Difco);10mM NaCl; 2.5mM KCl;10mM $MgCl_2$;10mM $MgSO_4$;20mM glucose (BDH)

Water : all water used in this thesis was deionised with the Milli-Q system (by Millipore)

Glycerol Broths : 50% TY, or LB (above) and 50% glycerol. The glycerol was added after cells were grown in the TY or LB broth. Glycerol broth was stored at -20°C.

Beads : "PROTECT" bacterial preservers (Technical Service Consultants Ltd.). A colony was picked and added to the cryopreservative fluid, the tube (containing the cryopreservative and beads) was inverted and excess liquid was withdrawn. Beads were stored at -20°C or -70°C.

2.2   Buffers

All reagents in section 2.2 were obtained from BDH Chemicals ltd.

Tris, EDTA (TE) buffer : 10mM Tris(hydroxymethyl)aminomethane (Tris);1mM Ethylenediaminetetraacetic acid (EDTA). The pH was adjusted to 8.2 with conc.hydrochloric acid (HCl).

10 x TBE buffer : 0.9M Tris.HCl (pH 8.4);25mM Ethylenediaminetetraacetic acid (EDTA);0.8M orthoboric acid

2.3   Bacterial Strains and Maintenance

2.3.1   Bacterial Strains

All the strains used in this study are listed in Table 1, along with some of their antibiotic resistances and references.

2.3.2   Cultivation of Bacteria

Rhizobium leguminosarum strains were taken from slopes, incubated in TY broth (section 2.1) for 2 days on a shaker at 28°C, streaked for single colonies on TY plates, inoculated onto *Trifolium repens* (white clover) seeds (section 2.4), isolated from nodules (section 2.5), then streaked again for single colonies. This ensured the presence of the Sym plasmid. Single colonies were incubated in TY broth for 2 days on a shaker at 28°C prior to DNA isolation.

Rhizobium loti strains were taken from slopes, streaked for single colonies on TY plates (section 2.1), and a single colony inoculum was incubated in TY broth for 3 days at 28°C prior to DNA isolation.

Agrobacterium tumefaciens and *Ag.rhizogenes* strains were taken from slopes, streaked for single colonies on LB plates (section 2.1), incubated for 2 days at 37°C, and a single colony inoculum was incubated for 2 days on a shaker in LB broth at 37°C prior to DNA isolation.

Escherichia coli strains were taken from slopes, streaked for single colonies on LB plates (section 2.1), incubated for 1 day at 37°C, and a single colony inoculum was incubated for 1 day on a shaker in LB broth at 37°C prior to DNA isolation.

2.3.3 Maintenance and Storage of Bacteria

Single colonies were isolated from plates up to 3 weeks old, which were stored at 4°C. All bacteria were stored short-term (approximately 2 months) on slopes at 4°C, and long-term at -20°C on beads, or in glycerol broth (section 2.1), and were freeze-dried.

2.4 Plant Inoculation

Reference : Vincent.1970

Materials for Plant Inoculation

Ethanol : 95%

Sterile water : deionised by Milli-Q sytem (Millipore) and autoclaved

Mercuric chloride : 0.1% in deionised water

TY plates : section 2.1

Seeds of *Trifolium repens* (white clover)

Thornton's agar slopes : section 2.1

Glass tube : diameter 16mm

Nylon mesh

Fine grade sandpaper

Temperature-controlled room : set to 28°C, artificial lighting 12 hours/day

Method

Clover seeds from *Trifolium repens* were sterilised two days ahead of inoculation as described below.

A piece of nylon mesh was secured over the end of a wide (16mm diameter) glass tube, the clover seeds were scarified by rubbing briefly between two pieces of fine sandpaper and were added to the tube with the mesh at the bottom. The seeds were added to give a layer one seed thick.

Ten petri dishes were prepared as follows : to dish 1, 95% ethanol was added;to dish 2, 0.1% mercury chloride was added;to dishes 3,4,5,6,7,8 and 9 sterile water was added. The last petri dish was left empty.

The seeds were momentarily immersed in the ethanol before being immersed in the mercuric chloride for 10 min. The seeds were then washed thoroughly in successive changes of sterile water and finally a heat sterilised scalpel blade was used to slit the nylon mesh, thus releasing the seeds into the empty sterile petri dish.

To check the affect of this surface sterilisation technique, a seed was rolled on a TY plate, and a seed which was not surface sterilised was also rolled on a TY plate. No growth around the sterilised seed showed that the method was effective in eliminating or greatly reducing the microflora on the seed surface which could otherwise grow on TY plates. No growth of contaminating microflora was observed on these plates, but fungal growth was observed when a non-sterile seed was put on a TY plate.

A heat sterilised loop was cooled, used to transfer the seeds to a water agar plate and then to spread them evenly over the plate's surface. The plates were incubated at room temperature in the dark for 2-3 days. Germinated seeds with small roots were used for the following procedure.

2 X 500μl of sterile water was transferred to a sterile petri dish, a sterile loop was used to transfer an inoculum (1-2 loops) of rhizobia

into one of the drops of water, making it turbid. A loop was flamed and cooled in the second drop of water, used to transfer a seedling from the water agar to the bacterial suspension and then to a slope of Thornton's agar (section 2.1). The tube was inoculated with 50µl of the bacterial suspension and the process was repeated for all the other seedlings used. The seedlings were incubated at 22°C for 4-8 weeks, and checked for nodules at weekly intervals after the first 2 weeks.

Once the nodules were big enough (usually after 6 weeks of incubation), the bacteria were isolated from within them (section 2.5).

2.5   Isolation of Bacteria From Nodules

Reference : Vincent.1970

Materials for Isolating Bacteria from Nodules

Thornton's agar : section 2.1

TY broth and TY plates : section 2.1

Ethanol : 95%

Mercuric chloride : 1% in deionised water

Glass tube : 16mm in diameter

Nylon mesh

Sterile scalpel blade

Petri dishes

Method

The nodules were isolated from the plant root by excision with a sterile scalpel blade and surface sterilised in the same manner as the seeds were before plant inoculation (section 2.4) with the exception that the nodules were not scarified. The nodules were aseptically transferred to a sterile petri dish which contained 50µl drops of sterile TY, each nodule was placed in a drop and each nodule was crushed with a sterile glass rod, thus releasing the nodule bacteria into the TY medium. Each inoculated drop was transferred to a TY plate with a sterile loop, and streaked for single colony growth.

2.6   Genomic DNA Isolation

Reference : Jarvis et al.1989

Materials for Genomic DNA Isolation

TY broth : section 2.1

TE buffer : 10mM Trizma base (pH to 8.0 by adding conc.HCl);1mM
         EDTA

TE$_{25}$: 10mM Trizma base (pH to 8.0 by adding conc.HCl);25mM
         EDTA

TES : 10mM Trizma base (pH to 8.0 by adding conc.HCl);25mM
         EDTA;150mM NaCl

Sarkosyl/pronase : 10% Sarkosyl (in deionised water);5mg/ml
Protease (in TE$_{25}$). Before starting the DNA isolation, 6.5ml of the
sarkosyl/protease mixture was prepared and incubated at 37°C for one
hour, divided into 0.6ml aliquots and stored at -20°C in 1.5ml
Eppendorf tubes until needed.

Lysozyme : 2mg/ml Lysozyme (in TE$_{25}$). The lysozyme solution was
stored in aliquots of 0.5ml in 1.5ml Eppendorf tubes at -20°C

RNAse stock solution : 10mg/ml DNAse-free RNAse in deionised,
sterile water. A volume of 10ml of RNAse stock solution was boiled
for 5 minutes to destroy DNA activity, aliquoted into 0.5ml volumes
and stored at -20°C.

Phenol : 500g of solid phenol was taken, to which 300ml of warm
25mM NaCl was added. The NaCl had been previously heated to
boiling point before addition to the phenol. A mass of 10g of Tris-base
(Sigma), and 0.9g of 8-hydroxyquinoline were added. The tris-
equilibrated phenol was stored at 4°C.

Chloroform

Sterile sodium acetate : 3M sodium acetate (pH 5.2), filter sterilised
as in section 2.19

Ethanol : 95%

Incubater : set at 28°C for rhizobia, with shaker

Dessicator and vacuum system

<u>Method</u>

Rhizobium strains were inoculated for 48 hours at 28°C in TY broth, *E.coli* and *Agrobacterium* strains were inoculated for 24 hours at 37°C in LB broth, and put on a shaker.

A volume of 30ml of cells was centrifuged at 6,400g (Hereaus Megafuge 1.0, rotor #3360) for 10 min in a Falcon tube. This step was not necessary for some *Agrobacterium* strains, or for *E.coli*. *Rhizobium* strains required it, however, as they had plenty of extracellular polysaccharides The pellet was resuspended in 1.0M NaCl, put on a shaker in the cold for 30 min, the cells were centrifuged at 6,400g for 10 min and the pellet was resuspended in 25ml TES. The cells were centrifuged again at 6,400g for 10 min, the pellet was resuspended in 5ml TE buffer, 0.5ml of lysozyme solution was added and the cells were incubated at 37°C for 20 min. DNAse-free RNAse was also added to a final concentration of 10 µg/ml, 0.6ml of sarkosyl/pronase was added and the suspension was incubated for 1 hour at 37°C. The solution should have become quite viscous by the end of this incubation. To this viscous lysate, an equal volume of phenol was added and the mixture was shaken briefly and centrifuged at 4,500g to separate the two phases. The top (aqueous) phase was removed with a 1-5ml auto-pipette, and transferred to a new Falcon tube. A minimum of interphase material was transferred into the new tube, as this was contaminating material to be avoided.

This phenol extraction was repeated one or two more times until the aqueous phase was clear and there was little or no interphase material remaining. A final extraction was carried out, but chloroform was used instead of phenol, and the mixture was centrifuged at 2,000g for 3 min to separate the phases.

The aqueous phase was transferred into a clean Falcon tube, 1/8 volume of 3M Na-acetate, and 2.5 volumes of 95% ethanol were added and mixed by inversion. If plenty of DNA was present it would precipitate out as white threads. If a precipitate was not seen, the DNA

was left overnight in the ethanol at -20°C. Otherwise, the DNA was centrifuged straight away.

The DNA was centrifuged at 6,400g for 20 min at room temperature, the ethanol was decanted, the pellet washed briefly with 70% ethanol and dried under a vacuum (in a dessicator for approx. 20 min, or until pellet was dry). Finally the DNA was resuspended in H_2O, or TE buffer, depending on how susceptible it was to degradation and was stored at 4°C temporarily, or at -70°C long-term.

The concentration of DNA is given by the following equation :

$$[DNA] = \frac{A(258) - A(300) \times DF}{0.024}$$

Where : A(258) is the absorption of the sample at 258nm.
A(300) is the absorption of the sample at 300nm.
DF is the dilution factor.

The DNA concentration is in $\mu g/\mu l$.

The purity of the DNA with respect to residual protein contamination was determined by the following ratio ;-

$$\frac{A(258)}{A(280)}$$

Where : A(280) is the absorption of the sample at 280nm.

If the number was above 1.8 and close to 2.0, the DNA was sufficiently pure. Values closer to 1.5 indicated that further purification was required.

2.7.1 A Small-Scale Plasmid Preparation by Alkaline Lysis.

Reference : Sambrook et al.1989

Materials for Alkaline Lysis

Luria broth : Section 2.1

Tris,EDTA,Glucose (TEG) buffer : 50mM glucose;10mM EDTA;25mM Trizma base (pH to 8.0 by adding conc HCl)

NaOH/SDS solution : 0.2M NaOH; 1% Sodium Dodecyl Sulphate (SDS)

Potassium-acetate solution : 3M Potassium-acetate; 2M glacial acetic acid, pH 4.8

TE : Section 2.2

Antibiotic stock solutions : Appendix 1

Chloroform

Phenol : Section 2.6

Ethanol : 95%

Incubator : with shaker at 37°C.

Method

2ml of Luria broth was inoculated with a bacterial colony of *E.coli* strain HB101 (Table 1), (section 3.2), or *E.coli* strain DH-1 (Table 1), (section 3.3), and grown overnight at 37°C. When the medium was inoculated with *E.coli* HB101, and the strain contained the plasmid construct pPF210 (fig 12), Tetracycline (Tc), Kanamycin (Kan), and Streptomycin (Sm) were added to final concentrations of 25μg/ml, 50μg/ml and 50μg/ml respectively. When inoculation was performed with *E.coli* strain DH-1, and the strain contained the plasmid construct pPF310 (fig 13), Ampicillin (Amp) was added to a final concentration of 25μg/ml.

a 1.5ml Eppendorf tube containing 1.5ml of culture was centrifuged at 13,000rpm for 1 min. The supernatant was removed by aspiration, the pellet was resuspended by vortexing in 100μl of Tris,EDTA,Glucose (TEG) and the suspension was stored for 5 min at room temperature. Freshly prepared NaOH/SDS solution (200μl) was added to the suspension, mixed by inversion, and stored on ice for 5 min. To the suspension, 150μl of ice-cold potassium-acetate solution was added

and the mixture was vortexed gently for 10 seconds. The suspension was stored on ice for 5 min, centrifuged for 5 min at 13,000rpm and the supernatant was transferred to a fresh tube. Equal quantities of phenol and chloroform were added and the mixture was vortexed thoroughly and then centrifuged for 2 min at 13,000rpm. The supernatant was transferred to a fresh tube, 2 volumes of 100% ethanol were added, the mixture was vortexed and left to stand at room temperature for 2 min. The tube was centrifuged for 5 min at 13,000 rpm to precipitate the DNA, and the pellet was washed with 70% ethanol. Finally the pellet was resuspended in TE buffer.

The plasmid preparation was usually stored at 4°C and was used within three days.

2.7.2 Large-Scale method of Plasmid Preparation, Using a Caesium chloride (CsCl) Gradient

Reference : Ish-Horowitz and Burke,1981

Materials For Plasmid Purification With a CsCl Gradient

Ethidium bromide stock (EtBr) : 10mg/ml

Na-acetate : 3M, filter sterilised (section 2.19)

Luria Broth : Section 2.1

Antibiotic stock solutions (Appendix 1)

Tris.HCl : 1M, pH to 8.0 with conc HCl

EDTA : 250mM

NaOH : 5M

Sodium Dodecyl Sulphate (SDS) : 10%

Solution I : 50mM glucose;25mM Tris.HCl;10mM EDTA. Solution I was brought to pH 8.0 with conc HCl

Solution II : 0.2M NaOH;1% SDS

Solution III : 60ml of 5M K-acetate;11.5ml of glacial acetic acid

Lysozyme : 2mg/ml dissolved in TE buffer (section 2.2)

CsCl

Isopropanol

Cheese-cloth : approx. 15cm x 15cm

Ethanol

Refractometer

Ultracentrifuge tubes : Sorvall, to fit in a TV865 rotor

Ultracentrifuge rotor : TV865, Du Pont

Crimping device : Ultracrimp, Du Pont

Syringes and spare needles : 1ml capacity

Ultracentrifuge machine : OTD-Combi, Du Pont

Method

A culture of *E.coli* was inoculated into 10ml of Luria broth (section 2.1) and incubated overnight at 37°C.

The overnight culture (5ml) was used to inoculate Luria broth (1L), the inoculum was incubated at 37°C to mid-log phase (OD_{600} = 0.4). With chloramphenicol-sensitive cells which had plasmids that were able to amplify in the presence of chloramphenicol, 150mg of chloramphenicol was added. This greatly increased the copy number of the targeted plasmids. Whether chloramphenicol was added or not, the cells were incubated at 37°C overnight on a shaker.

Cells were harvested by centrifugation,using a Sorvall GSA rotor at 6,500g for 10 min at 4°C, resuspended in 30ml of Solution I and 3ml of lysozyme solution. The cells were allowed to stand for 5 min at room temperature. Solution II (60ml) was added, mixed by inversion and the solution was allowed to stand on ice for 5 min. Solution III (45ml) was added, mixed by vortexing and the solution was allowed to stand on ice for 5 min. The suspension was centrifuged in a GSA rotor at 5,700g for 10 min at 0°C, and the supernatant decanted into a clean GSA bottle. Cold (-20°C) isopropanol (0.6 volume) was added and the solution was allowed to stand at room temperature for 5-10 min. To avoid large fragments of pelleted debris getting into the clean GSA bottle when decanting, the supernatent was decanted through a

cheese-cloth. The supernatant was then centrifuged at 6,500g for 20 min at 0-5°C, and the pellet washed with 95% ethanol at room temperature and dried under vacuum at room temperature for approx. 15 min. Finally the DNA pellet was resuspended in 30ml of 50mM Tris HCl and 20mM EDTA (50/20 TE - pH=8.0) and 30.7g of CsCl was added, and allowed to dissolve at room temperature. Ethidium bromide (1.9ml of 10mg/ml) was added to the solution after 20 min.

From here on the CsCl prep was kept away from strong light.

The solution was centrifuged in a GSA rotor at 10,200g for 20 min at 15-20°C. The precipitate was discarded and the refractive index was checked with a refractometer (see below). Where necessary, the CsCl concentration was adjusted to give a reading of 1.3885. Then the supernatant was divided into 4.5ml quantities in Sorvall centrifuge tubes, balanced to 2 decimal places, and placed into a Sorvall vertical TV865 rotor. Using a torque wrench, the tubes were secured within the rotor, the rotor was put in the OTD-Combi Sorvall ultracentrifuge (Du Pont), and centrifuged at 50,000rpm overnight.

The brake was applied at 1-2000 rpm to stop the rotor, then the brake was switched off to allow the rotor to coast to a stop. The tubes were transferred to the dark room, and the DNA bands were visualised with long wavelenght UV light. A chromosomal band and a plasmid band were observed. The plasmid band was **below** the chromosomal band. The tube was pierced near the top with a syringe needle. This allowed air flow into the tube, and allowed liquid to be drawn off lower down. To avoid leaking ethidium bromide over the bench area, the tube was put into a clamp, and suspended over a beaker. Then a second needle was inserted directly under the plasmid band with a syringe attached and the plasmid band was drawn off. A minimum of caesium chloride was drawn into the syringe. About 0.2ml of solution was drawn from each tube. This was added to an Eppendorf tube. Ethidium bromide was extracted by topping up each Eppendorf tube with isopropanol which had been saturated with CsCl and TE buffer. The isopropanol separated out as an upper layer, and most of the ethidium bromide dissolved in it. This upper layer was then discarded and the procedure was repeated twice. Further steps were undertaken in normal light.

was repeated twice. Further steps were undertaken in normal light. The solution was dialysed against three changes of 500ml 50/20 TE buffer over a period of 24 hours to remove the CsCl and OD readings were taken to calculate the purity and concentration of the plasmid DNA (section 2.6)

When required, the plasmid preparations. If the volume was small enough, it was centrifuged in 1.5ml Eppendorf tubes at 13,000rpm. If not, it was centrifuged in SS34 tubes, at 7,800g. In both cases centrifugation was continued for 25 min at 4°C.

Operation of Abbe Refractometer

The two halves of the cell were opened, a few drops of CsCl solution added and the cell closed. Two rectangles were observed through the eyepiece. A dark rectangle was at the bottom with a lighter rectangle above it. The rectangles were focussed with a focussing knob. A lower knob was then used to line up the two rectangles with the cross hairs The refractive index was read from the scale which was visible through the eyepiece. The required refractive index was 1.3885. The refractometer was washed and dried thoroughly between readings.

2.7.3   Eckhardt Procedure For Large Plasmid Separation

Reference : Eckhardt.1978

Materials for the Eckhardt Procedure

TY broth : Section 2.1

Antibiotic stocks : Appendix 1

10 x TBE buffer : Section 2.2

10 x TE buffer : Section 2.2

Lysozyme solution : 2mg/ml dissolved in TBE and stored in 50ul aliquots at -20°C

0.8% agarose : Sea Kem, dissolved in TBE buffer (section 2.2)

EtBr stock : 10mg/ml

MgSO₄ stock : 1M $MgSO_4$ in deionised water

RNAse stock solution : 20mg RNAse;2ml 0.4M Na-acetate, pH 4.0. DNAse was eliminated (section 2.6)

Solution I : 10µl Ficoll 400,000;0.05% Bromophenol blue;4mg Lysozyme powder;0.02ml RNAse stock solution;20.0ml 1 x TBE, pH 8.2 (section 2.2). Solution I was stored at 4°C

Solution II : 0.2% SDS;10% Ficoll 400,000;40mg Proteinase K;10 ml 1 x TBE, pH 8.2. Solution II was dispensed in 500ul aliquots and stored at -20°C

Solution III : 0.2% SDS;5%Ficoll 400,000;20ml 1 x TBE, pH 8.2. Solution III was stored at 4°C

Shaker in a 28°C incubater

Horizon electrophoresis equipment : BRL minigel box (58mm x 58mm)

Method

A single colony was picked from a plate and 5ml of TY broth, inoculated and incubated for 48 hours at 28°C. These conditions were ideal for rhizobia and similar slow-growing bacteria, but for *E.coli*, *Agrobacterium* and fast-growing bacteria this step was avoided.

An inoculum of 50µl of a given *Rhizobium* strain from a 48 hour culture was added to 5ml of TY broth (section 2.1), or a single colony of a given *Agrobacterium* strain was added to 5ml of LB (section 2.1). All bacterial strains were incubated on a shaker at 28°C for 18 hours.

All reagents were removed from cold storage before starting the plasmid isolation and brought to room temperature, except for the Lysozyme and Solution II, which were put on ice until 15 min before use. A 0.8% agarose gel was poured prior to the start of the procedure (see section 2.11) and was allowed 1 hour to set.

Aliquots of cells from the broth cultures were dispensed into 1.5ml Eppendorf tubes in the following volumes ;- *Rhizobium* strains, 0.8 ml culture at OD_{600} of 0.6;*Agrobacterium* strains, 0.4 ml culture at OD_{600} of 0.6

The broth was centrifuged in the Eppendorf tube for 3 min at 13,000g to pellet the cells, during which time the lysozyme was thawed. The supernatant was removed from the cell pellet with a pipette. The pellet was resuspended in 1ml TE buffer (section 2.2) and the suspension was centrifuged for another 3 min at 13,000g to pellet the cells. Lysozyme (50µl) was added to Solution I (500µl). The supernatant was removed carefully with a pipette, the pellet was resuspended in 20µl of Solution I and lysozyme, the cell suspension was immediately transferred to a well in the agarose gel and the cell suspension incubated in the well for 15 min. Solution II was incubated in a 37°C waterbath to redissolve the Sodium Dodecyl Sulphate (SDS). After 15 min Solution II (20µl) was added to the well, gently mixed with the pipette tip by stirring carefully once across the well and back again and left for another 15 min. Finally the suspension was overlaid with 20-40µl of Solution III.

The gel box was transferred to the cold room, the dams were removed and the gel was carefully flooded with cold 1 x TBE buffer (section 2.2). The terminals were connected with the negative terminal closest to the wells, electrophoresed at 20 volts for 1 hour, then overnight at 100V, or for 18 hours if the plasmids were greater than 50kb. The gel was run for as few as 4 hours if the plasmids were smaller than 20kb. The following day, the gel was removed from the tray and stained/de-stained (as in section 2.11).

2.8   Restriction Enzyme Digests

References : Maniatis et al.1982
              BRL and Biolabs catalogues

All enzymes were used with buffers recommended by the suppliers.

Buffers

React 1 : Tris.Hcl,50mM (pH 8.0);MgCl$_2$,10mM

React 2 : Tris.Hcl,50mM (pH 8.0);MgCl$_2$,10mM;NaCl,50mM

React 3 : Tris.Hcl,50mM (pH 8.0);MgCl$_2$,10mM;NaCl,0.1M

React 4 : Tris.Hcl,20mM (pH 7.4);MgCl$_2$,5mM;KCl,50mM

React 7 : Tris.Hcl,50mM (pH 8.0);MgCl$_2$,10mM;NaCl,50mM;KCl,50mM

React 10 : Tris.Hcl,0.1M (pH 7.6);MgCl$_2$,10mM;NaCl,0.15M

<u>Enzymes</u>

EcoR1 (BRL) used buffer **React 3 (BRL)** and was incubated at 37°C.

Hind III (BRL) used buffer **React 2 (BRL)** and was incubated at 37°C.

Sma I (Biolabs) used **Bialabs Buffer 4** and was incubated at 25°C.

Bgl II (BRL) used buffer **React 3 (BRL)** and was incubated at 37°C.

Sal 1 (BRL) used buffer **React 3 (BRL)** and was incubated at 37°C.

Cla 1 (BRL) used buffer **React 1 (BRL)** and was incubated at 37°C.

Pvu 1 (BRL) used buffer **React 7 (BRL)** and was incubated at 37°C.

Kpn 1 (BRL) used buffer **React 4 (BRL)** and was incubated at 37°C.

Bgl II (BRL) used buffer **React 3 (BRL)** and was incubated at 37°C.

Sal 1 (BRL) used buffer **React 10 (BRL)** and was incubated at 37°C.

EcoRI/Sma I double digests were digested in **Biolabs Buffer 4** (as is normal for Sma I) at 37°C for 1 hour (with only EcoR1 present), then at 25°C for another hour with Sma I also added.

EcoRI/Hind III double digests were carried out in **BRL React 4** buffer, which was not ideal for either enzyme (both enzymes operated

at only 80% efficiency), and so longer incubation (2-3 hours) was required.

2.9.1   Purification, In An Eppendorf Tube, of DNA From Gels

Materials

0.8% agarose gel : Sea Kem by FMC, dissolved in TBE (section 2.2)

1.5ml Eppendorf tubes

Siliconised glass wool

This method was used for small volumes of agarose gel slices (200µl or less). See section 2.9.2 for a description of how to excise desired DNA bands from gels.

Using a hot wire, a hole was made in the bottom of a small (500µl) Eppendorf tube, the Eppendorf tube was packed with siliconised glass wool to provide a filter which did not allow passage of agarose under centrifugation, an agarose slice containing DNA was placed in the small Eppendorf tube, and this tube was placed inside a large Eppendorf tube (1.5ml). The tubes were centrifuged at 6,500 rpm for 30 sec. The gel slice should collapse during this treatment, and liquid should accumulate in the large Eppendorf tube. If this did not occur, the slice was centrifuged for a further 20 sec at the same speed (6,500rpm). Some agarose gels contain substances which inhibit enzymatic reactions, and so the DNA solution was purified by microdialysis (section 2.10).

2.9.2   DNA Purification using Promega "Magic PCR Preps"

Reference : Mezei,1991

Materials for DNA Purification with Promega PCR Preps

Promega *Magic PCR Preps* kit (A7170) : *PCR Preps* resin;*PCR Preps* buffer;*PCR Preps* mini-columns

0.8% agarose gel slice : Sea Plaque by FMC, dissolved in TE buffer (Section 2.2) with DNA bands

Isopropanol : 80% in deionised water

Deionised water : sterilised by autoclaving (Section 2.19)

Syringe : 3ml capacity

<u>Method</u>

This method was used for DNA direct from PCR reactions or restriction enzyme digests, or from Sea Plaque agarose gels.

Sea Plaque (FMC) gels were used for isolating bands of DNA, as they had a comparatively low melting point. A volume of 350µl of agarose, or less was heated to 70°C until melted, and 1ml of *PCR-preps* resin was added. The slurry was treated in exactly the same way from now on as if it had come from a *PCR prep* or a restriction digest.

DNA solution (30-300µl) was added to a 1.5ml Eppendorf tube, with 100µl of *PCR-preps* buffer and 1ml of *PCR-preps* resin was and the resulting suspension was vortexed gently for 10 seconds three times over a 1 minute period. The slurry was added to a 3ml syringe attached to a *PCR-preps* mini-column with a 1.5ml Eppendorf tube on its outlet. Using the syringe plunger, the slurry was slowly pushed through the mini-column into the Eppendorf tube. The Eppendorf tube and it's contents were discarded and the resin column washed with 80% isopropanol by adding 2ml of the isopropanol to the syringe and pushing through the mini-column into a second 1.5ml Eppendorf tube. The mini-column had to be removed from the Eppendorf tube before removing the syringe plunger so that the resin in the column wouldn't be sucked back up into the syringe. Finally the column was removed from the Eppendorf tube and the syringe barrel, attached to a third 1.5ml Eppendorf tube, and centrifuged at 6,500rpm for 20 sec.

Residual traces of isopropanol were removed by leaving the mini-column on the bench for 15 min.

DNA was eluted from the column by the addition of sterile H_2O (40-60µl). The column was left on the bench for 1-2 min, attached to a clean, sterile 1.5ml Eppendorf tube and centrifuged for 20 sec to collect

the DNA. The DNA was stored at 4°C or at -20°C. It could be eluted in TE buffer (section 2.2) instead of H_2O.

2.9.3   Freeze-Squeeze Method of DNA Purification From Gels

Materials

0.8% agarose gel : Sea Kem by FMC, dissolved in TE buffer (section 2.2)

Sterile scalpel blade

Long wave UV source

Syringe : 3ml capacity

Method

This method was used for 1ml to 2.5ml of agarose gel.

Under long wave UV light, an area around the DNA bands of interest was excised with a sterile scalpel blade. This was done as quickly as possible, since even the longer wave UV light will damage the DNA. The agarose was placed in a 3ml syringe barrel, and stored overnight at -70°C.

The following day, the liquid fraction was squeezed into one or more Eppendorf tubes (1.5ml) with a fair amount of force. As the gel was frozen solid, the tube had to be rolled frequently in one's hands, the plunger squeezed, and the barrel rolled again, until the last drop of liquid was collected.

The DNA was purified by microdialysis (section 2.10). Sea Plaque and Sea Kem gels (FMC) are better grade gels which are supposed to have less inhibitors than other lower grade agarose gels. Therefore microdialysis was not always required.

2.10   Microdialysis

Materials for Microdialysis

TE buffer : Section 2.2

Deionised water

Millipore membrane : 0.025μm, diameter 50mm

<u>Method</u>

A petri dish was half filled with the dialysis solution (usually deionised water) and a small magnetic stirring bar was added to the dish. Enough dialysing solution was present to submerge the stirring bar, with a few millimeters to spare between the stirring bar and the surface of the liquid. A 0.25μm Millipore membrane was floated on the surface, up to 500μl of the sample was added to the upper surface of the membrane, and the dialysis solution was stirred very slowly to set up a current under the membrane. The water in the petri dish was changed when deemed necessary (usually once, or not at all), and dialysis was usually completed within 30 min. The longer the dialysis time, the greater the increase in the sample volume.

When the volume had become too great, ethanol precipitation was required.

2.11   Running and Staining Agarose Gels

Reference : Sambrook et al.1989

<u>Materials for Running Gels</u>

TBE buffer : section 2.2

Sea Kem or Sea Plaque agarose : FMC

Loading dye : 50% sucrose;0.1M EDTA;0.05% Bromophenol blue, stored at 4°C

Ethidium bromide : 10mg/ml stock, stored at 4°C in the dark

Destaining solution : 1mM $MgSO_4$ in deionised water

Gel box and apparatus : BRL, Mini- (58mm x 58mm) or Horizon (11cm x 14cm) gel boxes

Power pack

Photographic equipment and Polaroid 667 film : if a picture was
required

<u>Method</u>

Varying concentrations of agarose were used, and different gels were
also used. The most common gel was 0.8% Sea Kem agarose (FMC),
but when small bands of 500bp or less had to be resolved, 2% agarose
was used. If low melting temperature gels were required (for further
DNA isolation) Sea Plaque (FMC) was used.

The agarose was melted in TBE buffer (section 2.2) in a microwave,
the agarose was cooled to 55°C, poured into a gel box tray (either a
BRL mini-gel or a BRL Horizin gel box) and allowed to set for an hour
with a gel comb in it, to provide the wells. After the gel was set, it was
flooded with TBE buffer and loaded with the DNA samples mixed with
3-5μl of loading dye. Usually 1μg of DNA or less was added for
genomic restriction digests and a DNA ladder was commonly added so
that the size of DNA fragments could be easily compared. The
electrodes were connected to a power pack with the black, negative
electrode nearest to the wells and the power was turned on with the
volts set at 60V. After 15 minutes, the voltage was increased to 90V
and run for one and a half hours with the mini-gel, or three and a half
hours with the Horizon gel box (or until the loading dye was near the
end of the gel). After the gel had run, the gel was removed from the
tray and placed in water containing a few drops of ethidium bromide
(EtBr) to stain it. The ethidium bromide solution had a final
concentration of 1μg/L EtBr dissolved in deionised water (500ml).
After 15 min in the EtBr, the gel was transferred to destaining solution
for 30-90 min. The destaining solution comprised of 500ml of
deionised water with 500μl of 1M $MgSO_4$ added to it. The gel was
then photographed under UV light (unless the bands had to be intact
and were to be isolated for other procedures) using Polaroid Type 667
black and white film.

2.12.1   Southern Blotting

Reference : Southern.1975

Materials for Southern Blotting

Depurinating solution : 0.25M HCl

Denaturing solution : 0.5M NaOH;0.5M NaCl

Neutralising solution : 0.5M Trizma base;2M NaCl, pH 7.2

20 x SSC : 175.3g/L NaCl;88.2g/L Na-citrate, pH 7.0

2 x SSC stock : 10-fold dilution of 20 x SSC

Nylon membrane : Hybond-N (Amersham)

Paper towels

Whatman filter paper : 3MM

Pyrex dish : 30 x 20cm

Gladwrap

Method

An intact, unbroken gel was required.

The gel was soaked in depurinating solution (0.25M HCl) for 15 min in a suitable tray. The loading dye was used as an indicater of acid penetration as the bromophenol blue in it went yellow at low pH. After 15 min the HCl was poured off, the gel rinsed with deionised water and transferred to denaturing solution (NaOH/NaCl) for 15 min. The loading dye in the gel returned to it's original blue colour. The gel was rinsed again with deionised water and transferred to neutralising solution (Tris/NaCl) for 15 min.

Four layers of 3MM Whatman filter paper were cut to approximately 15x22cm and laid in the bottom of a pyrex baking dish (31 x 20cm). A piece of Gladwrap was placed over the whole dish so that it could be pressed down on the paper and overlap the edges of the dish. The Gladwrap was smoothed out and a hole slightly smaller than the gel was cut in it. The wells overlapped some of the Gladwrap. The Whatman 3MM paper was wetted with 20 x SSC, and the Gladwrap was pressed down so that there were no bubbles between the Gladwrap and the paper. A piece of nylon membrane was cut to a size slightly

larger than the hole in the Gladwrap and floated on the surface of a dish of deionised water. The gel was placed over the hole in the Gladwrap so that all four edges overlapped the hole. No air bubbles were allowed between the gel and the Whatman paper and any that remained were pushed out with gloved fingers. The wetted membrane was placed on the gel so that it overlapped the gel slightly on all four sides. Bubbles between the membrane and the gel were pushed out with gloved fingers. A sheet of Whatman 3MM paper slightly smaller than the membrane which had been previously wetted with 20xSSC was placed on the membrane, two sheets of Whatman 3MM paper of the same size were placed on this sheet and finally a stack of paper towels 5-8cm deep was placed on the Whatman 3MM paper. The towels were torn in two and stacked criss-cross in bundles of about 10 sheets, and a weight (approximately 200g) was put on top of the towels. This weight maintained firm contact between all parts of the assembly, without squashing the gel excessively.

The whole apparatus was left overnight after adding enough 20xSSC underneath the Gladwrap to keep the 3MM paper at the tray's bottom moist for several hours.

Nitrocellulose membranes were washed briefly with 2xSSC, placed between two sheets of Whatman 3MM paper, baked for 2 hours at 80°C and stored at room temperature until required. Nylon membranes were exposed to a UV light from a trans-illuminator for 3 min and stored at room temperature.

2.12.2   Vacuum Blotting

Reference : Southern, 1975 with modification to apparatus (uses a vacuum rather than capillary action, as in section 2.12.1)

Materials for Vacuum Blotting

Depurinating solution : 0.25M HCl

Denaturing solution : 0.5M NaOH;0.5M NaCl

Neutralising solution : 0.5M Trizma base;2M NaCl, pH 7.2

20 x SSC : 175.3g/L NaCl;88.2g/L Na-citrate, pH 7.0

2 x SSC stock : 10-fold dilution of 20 x SSC

Nylon membrane : Hybond-N (Amersham)

Vacuum blotting apparatus : Pharmacia LKB.VacuGene XL

Vacuum pump : Pharmacia LKB.VacuGene pump

Method

An intact gel was required for this procedure. The blotting apparatus was set up so that it was connected to a vacuum pump via a water trap.

A sheet of Whatman 3MM filter paper was placed on top of the porous base plate and wetted with 2xSSC, then a plastic mask which had been pre-cut to fit the whole of the base with a hole just smaller than the gel was laid on top of the paper with one side of the mask a little longer so that the wells in the gel would be covered by the mask. Four spots were put on the paper with a marker pen at the corners of the hole in the mask. The mask was removed and a piece of nylon or nitrocellulose membrane a little larger than the hole in the mask was placed on the four pen marks. The mask was replaced on the membrane, and a gel placed on the mask. The gel had to cover the mask completely on all four sides and the wells had to lie on the plastic mask. Once the gel was in place and the tray border clamped down, a vacuum (about 50mbar) was applied to the system.

The depurinating solution was placed on the gel, and the system left under vacuum for 15 min. If the gel started to dry out on top, more acid was added. After 15 min, the acid was sucked off through a second pump outlet and the wash was repeated using first the denaturing solution and then the neutralising solution. 20xSSC was finally placed on the gel, the vacuum applied for 45 min. The 20xSSC was topped up every 10 or so min to keep the gel from drying out. After 45 min, the 20xSSC was sucked from the apparatus, then the gel was put into Ethidium bromide solution (Section 2.11). By staining the gel the efficiency of the blotting was roughly gauged. The membrane was washed briefly with 2xSSC, baked (if it was nitrocellulose), or UV fixed if it was nylon (section 2.12.1) and stored as in section 2.12.1

2.13   Colony Hybridisation

Reference : Grunstein and Hogness.1975

Materials For Colony Hybridisation

Plates with bacterial colonies

Chloroform

Ethanol : 90%

Proteinase K

Stock 2 x SSC : Section 2.12.1

NaOH : 5M

Tris.HCl : 1M, pH 7.4

NaCl : 3M

Nitrocellulose membranes : Biodyne, 82mm diameter

Glass and plastic petri dishes

Pyrex dishes

Filter paper : Whatman 3MM

Method

Biodyne membranes were washed three times by placing them in boiling water for 1 min, and autoclaved at 120°C for 10 min between two sheets of filter paper. The sterile membranes were placed on LB (section 2.1) plates containing bacterial colonies, and incubated for 1 day at 30°C.

Each membrane was placed in a Pyrex dish on a piece of Whatman 3MM filter paper (15cm x 25cm) soaked with 0.5M NaOH for 7 min and then transferred to a second dish containing a similar piece of Whatman 3MM filter paper soaked with 1.0M Tris.HCl (pH 7.4) for 1 min. The second wash was repeated. The membrane was transferred to Whatman 3MM filter paper (15cm x 25cm) soaked with 1.5M

NaCl/0.5M Tris.HCl (pH 7.4) for 5 min, and then allowed to dry for approximately 15 min until it began to curl. The neutralised membrane was gently placed in 20ml of 1 x SSC containing 2mg of Proteinase K (sigma) equilibrated to 37°C, incubated at 37°C for 30 min, and the solution removed with a pasteur pipette. The filter was transferred to a petri dish and washed carefully in 90% ethanol. The ethanol was removed by aspiration and the membrane transferred to a glass dish. Chloroform (50ml) was added and removed by aspiration after 20 sec. This chloroform wash was repeated four more times. Finally the filter was dipped in 0.3M NaCl, to remove the loose cellular debris, baked in a vacuum oven at 80°C for 1 hour and stored between two sheets of Whatman 3MM filter paper at room temperature.

2.14.1   DNA Labelling With The Multiprime Method

References : Feinberg and Vogelstein.1983
                Feinberg and Vogelstein.1984

Materials for Multiprime DNA Labelling

Amersham Multiprime kit (RPN1601Z) : Solution I (buffer and dNTP's);Solution II (primer);Klenow fragment;Control DNA

α_{32}**Phosphate deoxycytosine triphosphate (α_{32}P-dCTP)** : 3000 Ci/mmol

DNA : probe template, 50ng

HCl : 2M

Sephadex G-50 slurry : 1g Sephadex in 20ml spin column wash buffer

Spin column wash buffer : 0.01M Tris.HCl, pH 8.0;1mM EDTA;0.03%β-mercaptoethanol;H_2O to 100ml

Radiation protective equipment for handling α_{32}P (perspex shields, disposable gloves etc)

Polyethyleneimine Cellulose Ion Exchange Resin (PEI) paper

Scintillation vials

Syringes : 1ml

Method

Template closed circular double stranded (ds) DNA was linearized and added to the labelling mixture in restriction buffer to make a final volume of 30µl and a total of 25-50ng of DNA in the final reaction. The DNA was denatured by boiling in a water bath for 2-3 min, immediately put on ice to cool rapidly, and briefly centrifuged in a 1.5ml Eppendorf tube at 13,000rpm. The following components were added to another 1.5ml Eppendorf tube in the following order ;- Template DNA solution (30µl);Buffer/dNTP solution (10µl);Primer solution (5µl);α^{32}P-dCTP, 3,000 Ci/mmol (3µl);Enzyme solution (2µl). The solution was mixed gently, centrifuged briefly at 13,000rpm, and incubated in a water bath at 37°C for 45 min. If more than 50% of the label was incorporated into the DNA, the probe was used directly for hybridisation.

Incorporation of α^{32}P-dCTP was checked with Polyethyleneimine cellulose ion exchange resin (PEI) paper (1 x 4cm). The reaction mix (1µl) was spotted approximately 1cm from the base of the paper, and the PEI paper was placed upright in a beaker containing a few ml of 2M HCl and the strip was left for 5-10 min to allow the liquid to rise up the paper by capillary action. Then the paper was cut in half, the two portions were placed in different scintillation vials and the radio-active emission of each portion measured using a liquid scintillation counter (Beckman LS 7000). Command tower program code 10 was used with channel 1, as explained in the manual. The vial with the lower portion of the PEI strip contained the incorporated label, and the vial with the upper portion the unincorporated label. The two readings were compared to give an indication of the percent incorporation and the intensity of labelling, or the specific activity (SA) of the probe, measured in counts per minute per ng DNA. If the percentage incorporation was low, unincorporated label was separated from the labelled DNA on a Mini-Spin column.

Mini-Spin column Procedure

A 1ml syringe plunger was used to push a little glass-wool into the bottom of a 1ml syringe barrel, and a hot wire was used to make a hole in the cap of a 1.5ml Eppendorf tube, the hole was immediately

enlarged with the 1ml syringe so that a close fit was obtained. The syringe and Eppendorf tube were attached so that any liquid passing through the syringe barrel would flow straight into the Eppendorf tube. This assembly was placed in a 50ml Falcon tube, 1ml of Sephadex G-50 slurry was added, the assembly centrifuged at 500g for 5 min (Heraeus Megafuge 1.0, Sepatech) and the liquid which was centrifuged down into the 1.5ml Eppendorf tube discarded. This process was repeated until the syringe barrel contained 0.8-0.9ml of Sephadex G-50 resin, after which another 1.5ml Eppendorf tube was attached to the syringe barrel, the labelling mixture was added to the column and allowed a fewminutes contact with the Sephadex G-50 resin column wash (350μl) buffer was added and the assembly centrifuged at 500g for 5 min. Finally the effluent was collected in a 1.5ml Eppendorf tube, and used forhybridisation. The probe was stored at -20°C.

2.14.2   Ready-To-Go DNA Labelling

References : Feinberg and Vogelstein.1983
Feinberg and Vogelstein.1984
Pharmacia catalogue

Materials

Ready-To-Go kit : 27-9251-01, Pharmacia

DNA : probe template, 50ng

α^{32}**P-dCTP** : 50μCi

All equipment necessary for safe operation whilst using radioactive materials

Sephadex G-50 slurry : 1g Sephadex in 20ml column buffer

Spin column wash buffer : 0.01M Tris.HCl, pH 8.0;1mM EDTA;0.03%β-mercaptoethanol;H_2O to 100ml

Syringes : 1ml

Method

The template DNA (25-50ng) was dissolved in H_2O to give a volume of 45µl, denatured by boiling in a water bath for 2-3 min, immediately put on ice, allowed to cool, and centrifuged for 10 sec at 13,000rpm.

The following components were added to the **Ready-To-Go** reagent mixture ;- denatured template DNA (45µl and 25-50ng) and α^{32}P-dCTP(50µCi and 5µl), the mixture was mixed gently by pipetting up and down several times and incubated at 37°C for 20 min. See section 2.14.1 for the method of checking the specific activity of the labelled DNA and the percentage incorporation of radio-labelled dCTP in the probe and the method of removing unincorporated label (Section 2.14.1).

2.15   Hybridising and Washing

Reference : Sambrook et al.1989

Materials

2 x SSC : Section 2.12

0.1 x SSC : 20-fold dilution of 2 x SSC

Hybridisation tubes

Hybridisation oven : Bachofer D-7410

Plastic box : large enough to contain the membrane

Hybridisation buffer : 1M HEPES buffer, pH7.0, 25ml;20 x SSC, 75ml;Herringsperm DNA, 10mg;20% SDS, 2.5ml;Ficoll 70 000, 1g;Bovine Serum Albumin (BSA), 1g;Polyvinyl pyrollidine (PVP) 10, 1g;H_2O to 500ml

Method

The hybridisation buffer was stored at 4°C. Before use the buffer was put in a waterbath at 37°C for 10 min. With clean gloves and tweezers, the nylon membrane was placed in a tube, and pre-hybridised in the hybridisation oven with about 80ml of hybridisation buffer for at least 2 hours. Alternatively, the membrane was pre-hybridised in a box which had been placed on a shaker. This allowed better flow and

contact of the buffer's components with the membrane. After two hours, the pre-hybridisation buffer was discarded and a further 40ml of hybridisation buffer added to the tube. The probe was placed behind perspex shields to thaw out, boiled for 3-5 min to denature the DNA, centrifuged briefly at 13,000rpm and put on ice. The probe was added (A minimum of 1×10^7cpm) to the hybridisation tube, and the membrane was incubated in the hybridisation oven overnight at the desired temperature (usually 60-65°C).

Washing

After hybridising overnight at 63°C, the hybridisation tube was taken from the oven, the supernatant poured off, about 80ml 2xSSC added, and the tube returned to the oven for 15 min. This process was repeated. A third wash was carried out with 0.1xSSC, in a box on a shaking waterbath at the desired temperature and this process was repeated. Washing in a plastic box ensured better contact between the 0.1xSSC and the surface of the membrane. The 0.1xSSC washes controlled the washing stringency as they had the lowest concentration of Na^+ ions.

The **washing stringency** and hybridisation temperature were vital as they determined the conditions under which the probe DNA and the target DNAs would anneal, with a minimum of binding between the probe DNA and less homologous sequences. Sequences with near to 100% homology would still bind after hybridisation at high stringency, but low homology binding was eliminated.

Stringency ; the degrees Celsius below the melting temperature (Tm) of the probe/target DNA hybrid.

Calculation ;

$$TM = 16.6log[Na^+] + 0.41(\%GC) + 81.5 - 0.65(\%formamide) - \frac{500}{bp\ in\ duplex}$$

$[Na^+]$ varied for each buffer ;hybridising buffer, 0.55M;20xSSC, 3.67M;0.1xSSC, 0.018M

2.16   Ligation

References : Ausabel et al.1991

Maniatis et al.1982

Materials

T4 DNA Ligase : BRL

5 x T4 DNA Ligase buffer : see BRL catalogue

Plasmid and Insert mixes

Thermal cycler : Techne PHC-3

Method

The following ingredients were added together and incubated overnight at 13°C in the thermal cycler;DNA as discussed below;5x T4 DNA Ligase Buffer (3μl);T4 DNA Ligase (2μl);H_2O (to 15μl)

To ensure ligation of the insert into a plasmid, the mixture was kept as concentrated as possible (it was usually done in 15μl).

The quantities of DNA employed depended on the size of the one or more different fragmentsbeing ligated, as relative concentrations were important. Oftenequal quantities of plasmid and insert are used. The more concentrated the ligation mix, the more likely it is that an insert will be incorporated into the vector plasmid (the extreme being concatamerisation, whereby more than one insert was ligated and repeated copies of either the insert, or the fragment which the insert was going into,occurred). Dilute ligation mixes result in ligation of the two ends of a DNA molecule, thus limiting the opportunities for insertion. For insertion of a fragment into CAP-treated plasmids, more plasmid was added, as only a very small amount of plasmid should have self-ligated. See sections 3.2 and 3.3.

2.17   Removal of the 5' Phosphate from Linear DNA
(CAP Treatment)

Reference : Maniatis.1982

Materials

10x Calf Alkaline Phosphatase (CAP) buffer : 0.5M Tris.HCl, pH 9.0; 10mM $MgCl_2$;1mM $ZnCl_2$;10mM Spermidine.

Sodium chloride/Tris.HCl/EDTA (STE) 10 x buffer : 0.1M Tris.HCl, pH 8.0;1M NaCl;10mM EDTA.

TE buffer : see section 2.2.

Ethanol : 95%.

SDS : 10% stock solution.

Calf Intestinal Alkaline Phospatase (CAP) : BRL, 0.5 units/µl

Phenol

Chloroform

Prepared spin column : section 2.14.1

Method

For every µg of 3kb linear DNA, 1.2pmole of free ends are available for 5'phosphate removal. 1 unit of calf alkaline phosphatase will digest 100pmoles (80µg) when the following procedure is used. Therefore the enzyme is commonly used in excess.

Plasmid DNA (1µg) was linearized with the restriction enzyme of choice (section 3.3), extracted with phenol/chloroform (50:50), ethanol precipitated, dissolved in a minimum volume of 10mM Tris.HCl (pH 8.0), and the following added ;- 10X CAP buffer, 5µl;H_2O, 45µl;10-fold dilution of Calf intestinal Alkaline Phosphatase (CAP), 0.5µl. The mixture was incubated at 37°C for 30 min, and a second aliquot (0.5µl) of CAP enzyme added before incubating for a further 30 min. After this incubation, the following reagents were added ;- H_2O, 40µl;10x STE, 10µl;10% SDS, 5µl, the mixture was heated to 68°C for 15 min, the DNA solution was purified by phenol/chloroform extraction and two chloroform extractions (section 2.6), the aqueous phase was passed through a column of Sephadex G-50 resin equilibrated in TE (section 2.14.1) and finally, the DNA was ethanol precipitated (section 2.6). The phenol/chloroform extraction and spin column techniques were

sometimes replaced by passing the treated DNA through a *PCR-preps* mini-column (see section 2.9.3).

2.18   Preparation of Cells For Electroporation

References : Ausabel et al,1991
Dower et al,1988

<u>Materials</u>

***Escherichia coli* cells (either strain DH-1 or strain HB101)** : Table 1

Glycerol

Luria broth : section 2.1

Antibiotics : Appendix 1

HEPES buffer : HEPES buffer (1M, BDH) in sterile, deionised water, pH 7.0

<u>Method</u>

Two universal bottles, each containing 10ml of Luria broth (section 2.2) were inoculated with *E.coli* strain HB101, as in section 3.2, or DH-1, as in section 3.3 (table 1) and incubated overnight, with shaking at 37°C. Antibiotics were added to select for plasmid maintenance and against contaminant growth (sections 3.2 and 3.3). Two 500ml flasks of Luria broth were inoculated the with the two 10ml cultures. These broths were put on a shaker at 37°C until an OD_{600} of 0.45-0.55 was reached (approx 2-3 hours).

After the culture had grown to the desired OD_{600}, it was chilled briefly on ice, transferred aseptically to sterile GSA bottles and the cells centrifuged in a GSA rotor at 5,000 rpm (2,560 g) for 10 min at 4°C. The supernatant was poured off, the cells resuspended in 1 litre of ice-cold sterile 1 mM Hepes buffer (pH 7.0) and the cells centrifuged in a GSA rotor at 5,000 rpm for 10 min at 4°C. The cells were resuspended in 500ml ice-cold Hepes, centrifuged in a GSA rotor at 5,000 rpm for 10 min at 4°C and resuspended in 20ml ice-cold, sterile glycerol. The cells were then transferred aseptically to a sterile SS34 tube and centrifuged in an SS34 rotor at 6,000 rpm (2,806 g) for 10 min at 4°C.

The supernatant was poured off and the cells resuspended in 2-3ml ice-cold, sterile 10% glycerol, aliquoted into sterile tubes and stored at -70°C.

2.19   Filter Sterilisation

Materials

Parchment paper

Swinnex filter holders : Millipore, 25mm diameter

Millipore nitro-cellulose membranes : 0.22μm, 25mm diameter

Syringe : 10ml capacity

Method

This method was useful for filter sterilising any liquid which could not be heat sterilised.

A Millipore membrane (0.22μm) was placed inside the filter holder and autoclaved in parchment paper at 121°C for 15 min. Universal bottles were also pre-sterilised by autoclaving. The solution to be sterilised was added to a syringe barrel which was attached to the filter holder and the syringe plunger used to pass the solution through the membrane and into a universal bottle. The bottle was capped and stored under appropriate conditions.

2.20   Electroporation

References : Dower et al.1988
               Ausabel et al.1991

Materials

SOC medium : section 2.2

***Escherichia coli* cells** : prepared as in method 2.18 (either strain DH-1 or strain HB101, Table 1)

Ligation mix : containing plasmid with insert

Control plasmid DNA

Luria broth (LB) plates : section 2.1, antibiotics added (Appendix 1)

Luria broth (LB) plates with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) and isopropylthio-β-D-galactoside (IPTG) : used if *lac Z* was in the plasmid construct (section 2.22)

Gene Pulser : Bio-Rad

Gene Pulser cuvettes : Bio-Rad, 0.2cm electrode gap

<u>Method</u>

The Gene Pulser delivers a high voltage charge across the cells, disrupting their outer membranes, thus allowing DNA transfer into the cells through newly created pores.

The volts were set to 2,500 volts, the capacitance was set to 25μF and the resistance was varied. A resistance of 400Ω was usually high enough, particularly for *E.coli* strains such as DH-1. Reducing the resistance helped to prevent arcing (which results in a high current passing through the mixture, destroying the cells),but also reduced the efficiency of DNA transfer. The time constant was observed (by pressing the time constant button). At 2,500 V, a time constant of 4 or more msec was desirable. This was the number of msec during which the charge was applied to the cells. Time constants with lower values than this resulted in lower transfection efficiencies.

The cells, DNA to be inserted, 1.5ml Eppendorf tubes and cuvettes were put on ice. Approximately 300μl of prepared cells (see section 2.18) were divided into four tubes containing 75μl of cells in each. The concentration and amount of DNA in the sample to be transferred determined what volume was to be added to the cells. The two plasmid constructs electroporated in this thesis, pPF210 and pPF310 were treated differently as the latter was not purified after ligation and therefore   a much smaller volume of pPF310 was added.

The reactions for pPF210 electroporation were as follows ;- tube #1 : 75μl of cells (*Escherichia coli* strain HB101), 5μl of pBR328;tube #2 : 75μl of cells, 25μl of ligation mix;tube #3 : 75μl of cells,1 5 μ l   o f ligation mix;tube #4 : 75μl of cells, 10μl of ligation mix.

The reactions for pPF310 electroporation were as follows ;- tube #1 : 75μl of cells (*Escherichia coli* strain DH-5α), 2μl of PUC118;tube #2 : 75μl of cells, 3μl of ligation mix;tube #3 : 75μl of cells,5μl of ligation mix;tube #4 : 75μl of cells, 7μl of ligation mix.

The reactions were mixed gently with a pasteur pipette and kept on ice, the first sample was loaded into an ice-cold Gene Pulser cuvette, the outside of the cuvette was wiped and the cuvette immediately transferred to the white slide on the BIO-RAD electroporater. This slide was pushed into the electroporation chamber and the two buttons required to deliver an electric charge were pressed and held down until the machine produced a soft beeping sound. If a loud pop occurred, the machine had arced, and action was taken before the next sample was loaded to reduce the resistance, or eliminate any ions either outside or inside the cuvette. The lid was placed on the cuvette when it was in the chamber to prevent spreading cells inside the chamber if arcing occurred.

Immediately after applying the pulse, 1ml of SOC (section 2.1) medium was added to the cuvette (this was used as a recovery medium in which the electrically shocked cells could recuperate before growing and multiplying), the cellswere transferred to a 1.5ml Eppendorf tube and incubated at 37°C for 2 hours. After two hours incubation, the four tubes were plated out on LB with antibiotics to select for the recently electroporated plasmid. For the HB101 cells which were transfected with pPF210, Kan/Tc/Sm plates were used (section 3.2). TheD H - 1 cells which were transfected with pPF310 were plated on plates containing IPTG, X-gal and ampicillin (section 3.3).

2.21   Pick-and-Patching

Materials

LB plates : section 2.1, each with different antibiotics (sections 3.2 and 3.3)

Sterile toothpicks

Method

This is a simple method for transferring single colonies to more than one plate to determine their antibiotic resistance while keeping track of which colony is which.

Tooth-picks were sterilised in universal bottles. On the bottom, outer surface of each petri dish, eight rows and eight columns were drawn with a marker pen and a reference number was put in the first box of each row. Each colony to be patched was picked up with a tooth-pick and transferred to each plate in succession and finally to a control plate with no antibiotics. The colony was inoculated in the same number box each time, as indicated by the numbers in the rows on the bottom of each plate and each plate was incubated at 37°C for 2 days.

2.22   Replica Plating

Materials

LB plates : section 2.1, with different antibiotics in each plate and control plates with no antibiotics

Parchment paper

Velvet squares : approx. 18cm x 18cm

Wooden block : with a diameter slightly smaller than that of a petri dish

Metal ring : with a diameter slightly larger than that of the block

Method

This technique allowed the transfer of large numbers of single colonies from one plate to a number of other plates, whilst maintaining the position of each colony so that individual colonies could be identified later regardless of which plate they were on.

Velvet squares (10) were sterilised by autoclaving in parchment paper. A biohazard unit was sterilised and the airflow turned on. The plates with colonies on them, and the sterile plates were put in the biohazard unit with their lids ajar. For each plate with colonies on it a velvet square was required. A velvet sheet was placed on the wooden block.

The velvet sheet was pushed down firmly and held in place with the metal ring. All the plates were marked on the edge of the bottom of the plate for orientation. A plate from which colonies were to be transferred was placed on the velvet, upside down with the lid off and the mark on both the plate and block lined up. Once on the velvet, no sideways motion was allowed. The plate was pushed firmly onto the velvet to ensure that all areas of the plate had sufficient contact and carefully lifted off. A sterile antibiotic plate was put on in the same manner with the marks on the plate and the block lining up. This was repeated for each different antibiotic plate and finally for a plate with no antibiotics on it. The plates were incubated at 37°C for 2-4 days.

2.23   Blue/white Selection

References : Sambrook et al.1989
Horowitz et al.1974

Materials for Blue/White Selection

Luria plates : section 2.1

Soft Luria top agar : section 2.1

Isopropylthio-β-D-galactoside (IPTG) : dissolved to 100mM in deionised water, Sigma

5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) : 2%, dissolved in dimethyl formamide, BRL

Antibiotics :

Method

This method was used for selection of plasmids containing the *lac Z* gene, such as PUC 118.

Luria plates were made with Ampicillin (Appendix 1), 3.5ml of Luria soft agar, containing X-gal (50µl) and IPTG (20µl) was poured on top of the Luria plates, and allowed to set before the inocula were added.

After incubation at 37°C for 2 days *(E.coli)*, two different types of colonies were present ;- blue and white. The presence of the plasmid

was ensured by antibiotic selection. The presence of an insert was detected because it inactivated the β-galactosidase, and thus the colonies were white.

Sometimes a little background activity made cells with no β-galactosidase activity appear light blue, so negative and positive controls were used.

2.24   Thermal Cycling

Reference : Ausabel et al.1991

Materials for Thermal Cycling

Deoxynucleotide triphosphates (dNTPs) : Pharmacia (100mM)

Taq DNA polymerase : Life Technologies, 10 units/µl

DNA template : pPF210 (section 3.2), or pPF310 (section 3.3)

Primer DNA : as explained in sections 3.2 and 3.3

10 x Taq DNA polymerase buffer : 100mM Tris.HCl, pH 8.3;500mM KCl;0.2% gelatin;250mM $MgCl_2$, Life Technologies

Paraffin oil : Perkin Elmar Cetus

Method

Setting the thermal cycler.

A Techne thermal cycler, model PHC-3, was used. Three different files were run sequentially for all experiments regardless of the temperatures used in the following order;- #13, #10, #11. The temperatures and length of time at each temperature were set as follows ;- file #13,93°C for 3 min, 60°C for 30 sec, 72°C for 30 sec (1 cycle);file #10,93°C for 45 sec, 60°C for 30 sec, 70°C for 30 sec (40 cycles);file #11,04°C for 99 hrs (1 cycle).

The reactants were added to a 0.5ml Eppendorf tube in an ice bucket in the following order ;- sterile water (to make final volume of 20µl in the mixture);10 x buffer, 2µl;0.2mM dNTPs, 4µl;DNA (sections 3.2 and

3.3), 0.5-2µl of a 50µg/ml stock;primer, 0.5µl of a 20pmol/ml stock;taq DNA polymerase, 0.5µl. All stock solutions were made on the same day that the polymerase chain reaction was run, and stored at 4°C. The mixture was centrifuged briefly (13,000rpm for 3 sec), 15µl of oil added, the Eppendorf tube was flicked three times, centrifuged again (at 13,000rpm for 3 sec) and placed in the thermal cycler and immediately processed.

The reaction product (5µl) was separated by electrophoresis on a BRL Minigel apparatus containing 2% Sea Kem (FMC) agarose gel and TBE buffer (section 2.2). The gel was stained and destained as explained in section 2.11.

3   Results

Three separate areas, each containing *tra* gene DNA from the strain *A.tumefaciens C58* were cloned and used as probes to detect DNA from other *Agrobacterium* or *Rhizobium* strains with significant homology (greater than 75%) as shown by the presence of one or more bands on X-ray film.

This chapter describes how the *tra* gene probes were made and which *Agrobacterium* and *Rhizobium* strains tested contained DNA with areas homologous to these probes.

3.1   Results for Tra I

A sub-clone containing the construct pPF110 (fig 7) was supplied by Professor S.K.Farrand (University of Illinois). The *tra* 1 Hind III fragment #3 (Cook and Farrand,1992) had been sub-cloned (unpublished) into the broad host range plasmid pSA152 (Tait et al,1983) and placed into *E.coli* αDH-5 (Table 1). The *tra* region of this construct was used to probe a number of *Rhizobium* and *Agrobacterium* strains, the results of which are explained below.

3.1.1 Isolation of the Plasmid DNA from Sub-clone pPF110

The *E.coli* cells were grown in LB broth (section 2.1) with ampicillin added to 50µg/ml, and the plasmid pPF110 (fig 7) was isolated (section 2.7). The ratio of the absorptions at 258nm and 280nm (258/280) was approximately 1.9, indicating a clean preparation. The plasmid preparation was then stored in three 1.5ml Eppendorf tubes, two of which were stored at -70°C, and the third at 4°C. A volume (2µl) of the plasmid preparation was later digested with EcoR1 (section 2.8) and 2µl of the mixture was electrophoresed on a 0.7% Sea Kem gel. The approximate concentration of the DNA added to the gel was 125µg/ml as indicated by band intensities compared to the concentration markers (figure 9).

3.1.2 Preparation of Tra 1 for Radioactive Labelling with α^{32}P-dCTP

Figure 7 : The plasmid pPF110, consisting of a
9kb fragment containing *tra* DNA from
the *A.tumefaciens* plasmid pTiC58
in the broad host range plasmid pSa152.
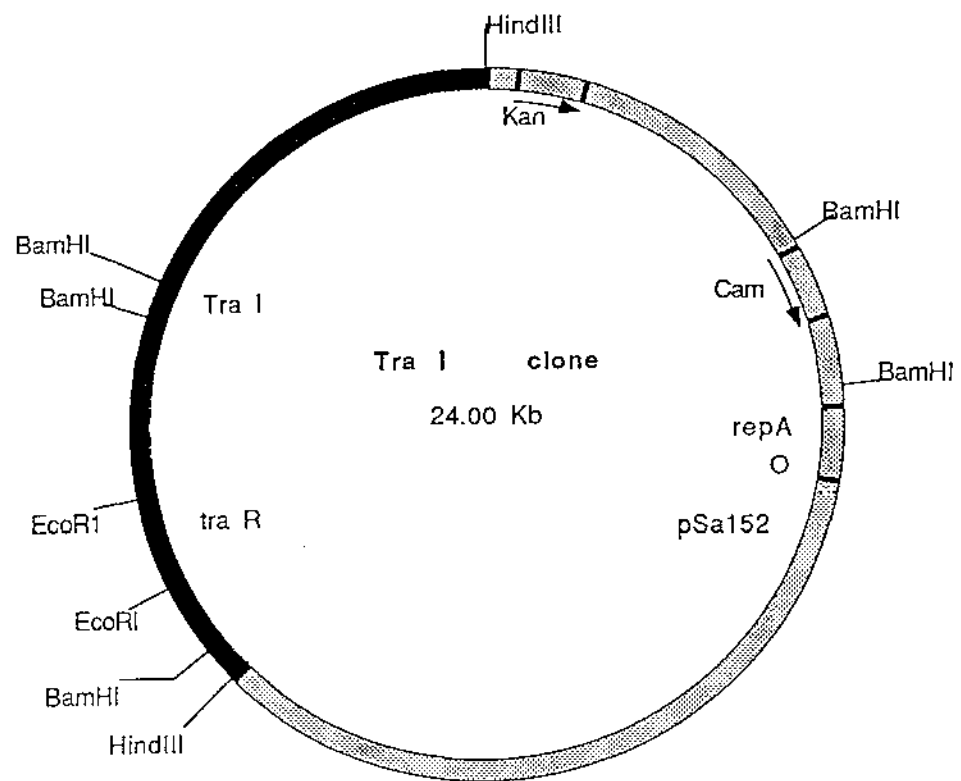
Figure 8 : The Lambda Hind III DNA ladder.
bp = base pairs

λ DNA/*Hind* III Fragments
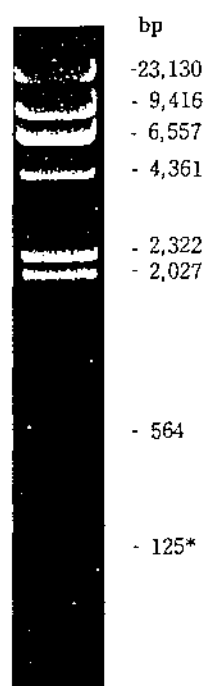
bp
- -23,130
- - 9,416
- - 6,557
- - 4,361

- - 2,322
- - 2,027

- - 564

- - 125*

Figure 9 : DNA concentration markers on a 0.7%
agarose gel. The wells were loaded
as follows : (1) 200ng 1kb ladder,
(2) 150ng pUC118, (4) 5ng concentration
marker pUC118, (5) 10ng concentration
marker pUC118.

LANES

1 2 3 4 5
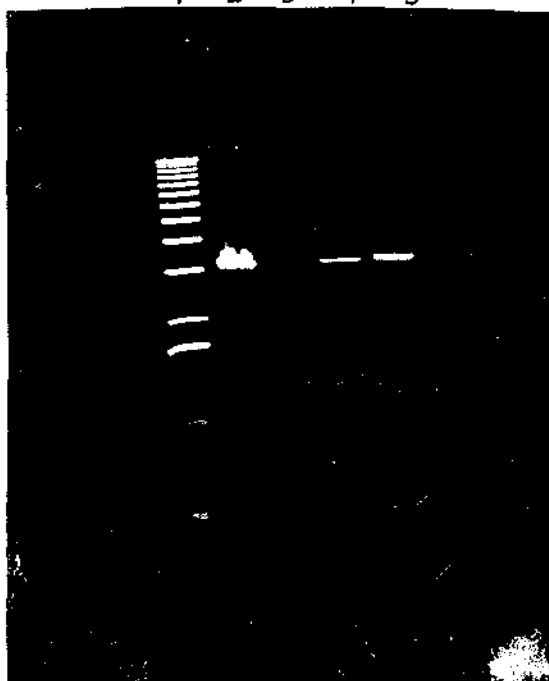
The entire pPF110 construct was digested with Hind III in sufficient quantity to add 50ng of DNA from the digest to the labelling system in a volume of less than 10μl. The restriction buffer components did not affect the subsequent labelling reaction, but it was considered advisable to add as little as possible to the latter reactions.

Plasmid pSA152 was derived from the *E.coli* plasmid pSA (Tait et al,1983), and contains gene responsible for resistance to kanamycin and chloroamphenicol. When the pSA152 portion of pPF110 (see fig 7) was to be excluded from the labelling reaction, pPF110 was digested with Hind III and the fragments were separated by electrophoresis (section 2.11). The fragment containing the *tra* region was then excised from the gel and purified (section 2.9.3). A Sea Plaque gel (FMC) was poured in a Minigel apparatus (BRL), but first all but one of the teeth in a comb were taped up to provide one long well. The 1kb ladder was added to the tooth which was left untaped. The lower band (9kb) was excised from the bulk of the gel using a sterile scalpel blade and added to a sterile 1.5ml Eppendorf tube before labelling (2.9.3). About 6μg of DNA was added to the long well to provide a good yield at the end of the procedure. Approximately 3.5μg was left in a final volume of 35μl for subsequent use as a probe.

3.1.3 Radioactive Labelling of DNA

For both the 9kb *tra* 1 fragment and the entire pPF110 construct, 50ng of DNA was added to Ready-To-Go kits (Pharmacia) and labelled (section 2.14.2). The percent of radioactivity incorporated into the entire pPF110 plasmid probe was 59% The percent of radioactivity incorporated into the 9kb *tra* 1 fragment was 58% The number of counts in the entire pPF110 plasmid probe was 9.8×10^7 counts. The number of counts in the 9kb *tra* 1 fragment was 3.8×10^7 counts.

3.1.4 Labelling the 1kb Ladder

25ng of DNA was labelled with a Pharmacia Ready-To-Go kit (section 2.14.2). The entire probe produced 3.4×10^8 counts, and the percent of radioactive incorporation was 69%

3.1.5 Provision of DNA from *Agrobacterium* and *Rhizobium* strains

Genomic DNA was isolated (section 2.6) from the strains listed in figures 11A and 12A. However, before the genomic DNA of *R.leguminosarum* bv trifolii strain 2163 was isolated, the cells were inoculated onto white clover seeds (section 2.4). The cells, after forming nodules, were isolated from these nodules (section 2.5) and the Eckhardt procedure for large plasmid isolation (section 2.7.3) was performed. The inoculation and isolation ensured the presence of the Sym plasmid, without which nodulation would not have occurred, and the plasmid isolation confirmed it's presence. Figure 10 shows the plasmid profile of *R.leguminosarum* bv trifolii strain 2163, which has five plasmids (the lower band is a doublet containing the Sym plasmid) and the plasmid profile of *R.leguminosarum* bv trifolii strain PN165. The latter strain is a derivative of 2163, but has been cured of the Sym plasmid. Therefore only four plasmids are present. The Eckhardt procedure was performed on *Agrobacterium* strains to ensure that they still contained their plasmids, which they did (figures not shown).

3.1.6 Southern Blots for Probing with pPF110

The genomic DNA of different bacterial strains was digested (Section 2.8), then electrophoresed (Section 2.11) in the gel shown in figure 11A. The gel was then transferred by vacuum blot (Section 2.12.2) to a nylon membrane (Hybond-N, Amersham).

The gel was stained and destained (section 2.11) before blotting (section 2.12.2), and restained after blotting to check the efficiency of transfer. No bands were visible after blotting, indicating that virtually all the DNA had transferred from the gel onto the membrane.

Figure 12A shows further genomic EcoR1 digests, digested, electrophoresed and blotted in exactly the same way as the DNA shown in figure 11A.

3.1.7 Hybridisation of the Probes to the Southern Blots

After hybridisation (section 2.15) the blot of the gel shown in figure 11A was autoradiographed (fig 11B). Only the positive controls (lanes

Figure 10 : Eckhardt gel of *Rhizobium leguminosarum*
bv trifolii strain ICMP2163 and *Rhizobium*
leguminosarum bv trifolii strain PN165.
For both strains, wells were loaded with
0.7ml of cells at an optical density
(OD) of 0.600 at a 600nm wavelength.
Lane 1 contains ICMP2163 and lane 2, PN165.

LANES
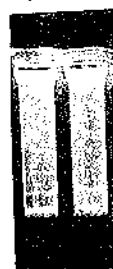
Figure 11 : DNA from *Agrobacterium* and *Rhizobium* strains digested with EcoR1 and separated (1μg each) by electrophoresis in 0.7% agarose, then blotted and probed. The wells were loaded as follows : (1) 200ng 1kb ladder, (2) *Rhizobium leguminosarum* bv trifolii strain ICMP2163::Tn5, (3) *Rhizobium leguminosarum* bv trifolii strain ICMP2163 ,(4) *Agrobacterium tumefaciens* C58 strain 2-17, (5) *Rhizobium leguminosarum* bv trifolii strain PN165

A : agarose gel. B : autoradiograph.
The final wash was carried out in 0.1 x SSC at 47°C.

LANES

1 2 3 4 5

A
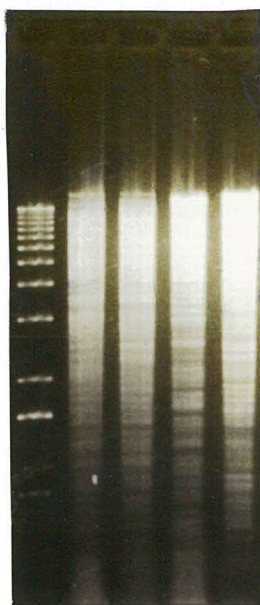
LANES

2 3 4 5 6

B

Figure 12 : DNA from *Agrobacterium* and *Rhizobium*
strains digested with EcoR1 and separated
(1μg each) by electrophoresis in 0.7%
agarose, Southern blotted onto nitro-
cellulose and probed with the HindIII
fragment of pPF110. The wells were loaded
as follows : (1) *A.tumefaciens* C58 strain
2-17, (2) *Rhizobium leguminosarum* bv
trifolii strain PN165, (3) *Rhizobium
leguminosarum* bv trifolii strain ATCC14480,
(4) *Rhizobium leguminosarum* bv trifolii
strain ICMP2163, (5) *Rhizobium loti* strain
ATCC33669, (6) *Agrobacterium tumefaciens*
strain LMG64 (7) 200ng 1kb ladder,

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

LANES

1 2 3 4 5 6

A

LANES

1 2 3 4 5 6 7

B

2 and 4) showed bands. *R.leguminosarum* bv trifolii strain 2163, and *R.leg* bv trifolii strain PN165 did not show any bands, indicating a lack of homology with the probe DNA. The Tm (the temperature at which 50% of the DNA anneals) for the *tra1* probe is 77°C in 0.1 x SSC, and 99°C in hybridisation buffer. The hybridisation and the final wash were carried out at 30°C below the Tm.

After hybridisation (section 2.15) the blot of the gel shown in figure 12A was autoradiographed (fig 12B). Not only did the positive controls show bands, but a single band was seen in both *A.tumefaciens* strain LMG64, and in *R.leguminosarum* bv trifolii strain 14480. The hybridisation and the final wash were carried out at 35°C below the Tm.

3.2 Results for Tra II

An area on the Ti plasmid pTiC58 containing the *oriT* site (Cook and Farrand,1992) was ligated into the plasmid pBR328 (Bolivar,1977), electroporated into *E.coli* strain HB101 (Table 1), and used as a probe to locate bands with significant homology (60% or greater) to itself among a number of *Rhizobium* and *Agrobacterium* strains. The fragment corresponded to the EcoR1 *tra* II #20 fragment (fig 13) and contained a Tn5 insertion (Jorgenson et al,1979). The final construct, pPF210 is shown in figure 14. The following steps were involved in this process.

3.2.1 Isolation of Genomic DNA from *A.tumefaciens* C58 strain 15-26

Strain 15-26 is a C58 mutant with a Tn5 insertion located within the *tra* II region of the plasmid pTiC58 (fig 13). The cells were grown for 24 hours in 40ml of LB (section 2.1) with 50µg/ml of kanamycin (Appendix 1) added, and the total DNA was isolated (section 2.6). The ratio of the absorptions at 258nm and 280nm was approximately 1.8, indicating that the DNA was clean enough for digestion with the restriction enzyme EcoR1. There was approximately 750µg/ml of DNA (section 2.6) in the preparation.

Figure 13 : The restriction map of the *tra I* and *tra II* areas of the pTiC58 plasmid (von Bodman,1989). The positions of Tn5 insertions which disrupt conjugal plasmid transfer are indicated by the vertical lines from the mutant strains to the map.

traII   tra I

Tra⁻15—28
Tra⁻3—16
Tra⁻16—24

Tra⁻4—32
Tra⁻⁰10—23
Tra⁻17—60
Tra⁻2—17
Tra⁻12—8

acc

| HindIII | 20 | 21a | 3 6 | 29 38 | 21b | 4 | 3 | 4 2 | 31 1 | 11 |

| BamHI | 22 26 | 7 | 18 33 | 4 | 10 | 24 35 | 14 | 5 |

| SmaI | 3b | 3a | 19 | 9 | 22 |

| EcoRI | 5 | 10 | 25 | 27 | 34 | 20 | 4 | 33 | 21 | 28 | 16 |

kb   100   105   110   115   120   125   130
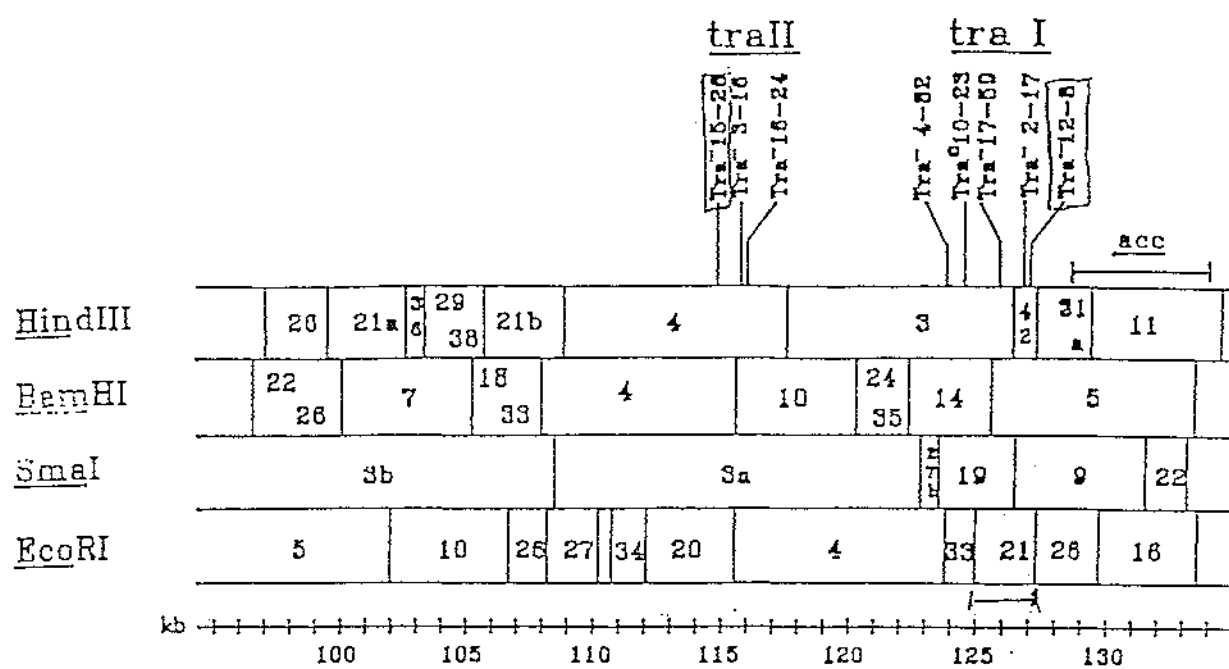
Figure 14 : A restriction map of the plasmid pPF210. The plasmid contains the plasmid pBR328 (Bolivar,1977) with an insert containing Tn5 DNA (Jorgenson et al,1979) with flanking *tra II* regions (von Bodman,1989).

EcoR1

Hind III

Amp

Cam

Tra II

pBR328

Tc

pPF210

13.80 Kb

Hind III

Cam

Tra II

Tn5

EcoRi

Kan

Kpnl
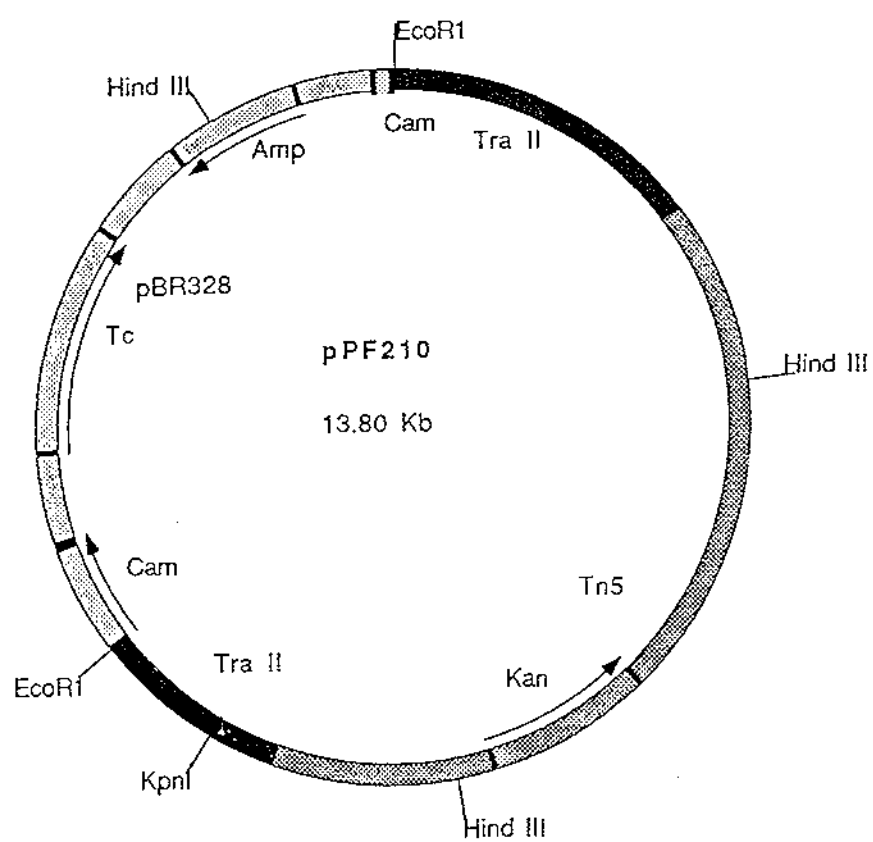
Hind III

Figure 15 : 1kb ladder DNA. bp = base pairs.

1 Kb DNA Ladder

bp

| 12,216 |
| 11,198 10,180 9,162 |
| 6,108 7,126 8,144 |
| 5,090 |
| 4,072 |
| 3,054 |
| 2,036 |
| 1,636 |
| 1,018 |

— 506, 517
— 396
— 344
— 298
— 220
201
154
134
75

Hinf I fragments
of the vector
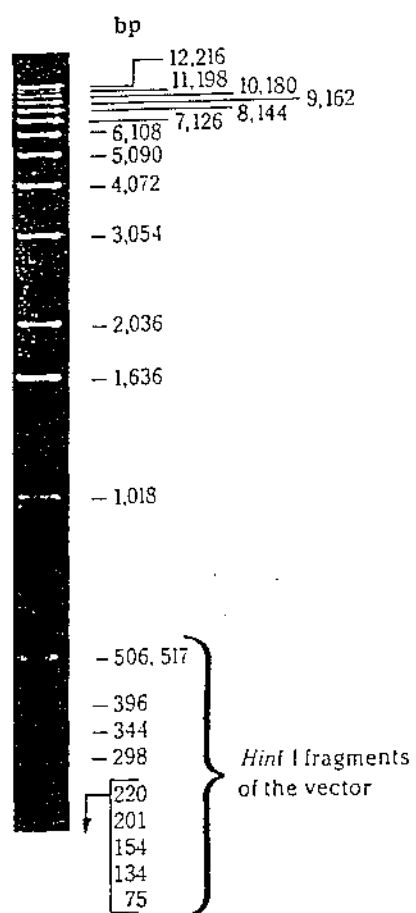
3.2.2 Digestion of Lambda DNA

Bacteriophage (Lambda Cl857Sam7) DNA (Amersham) was supplied at a concentration of 500ug/ml. The DNA (10µg) was digested with 20 units of Hind III restriction enzyme (section 2.8). 150ng of the digest was electrophoresed on agarose. Fig 8 details the sizes of the Lambda DNA bands.

3.2.3 Isolation of the Plasmid pKAN2

A plasmid preparation (section 2.7.2) of the plasmid pKAN2 (Scott,1982) was supplied. pKAN2 contains the Hind III internal fragment of Tn5 (fig 16) cloned into the E.coli plasmid pBR322 (Bolivar,1977)

3.2.4 Separation of the Tn5 Hind III fragment from pKAN2

The internal Hind III fragment of the Tn5 portion of pKAN2 was isolated by digestion with Hind III (section 2.8) followed by excision of the 3.3kb fragment and purification from a 0.7% agarose gel (section 2.9.1).

3.2.5 Radioactive Labelling of Lambda and Tn5 DNA

A Multiprime kit (Amersham) was used for the radioactive labelling of both the Tn5 Hind III fragment and the Lambda DNA (section 2.14.1). Both probes required the use of spin columns (section 2.14.1) which increased the percent of radioactive incorporation to approximately 75% The Tn5 DNA probe had a total of 4.7 x 10^8 counts in 400µl, whereas the Lambda DNA had a total of 9.6 x 10^8 counts in 400µl.

3.2.6 Digestion and Electrophoresis of DNA from C58 strain 15-26

Genomic DNA (1µg), isolated from strain 15-26 (section 3.2.1) was digested with EcoR1 (section 2.8), divided into 2 lanes (fig 17A) and electrophoresed (section 2.11) between 2 lanes of digested Lambda ladder DNA (section 3.2.2).

Figure 16 : A restriction map of the mobile genetic
element Tn5 (Jorgenson et al,1979).

Tn5
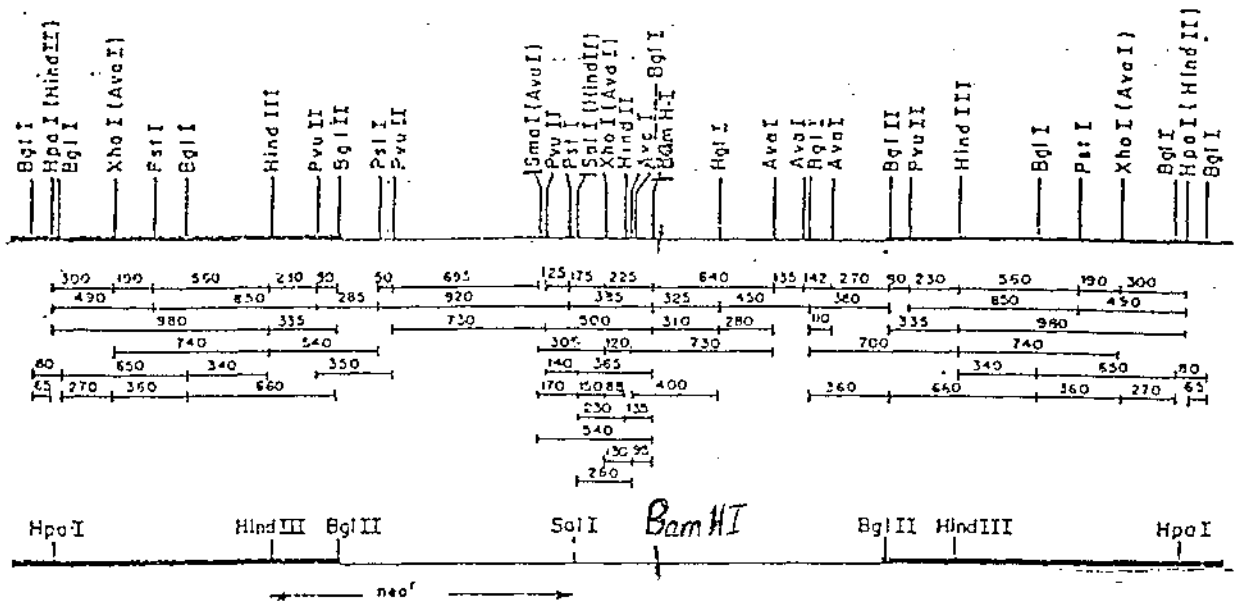
Figure 17 : DNA from *Agrobacterium tumefaciens strain C58* mutant tra-15-26 and C58 mutant tra-2-16 digested with EcoR1 and separated by electrophoresis in 0.7% agarose, Southern blotted onto nitrocellulose and probed with the internal Tn5 Hind III fragment of pKAN2. The wells were loaded as follows : (1) 100ng Lambda ladder, (2) 300ng *A.tumefaciens* C58 strain 15-26, (3) 500ng *A.tumefaciens* C58 strain 15-26, (4) 100ng Lambda ladder, (5) 100ng *A.tumefaciens* C58 strain 2-16, (6) 200ng *A.tumefaciens* C58 strain 2-16

A : agarose gel. **B** : autoradiograph. The final wash was carried out in 0.1 x SSC at 37°C.

LANES

1 2 3 4 5 6

A
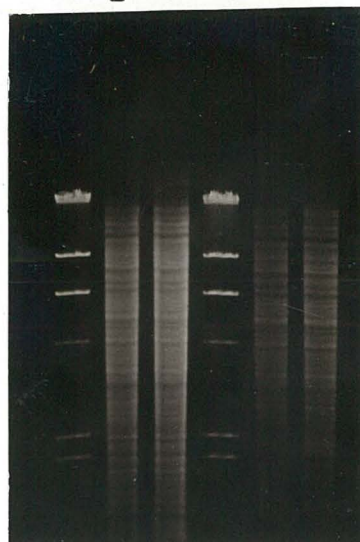
LANES

1 2 3 4 5 6

B
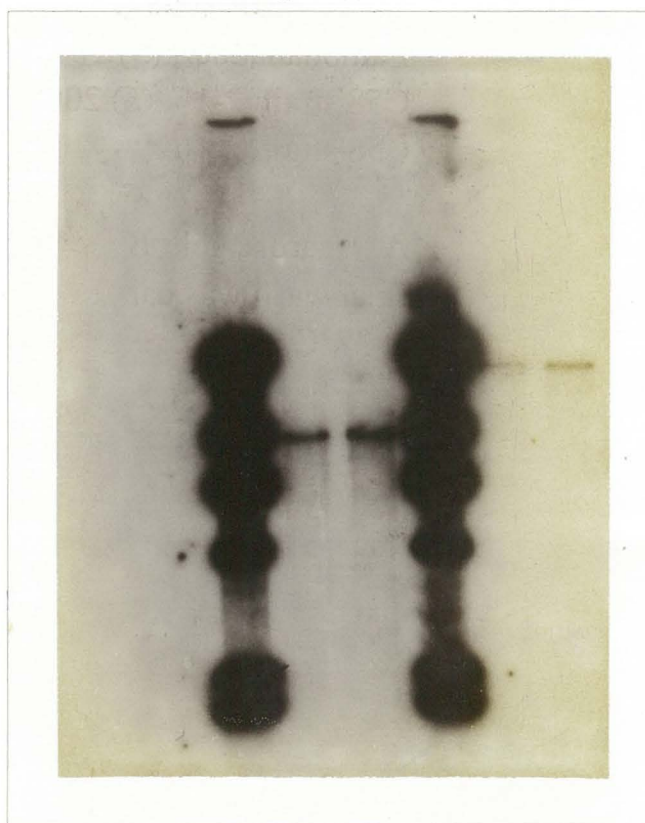
3.2.7   Southern Blot of C58 strain 15-26 DNA

The gel shown in figure 17A was blotted onto a nitrocellulose membrane (section 2.12.1) and the membrane was hybridised (section 2.15) with 40µl of the Tn5 probe and 30µl of the Lambda probe (section 3.2.5). The resulting autoradiogram (fig 17B) shows the presence of bands containing Tn5 in lanes 2 and 3. Comparison with the Lambda ladder indicated that the bands were approximately 9kb in length. Reference to figure 13 suggested that this was the expected size of the 3kb EcoR1 fragment (#20) with an insertion of the entire Tn5 DNA, which is 5.7kb in length, and has no EcoR1 sites (fig 16). This confirmed that the DNA on the membrane was from the correct bacterial strain, and that Tn5 DNA was still present in the *tra* II area, as expected (von Bodman et al,1989).

3.2.8   The Isolation of 8-10kb DNA Bands from Agarose Gels

The band containing the Tn5 DNA and bands of similar size were excised from a 0.7% Sea Kem gel (section 2.9.2), ethanol precipitated (section 2.6), resuspended in 50µl of deionised water and purified by microdialysis (section 2.10). Microdialysis increased the mixture's final volume to 80µl. Figure 18 indicates that there was still plenty of DNA after this process, as only 8µl of the dialysed mixture was added to lane 6. Lane 5 shows a further 10-fold dilution of the sample.

3.2.9   Preparation of Plasmid pBR328 for Ligation

The plasmid pBR328 is a derivative of pBR322 (Bolivar et al,1977), but has a chloramphenicol gene inserted into it (Bolivar,1977). A single EcoR1 site is located on the plasmid within this chloramphenicol gene. When placed in *E.coli* strain HB101, pBR328 also confers tetracycline and ampicillin resistance. A large scale preparation of the plasmid was carried out (section 2.7.2) and a yield of 500µg was obtained. The DNA preparation had a 258nm/280nm absorption ratio of approximately 1.9.

The plasmid DNA (2µg) was digested with EcoR1 (section 2.8) and 100ng was electrophoresed (section 2.11) alongside the undigested

Figure 18 : DNA electrophoresed on a 0.7% agarose
gel. The wells were loaded as follows :
(1) 200ng 1kb ladder, (4) Product from
polymerase chain reaction of *tra I* DNA
using Tn5 primers, (5) 1µl from a
mixture of genomic DNA bands 8-10kb
in size after it was digested with
EcoR1, excised from a Sea Plaque
agarose gel, and purified, (6) 10µl
of the mixture in lane 5.

LANES
1 2 3 4 5 6

DNA on a 0.7% agarose gel to confirm that the digestion was complete, and that the plasmid was linear (fig 19). Lane 2 shows the single, linear digested pBR328 DNA, which has a length of approximately 4.9kb compared to the Lambda ladder. The undigested DNA (lane 3) has both closed and open circular forms which travel through the gel at different rates. The digested DNA was purified by extraction with a 50:50 phenol/chloroform mixture (section 2.6), ethanol precipitation (section 2.6) and microdialysis (section 2.10). This ensured that no EcoR1 was still present in the mixture before ligation was carried out.

3.2.10 Ligation of the 8-10kb Fragments into pBR328

To prevent excess concatamerisation, or excessive re-ligation of pBR328 to itself (section 2.16) 100ng of the 8-10kb band DNA (section 3.2.8) was added to the ligation mixture with 25ng of prepared pBR328 (section 3.2.9). The total volume of the ligation mixture was 15μl. This provided twice as many insert (the 8-10kb DNA band mixture) ends available for ligation as there were for pBR328 (section 4), thus reducing the chances of self-ligation of pBR328. The total DNA concentration was about 8ng/μl, which also reduced the frequency of self-ligation of either the pBR328 DNA, or the insert DNA. The ligation was carried out at 13°C overnight (section 2.16).

The ligation mixture was purified by microdialysis (section 2.10) to eliminate ions present in the ligation buffer, which would otherwise cause arcing during electroporation (section 2.20). The final volume of the ligation mixture after microdialysis was 50μl.

3.2.11 Electroporation of Plasmid DNA into *E.coli* strain HB101

E.coli HB101 cells which had been prepared for electroporation (section 2.18) were placed in four sterile 1.5ml Eppendorf tubes with ligated DNA, or with undigested pBR328 and electroporated (Section 2.20). Tube #2 arced during electroporation, but was still placed into SOC medium (Section 2.1) and plated onto LB (Section 2.1) plates as were tube #1, tube #3 and tube #4. Results and the contents of each tube are shown in Table 2. Tube #1 was divided into four aliquots,

Figure 19 : DNA electrophoresed on a 0.7% agarose gel. The wells were loaded as follows :
(1) 200ng Lambda ladder, (2) 50ng pBR328,
(3) 50ng pBR328 after digestion with EcoR1.

LANES

1 2 3
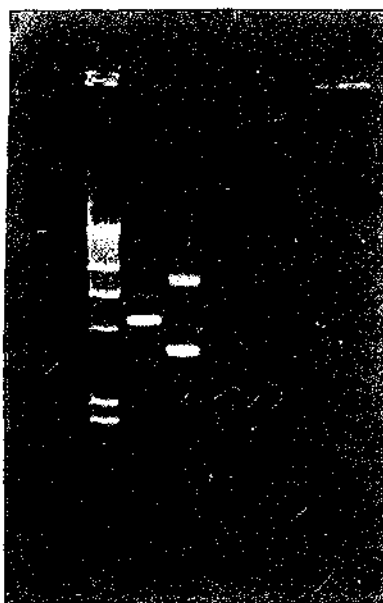
three of which were plated onto Luria plates containing tetracycline, and the fourth was plated on a Luria plate containing streptomycin. The figures given in Table 2 for the Tc plates of all four tubes were the average of three plates, not the total. One LB/Tc plate, with approximately 4,500 colonies on it was replica plated (Section 2.22) onto a plate containing chloramphenicol (Cam), then onto a plate containing streptomycin, tetracycline and kanamycin (Tc/Kan/Sm), and finally onto another Tc plate. The Cam and Tc replicas both contained approximately 4,500 colonies, whilst the Tc/Kan/Sm plate contained none. The Tc/Kan/Sm plate acted as a negative control, showing that HB101 cells (which are Sm resistant) containing pBR328 (Tc resistant) are unable to grow on LB plates containing 50µg/ml of kanamycin. The higher number of colonies on tube #1 Tc plates, compared with the numbers of colonies on Tc plates from tube #3, and tube #4 indicates that the plasmid pBR328 alone electroporates at a higher frequency than the plasmid plus the insert. Also, the frequency of Sm resistant colonies compared with the frequency of Tc resistant colonies indicates that approximately 0.1% of viable HB101 cells contained pBR328, and even less contained plasmids with an insert. The 197 Tc resistant colonies from tube #3, and the 148 Tc resistant colonies from tube #4 were patched onto LB/Tc/Kan/Sm, LB/Cam and finally LB/Sm plates. The results indicated that approximately 45% of tetracycline resistant colonies were also resistant to chloramphenicol (and therefore were presumed to have no inserts. Of the remaining chloramphenicol sensitive colonies, eight colonies grew on Tc/Kan/Sm plates. These colonies were presumed to contain the plasmid pBR328 with a Tn5 insert which had flanking *tra* II DNA (fig 14). They were chloramphenicol sensitive.

3.2.12 Confirmation of pPF210 with Plasmid Digests

Three Tc/Kan/Sm resistant colonies were incubated in Luria broth (Section 2.1) with tetracycline, streptomycin and kanamycin, and small-scale plasmid preparations were carried out (Section 2.7.1). Each of the three colonies were treated in exactly the same manner and all gave the same results when digested. Restriction digests of the plasmid preparations were made using ;- EcoR1, Hind III, and Sma I (Section 2.8). Figure 20 shows the results of these digests, from which

Figure 20 : Restriction enzyme digests of the
plasmid pPF210 on 0.7% agarose gel.
The wells were loaded as follows : (1)
10ng pPF210, uncut, (2) 30ng pPF210 after
EcoR1 digest, (3) 15ng pPF210 after Sma1
digest, (4) 30ng pPF210 after Hind III
digest, (5) 200ng 1kb ladder.

LANES

1 2 3 4 5
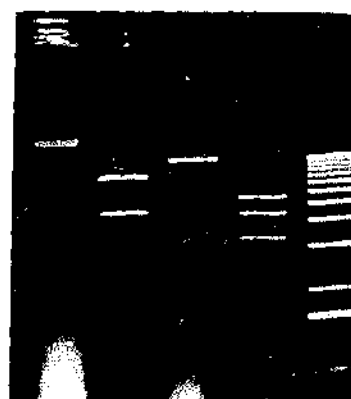
a plasmid map was constructed (fig 14). An EcoR1/Hind III double digest (Section 2.8) was also carried out (not shown) which produced five bands, each of the expected size. Fig 21 shows four of the five EcoR1/Hind III digest bands (Section 3.2.13). A large scale plasmid preparation (Section 2.7.2) was performed with one of the colonies, from which plenty of plasmid DNA (700µg) was obtained. The 258nm/280nm absorption ratio of the preparation was approximately 1.9 (Section 2.6).

3.2.13 Separation of the EcoR1/Hind III Fragment With the *Ori* Site

The *tra* II EcoR1 fragment #20 (fig 13) contains an *ori* (origin of plasmid tranfer) site which is important for transfer of the plasmid pTiC58 by transconjugation (Cook and Farrand,1992). Therefore, the 1.1kb Tn5 flanking region of the construct pPF210 (fig 14) was targeted as an area of interest as a probe. The plasmid DNA was digested with EcoR1 and Hind III (section 2.8), electrophoresed (section 2.11), the 2.2kb fragment (containing both *tra* II and Tn5 DNA) was excised from a Sea Plaque agarose gel (fig 21) and purified (section 2.9.3).

3.2.14 Amplification of the *Tra* II 1.2kb Fragment

Thermal cycling (section 2.24) was carried out with the plasmid pPF210 as the DNA template, and with the primers CAT-E1 and Tn5 17-mer (Appendix 2). The CAT-E1 primer directed DNA amplification from within the chloramphenicol gene of pBR328, across the EcoR1 site and into the flanking *tra* II DNA. The Tn5 17-mer primer directed DNA amplification from within the Tn5 DNA and across the flanking *tra* II DNA. Because the primer bound to two different areas on the plasmid (as it binds within the Tn5 inverted repeat), single stranded amplification of *tra* II DNA was presumed to occur within the 2kb *tra* II flanking region (at the top of fig 14). However, as no primer was used in the alternative direction, logarithmic amplification was not observed (fig 22). The resulting 1.2kb band is shown in figure 22 (lane 2) and contained approximately 1.1kb of *tra II* DNA. The 1.2kb DNA product was digested with EcoR1, which excised about 50bp of the pBR328 chloramphenicol

Figure 21 : DNA electrophoresed on a 0.7% Sea Plaque agarose gel. The wells were loaded as follows : (1) 200ng 1kb ladder, (2) 1µg plasmid pPF210 digested with EcoR1 and Hind III without the 2.4kb EcoR1/Hind III fragment containing the *oriT* site of pTiC58.

LANES

1   2
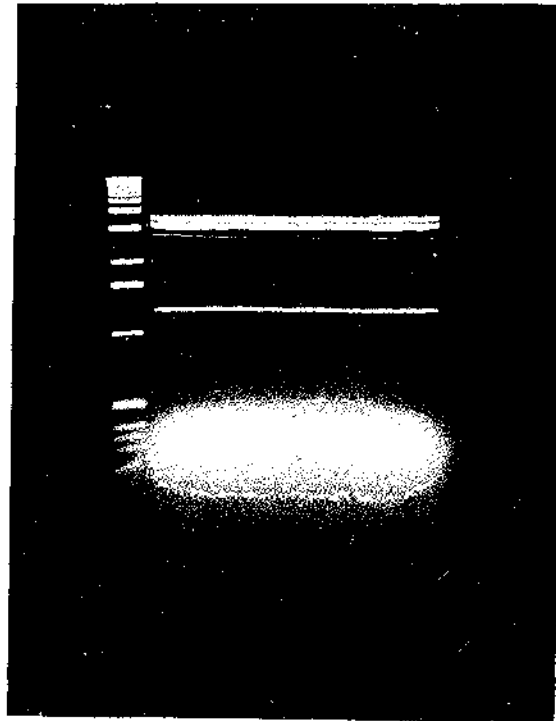
Figure 22 : DNA electrophoresed on a 0.7% agarose
gel. The wells were loaded as follows :
(1) 200ng 1kb ladder, (2) 1.1kb
polymerase chain reaction product (30ng)
containing the *oriT* site of pTiC58, (4)
300bp polymerase chain reaction product
(30ng) containing *tra 3* mating bridge
assembly DNA, (6) 200ng 1kb ladder.

LANES

1 2 3 4 5 6
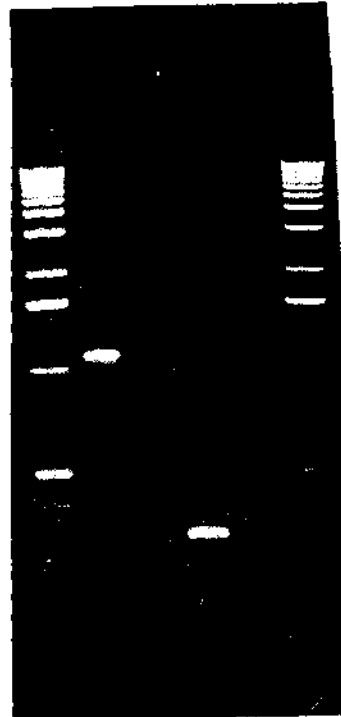
gene, and Kpn1, which excised approximately 400bp, containing about 50bp of Tn5 DNA from the fragment. The 400bp and the 800bp fragments were separated by electrophoresis (section 2.11) on a 2% Sea Plaque agarose gel, and the 800bp fragment was excised and purified (section 2.9.3). This fragment contained only *tra* II DNA (with the *ori* site in it).

3.2.15 Southern Blots for Probing with pPF210

Figures 23A and 24A show the genomic digests with EcoR1 of DNA from a number of *Agrobacterium* and *Rhizobium* strains after electrophoresis (section 2.11) on 0.7% agarose gels. These gels were blotted (section 2.12.1) onto nylon membranes (Hybond-N, Amersham), after which the gels were restained to confirm that the DNA had transferred from the gels to the membranes.

3.2.16 Radioactive Labelling of DNA

A Multiprime DNA labelling kit (Amersham) was used to make probes (section 2.14.1) of both the 2.2kb EcoR1/Hind III fragment (section 3.2.13) and the 800bp *tra* II fragment (section 3.2.14). Both probes had a high radioactive incorporation in the DNA (approximately 70%) and both had approximately 6 x 10⁷ counts in the total probe mixture.

3.2.17 Hybridisation of the Probes to the Southern Blots

Figure 23B shows the autoradiogram of the blot of the gel in figure 23A. The hybridisation and final wash were performed at low stringency (approximately 40°C below the predicted Tm), yet only the positive control (lane 6) and the *Rhizobium* strain containing Tn5 showed hybridisation above the background level. The 2.2kb EcoR1/Hind III fragment was used as the probe (section 3.2.13)

Figure 24B shows the autoradiogram of the gel in figure 24A. The hybridisation and final wash were carried out at low stringency (approximately 40°C below the predicted Tm), but only the positive controls (lanes 5 and 6) showed hybridisation. The 800bp *tra* II DNA fragment was used as the probe (section 3.2.14).

Figure 23 : DNA from *Agrobacterium* and *Rhizobium*
strains digested with EcoR1 and separated
by electrophoresis in 0.7% agarose,
Southern blotted onto nitrocellulose
and probed with the 2.2kb EcoR1/Hind III
fragment from pPF210. The wells were
loaded as follows : (1) 200ng 1kb, (2)
1μg *Agrobacterium tumefaciens* strain LMG64,
(3) 1μg *Agrobacterium rhizogenes* strain
ATCC15834, (4) 1μg *Rhizobium loti* strain
ATCC33669, (5) 1μg *Rhizobium leguminosarum*
bv trifolii strain ICMP2163::Tn5 (6) 1μg
Rhizobium leguminosarum bv trifolii strain
ICMP2163 (7) 1μg *A.tumefaciens* C58 strain
2-16

A : agarose gel. **B** : autoradiograph. The
final wash was carried out in 0.1 x SSC
at 37°C.

LANES

1 2 3 4 5 6 7

A

LANES

1 2 3 4 5 6 7

B

Figure 24 : DNA from *Agrobacterium* and *Rhizobium*
strains digested with EcoR1 and separated
by electrophoresis in 0.7% agarose,
Southern blotted onto nitrocellulose
and probed with pPF210. The wells were
loaded as follows : (1) 1μg Lambda ladder,
(2) 5μg *Rhizobium leguminosarum* bv trifolii
strain ICMP2163::Tn5 (3) 5μg *Rhizobium
leguminosarum* bv trifolii strain ICMP2163
(4) 3μg *A.tumefaciens* C58 strain 15-26
(5) 5μg *A.tumefaciens* C58 strain 15-26
(6) (1) 1μg Lambda ladder, (7) 1μg pPF210

A : agarose gel. **B** : autoradiograph. The
final wash was carried out in 0.1 x SSC
at 37°C.

LANES

1 2 3 4 5 6 7

A
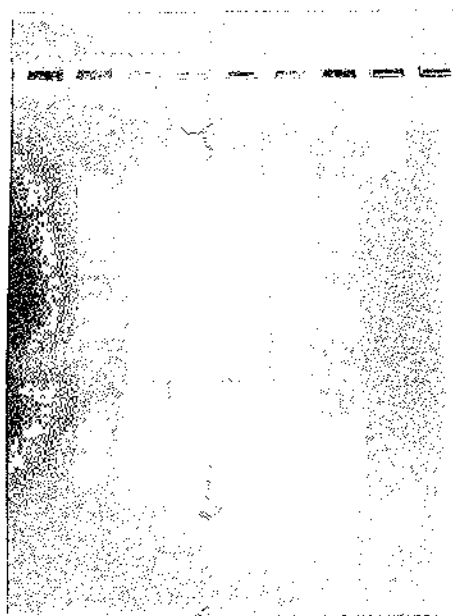
LANES

1 2 3 4 5 6 7

B
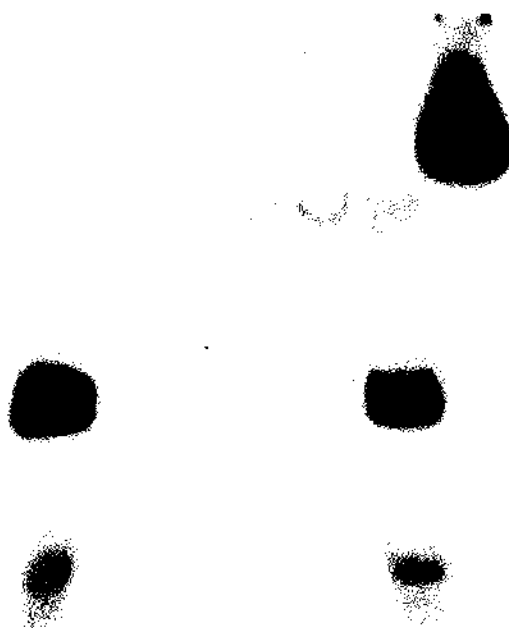
3.3 Results for Tra III

An area on the Ti plasmid pTiC58 containing possible genes responsible for mating bridge assembly in transconjugal transfer (personal communication,Farrand) was ligated into the plasmid pUC118 (Yannisch-Perron,1985), electroporated into *E.coli* strain DH-1 (Table 1) and used as a probe to locate bands with significant homology (60% or greater) to itself among a number of *Rhizobium* and *Agrobacterium* strains. The *tra* III portion of the probe corresponded to the 300bp to the right of the BamH1 tra III #3 fragment (fig 25) and flanked the BamH1 fragment on the right of the Tn5 map (fig 16). The final construct is shown in figure 26.

3.3.1 Isolation of Genomic DNA from *A.tumefaciens* C58 strain 2-16

Strain 2-16 is an *A.tumefaciens* C58 mutant with a Tn5 insertion located within the *tra* III portion of the plasmid pTiC58 (fig 25). The cells were grown for 24 hours in 40ml of Luria broth (section 2.1) with $50\mu g/ml$ of kanamycin added (Appendix 1), and the total DNA was isolated (section 2.6). The ratio of the absorptions at 258nm and 280nm (section 2.6) was approximately 1.8, indicating that the DNA was clean enough for digestion with the restriction enzyme BamH1. There was also approximately $600\mu g/ml$ of DNA (section 2.6) in each preparation.

3.3.2 Confirmation the the Presence of Tn5 DNA in Strain 2-16

The digestion of Lambda DNA, isolation of plasmid pKAN2 DNA, separation of the Tn5 Hind III fragment from pKAN2, and radioactive labelling of Lambda and Tn5 DNA was performed and is explained in section 3.2.2 through to section 3.2.5. All of these procedures were carried out once in order to confirm the presence of Tn5 DNA (fig 16) both in strain 15-26 (section 3.2.1) and strain 2-16 (section 3.3.1).

3.3.3 Digestion and Electrophoresis of DNA from C58 strain 2-16

Figure 25 : The restriction map of the *tra III* area
of the pTiC58 plasmid (von Bodman,1989).
The positions of Tn5 insertions which
disrupt conjugal plasmid transfer are
indicated by the vertical lines from the
mutant strains to the map.

tra III

noc

Tra 10-22
Tra 0-52
Tra 2-16
Tra 12-33
Tra 0-74
Tra 16-82

par inc cop

| IIII | 33 | 2 | | 30 b | 20 a | 13 | 8 | 13 | 32 | 25 b | 5 | |

| HI | 2 | 3 | 20 | 12 | 19 | 13 | |

| I | 23 | 11a | 27 28 | 14 | 4 | 11 | 18 | 19c | 21 | 6 |

| RI | 1 | 29 | 13 | 2 | 27 | 25 | 20 | 12 | |

kb ┤┼┼

5   10   15   20   25   30   35   40   45   50
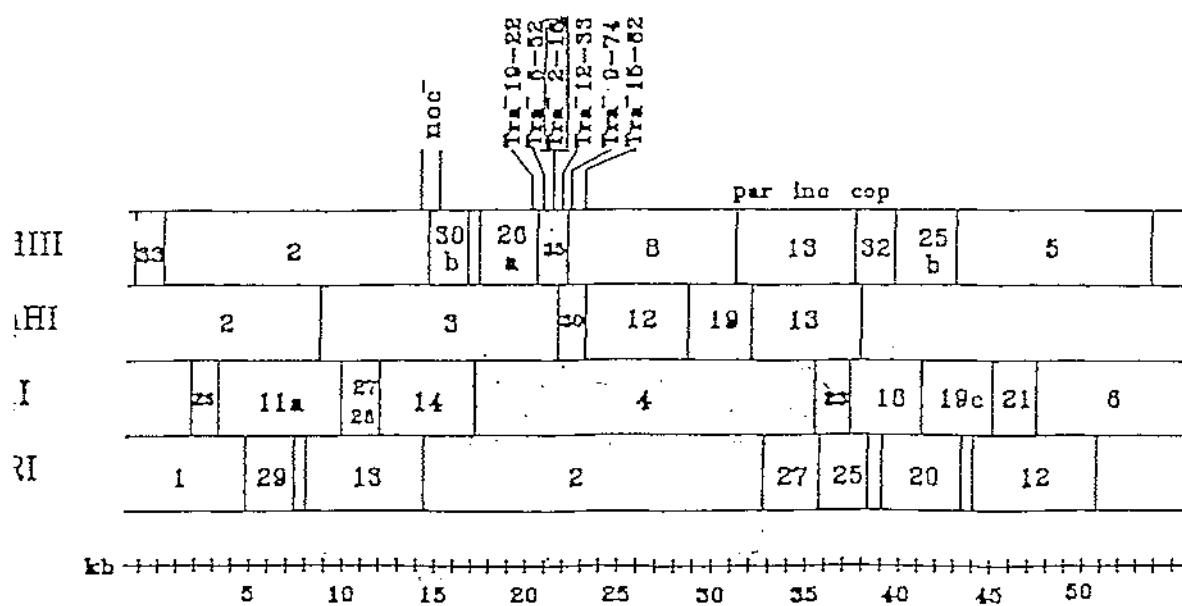
Figure 26 : A restriction map of the plasmid pPF310. The plasmid contains the plasmid pUC118 (Yannisch-Perron,1985) with an insert containing Tn5 DNA (Jorgenson et al,1979) and a flanking *tra III* region (von Bodman, 1989).
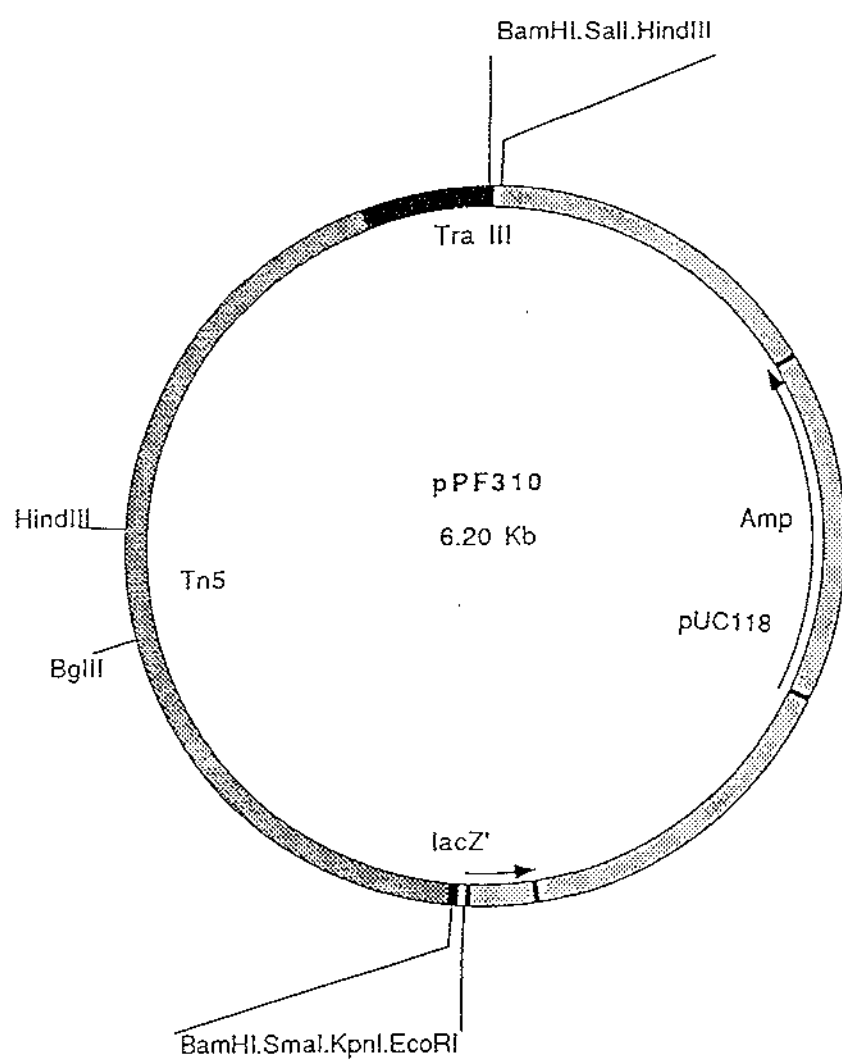
Genomic DNA (1μg), isolated from strain 2-16 (section 3.3.1) was digested with EcoR1 (section 2.8), divided into two lanes (fig 17A) and electrophoresed (section 2.11) on a 0.7% agarose gel.

3.3.4 Southern Blot of C58 strain 2-16 DNA

The gel shown in figure 17A was blotted onto a nitrocellulose membrane (section 2.12.1) and the membrane was hybridised (section 2.15) with 40μl of Tn5 probe and 30μl of Lambda probe (section 3.2.5). The resulting autoradiogram (figure 17B) shows the presence of bands containing Tn5 in lanes 5 and 6. Comparison to the Lambda ladder indicated that the bands were approximately 23kb in length. Reference to figure 25 indicated that this was the size which should have been predicted as an EcoR1 pTiC58 fragment of approximately 17kb was formed by digestion, and the Tn5 insertion within the fragment was 5.7kb (fig 16).

3.3.5 The Isolation of 2.5-4kb DNA Bands from Agarose Gels

The genomic DNA of C58 strain 2-16 was digested with Kpn1, EcoR1, Cla1 and Pvu1 (section 2.8). None of these enzymes had any sites within the Tn5 DNA (fig 16), which was useful as the regions flanking the Tn5 DNA could then be ligated, electroporated and selected for on kanamycin plates. However, a blot of genomic DNA (section 2.12.1) from C58 strain 2-16, which had been digested with each enzyme (i.e. separate digests of Kpn1, Cla1, EcoR1 and Pvu1, section 2.8) was probed with the pKAN2 Tn5 probe (sections 3.2.3 to 3.2.5). The results (not shown) indicated that all four enzymes digested the *tra* III region flanking the Tn5 DNA to fragments of greater than 20kb (the 5.7kb Tn5 DNA was included in the 20kb). This was considered too large a fragment to electroporate successfully into DH-1 cells.

Having confirmed that Tn5 was present within the *tra* III area (section 3.3.4), the genomic DNA of strain 2-16 was digested with BamH1 (section 2.8) and electrophoresed on a 0.7% Sea Plaque (FMC) agarose gel (section 2.11). Excision of 2.5-4kb bands was carried out (section 2.9.3) because the BamH1 band of interest was 3kb in length. A 3kb band was expected to contain approximately 300bp of *tra* III DNA (fig

25) and approximately 2.7kb of Tn5 DNA (figure 16). It was not known at the time in which orientation the Tn5 DNA had inserted into the *tra* III area, therefore it was also unknown whether an *E.coli* DH-1 cell (Table 1) receiving this 3kb BamH1 fragment inside the plasmid vector pUC118 would be kanamycin resistant.

3.3.6 Digestion of the Plasmid pUC118

pUC118 is a 3.2kb plasmid vector commonly used to clone ligated fragments into *E.coli* (Yanisch-Perron,1985). It contains a multiple cloning site, a gene which confers resistance to ampicillin, and a *lac* operon. When a fragment is inserted into the multiple cloning site, which is located within the *lacZ* gene, the gene is disrupted and β-galactosidase production is prevented. If there is no disruption of *lacZ* (that is, no insert is present), the presence of the plasmid in *E.coli* DH-1 cells (Table 1) will be seen when the cells are grown on plates containing X-gal and IPTG (section 2.23).

pUC118 DNA (Bio-Rad) was digested with BamH1 to linearise it (section 2.8), purified (section 2.9.3) and stored in a 1.5ml Eppendorf tube at 4°C until required. Digested and undigested pUC118 DNA (25ng of each) was electrophoresed on a 0.7% agarose gel (section 2.11) to confirm complete digestion to linear DNA.

3.3.7 Removal of the 5' Phosphate from Linear DNA

Removal of the 5' phosphate from linear DNA is commonly termed as CAP-treatment, as the enzyme Calf Alkaline Phosphatase (BRL) is required (section 2.17). This prevents self-ligation (Section 2.16) of the targeted DNA. Figure 27 indicated that this CAP-treatment and the subsequent ligation of pUC118 were carried out successfully. Lane 7 of figure 27 shows concatamerisation and circularisation of pUC118 after ligation with itself. Lane 8 of figure 27 shows that pUC118 DNA remained linear after ligation. The only difference in the treatment of the DNA in lanes 7 and 8 is that the DNA in lane 8 had been CAP-treated.

3.3.8 Preparation of *E.coli* DH-1 Cells for Electroporation

Figure 27 : DNA electrophoresed on a 0.7% Sea Plaque
agarose gel. The wells were loaded as
follows : (1) 200ng 1kb ladder, (7)
100ng linearised pUC118 after ligation,
(8) 10ng linearised pUC118 after ligation
and subsequent CAP-treatment.

LANES
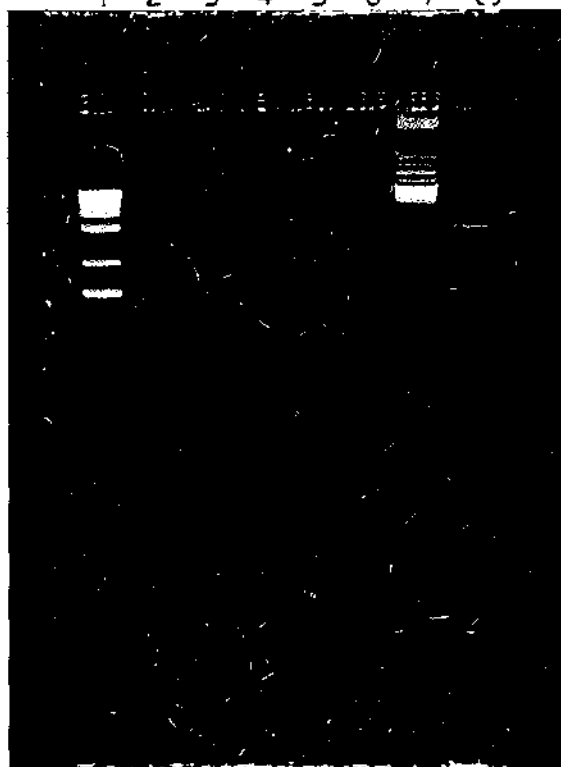
E.coli DH-1 cells (Table 1) were prepared for electroporation (section 2.18).

3.3.9 Ligation of *Tra III* DNA into pUC118

The mixture of 2.5-4kb bands containing the *tra* III area (section 3.3.5) was ligated into CAP-treated pUC118 (section 3.3.7). The ligation was carried out in a 15μl mixture with 60μg of insert DNA (the 2.5-4kb mixture) and 90μg of CAP-treated pUC118 DNA. Therefore ligation was carried out with a DNA concentration of 10μg/μl. This high concentration reduced the chances of self-ligation of the insert DNA. More pUC118 DNA than insert DNA was also provided because the plasmid had already been CAP-treated, therefore the chances of pUC118 self-ligating were greatly reduced and also more plasmid DNA correlated to a higher chance of insert DNA being incorporated into a plasmid.

3.3.10 Electroporation of Plasmid DNA into *E.coli* strain DH-1

E.coli DH-1 cells which had been prepared for electropoartion (section 2.18) were divided into four 75μl aliquots in four 1.5ml Eppendorf tubes and electroporated (section 2.20). No arcing (section 2.20) occurred in any tube. Each of the four tubes were plated onto four LB (section 2.1) plates containing ampicillin (Appendix 1), X-gal and IPTG (section 2.23). Tube #1, which contained pUC118 and DH-1 cells (1ml total) was diluted 100-fold before 200μl of the dilution was added to each of four plates. After incubation for 2 days thousands of blue colonies were observed. The mixtures from tube #2, tube #3 and tube #4 were plated and after 2 days gave rise to hundreds of ampicillin resistant colonies, most of which were white.

3.3.11 Selection of a Plasmid Containing Tra III DNA

Eighty white DH-1 colonies were selected from the ampicillin resistant colonies (section 3.3.10) and plated onto two fresh LB (section 2.1) plates containing X-gal, IPTG and ampicillin (section 2.23). All the colonies grew after 2 days incubation at 37°C and were still white.

These plasmids were presumed to have the plasmid pUC118 (which conferred ampicillin resistance) with an insert which disrupted the *lacZ* gene, thus keeping the colonies white on X-gal/IPTG plates. The colonies were also patched (section 2.21) onto LB plates containing kanamycin (50μg/ml). No colonies grew on kanamycin plates indicating that the portion of the Tn5 which contained the kanamycin resistance gene was not present.

3.3.12 Colony Hybridisation of DH-1 Cells

White, ampicillin resistant DH-1 colonies (section 3.3.11) were blotted onto nitrocellulose (Biodyne) membranes (section 2.13) and were probed with the Tn5 Hind III probe (section 3.2.5). Cells (*E.coli* HB101) containing the plasmid pPF210 (figure 14) were also blotted and probed with the Tn5 DNA probe. They served as a positive control, and they all hybridised (figure 28A). However, none of the DH-1 colonies showed any hybridisation with the Tn5 probe, even at a stringency 40°C below the predicted Tm (figure 28B).

3.3.13 Digestion of the 2.5-4kb Tra III Mixture with EcoR1 and Sma1

Because no colonies hybridised to the Tn5 probe (section 3.3.12) it was presumed that the BamH1 fragment containing the Tn5 fragment must have been only one band in a mixture of more than eighty different types of bands. Therefore the BamH1 mixture was digested with a Sma1/EcoR1 double digest (section 2.8). EcoR1 had no restriction sites in Tn5 (figure 16), or in the flanking BamH1 *tra* III region (figure 25). Sma1 had no restriction sites in the *tra* III flanking DNA, and was shown to also have no restriction sites in the Tn5 (fig 29B). Figure 29A shows a sample of the BamH1 DNA band mixture (lane 2), and a sample of the same mixture after it had been digested with Sma1 and EcoR1 (lane 3).

3.3.14 Southern Blot of BamH1 and EcoR1/Sma1/BamH1 Bands

A blot of the gel shown in figure 29A was carried out (section 2.12.1) and the blot was probed with the Tn5 probe (section 3.2.5). The autoradiogram shown in figure 29B indicated that Tn5 DNA was

Figure 28 : Colony hybridisations.

A : *E.coli* strain HB101 colonies
containing pPF210 probed with Tn5,
and exposed for 16 hours.

B : E.coli strain DH-1 ampicillin
resistant colonies were probed with
Tn5, and exposed for 4 days.

A

B
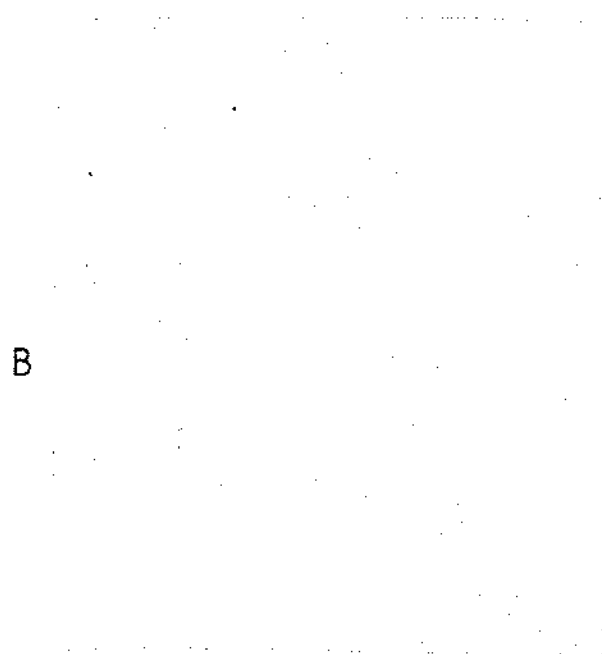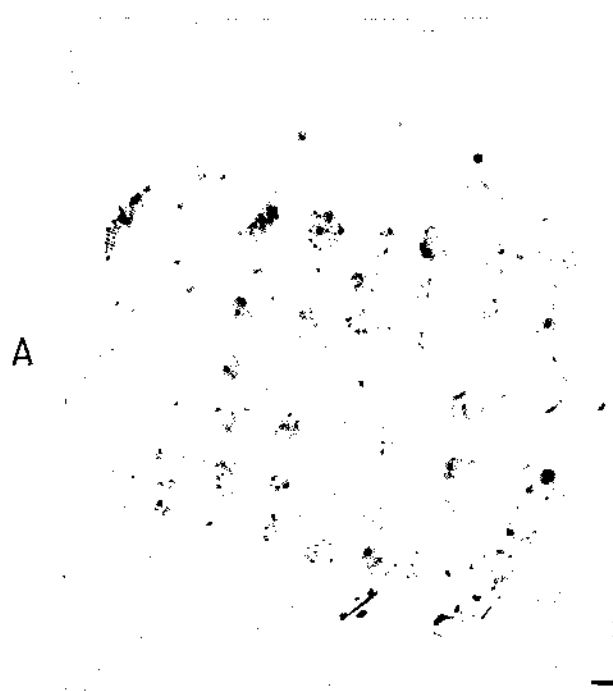
Figure 29 : DNA from *A.tumefaciens* strain C58 2-16 separated by electrophoresis in 0.7% agarose, Southern blotted onto nitro-cellulose and probed with the Tn5 internal HindIII fragment. The wells were loaded as follows : (1) 200ng 1kb ladder, (2) 100ng mixture of DNA bands 2.5-4kb in size digested with BamH1 (3) 100ng of the mixture in lane 2 after further digestion with EcoR1 and Sma1.

A : agarose gel. **B** : autoradiograph.

LANES

1 2 3

A
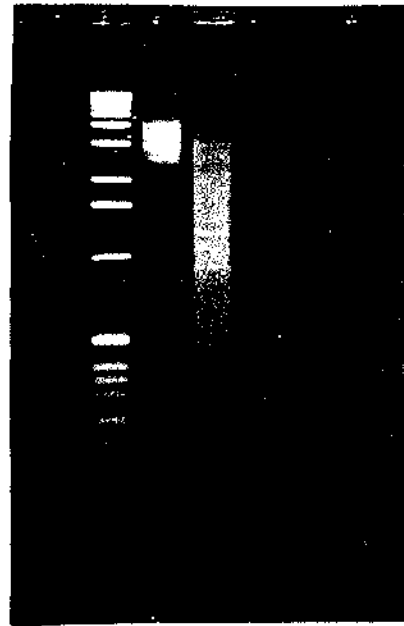
LANES

1 2 3

B

present in the BamH1 DNA mixture both before and after digestion with Sma1 and EcoR1. Also, the hybridising band was still 3kb in length after digestion, which was expected only if the Tn5 DNA had inserted with the right-hand BamH1 fragment of figure 16 (without the neomycin/kanamycin resistant gene) next to the right-hand side of BamH1 DNA fragment #3 (figure 25). The left-hand BamH1 fragment had a Sma1 site which would have digested the band, reducing it's size to 2.6kb.

3.3.15 Isolation of BamH1/Sma1/EcoR1-digested Tra III DNA Bands

The 2.5kb-4kb BamH1 DNA fragment mixture (section 3.3.5) was digested with a Sma1/EcoR1 double digest (section 2.8), electrophoresed on a Sea Plaque agarose gel (section 2.11) and bands between 2.7kb and 3.5kb in length were excised and purified (section 2.9.3). This mixture of DNA bands was then referred to as B/S/E-310 DNA.

3.3.16 Ligation of *B/S/E-310* DNA Bands into pUC118

The B/S/E-310 (section 3.3.15) DNA mixture (50ng) was ligated into CAP-treated (section 3.3.7) pUC118 (100ng) in a volume of 15μl (section 2.16). The ligation product was electrophoresed on a 0.7% agarose gel (section 2.11) next to a sample of unligated B/S/E-310 DNA and a decrease in mobility indicated that the ligation had been successful.

3.3.17 Electroporation of Plasmid DNA into *E.coli* strain DH-1

The ligation product (section 3.3.16) was electroporated (section 2.20) into *E.coli* DH-1 cells (Table 1) which had been prepared for electroporation (section 2.18). The cells were plated onto LB (section 2.1) plates containing ampicillin (Appendix 1), X-gal and IPTG (section 2.23). After incubation at 37°C for two days, thousands of white colonies and a few (approximately 1%) blue colonies had grown. Twenty white colonies were selected for plasmid isolation.

3.3.18 Isolation of Plasmid DNA from Ampicillin-Resistant Colonies

The twenty colonies selected in section 3.3.20 were grown overnight in 5ml of Luria broth (section 2.1) with ampicillin added (Appendix 1) at 37°C. Plasmid DNA was isolated (section 2.7.1), digested with EcoR1 (section 2.8) and was electrophoresed on a 0.7% agarose gel (section 2.11). Three of the twenty colonies contained plasmid DNA which was 6kb and had only one EcoR1 site (figures 30 and 31), which was expected if the cells contained the desired construct. BamH1 and Sma1 digests (section 2.8) were carried out on the plasmid DNA from these three colonies. All three plasmid preparations were electrophoresed (section 2.11) on a 0.7% agarose gel and all three plasmid preparations gave one 6kb Sma1 fragment and two 3kb (doublet) BamH1 fragments, as predicted from the restriction maps of Tn5 DNA (figure16), tra III DNA (figure 25) and pUC118 DNA (Yanisch-Perron,1985). Figure 31 shows the plasmid DNA of one clone (#16) after digestion (section 2.8) with a Bgl II/Sal1 double digest, a Bgl II/BamH1 double digest, a Hind III digest and undigested DNA. All the digests produced bands of predicted sizes (from the restriction maps of figure 16, figure 25 and of pUC118).

3.3.19 Construction of a Restriction Map of Plasmid pPF310

Having confirmed that the plasmid DNA of clone #16 (section 3.3.18) was of the correct size, and had the correct restriction sites in it (with six different restriction enzymes), a construct was produced (figure 26).

3.3.20 Isolation of Plasmid pPF310

A large-scale plasmid preparation of the plasmid DNA from clone #16 was carried out (section 2.7.2), and the ratio of the absorbance at 258nm compared to that at 280nm (section 2.6) was approximately 1.9, indicating a pure preparation.

3.3.21 Amplification of the Tra III 350bp Fragment

The tra III fragment of the construct pPF310 (figure 26) was amplified (section 2.24) using the pUC118 forward primer and the Tn5 17-mer primer (Appendix 2). Figure 22 shows the fragment (lane 4) which was approximately 300bp in length.

Figure 30 : EcoR1 digests of plasmids from ampicillin resistant colonies obtained by transform- ation with a ligation mixture containing pUC118 and pPF310, and electrophoresed in 0.7% agarose gel. The wells were loaded as follows : (1) 200ng 1kb ladder, (2) through to (7) 10ng plasmid.

LANES
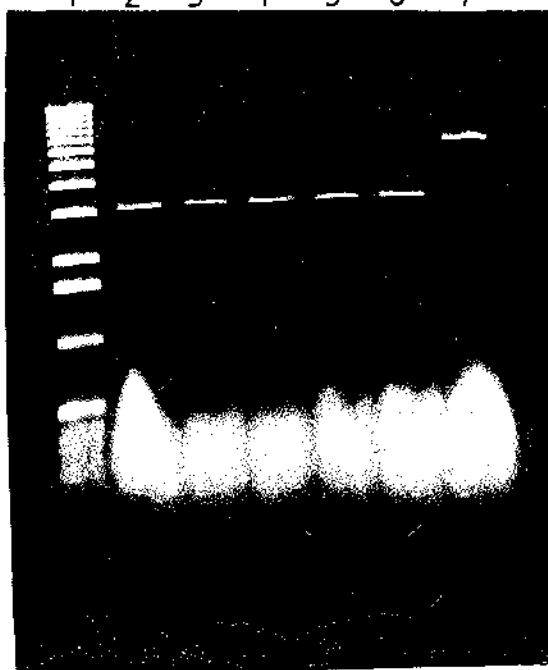
Figure 31 : Restriction enzyme digests of the plasmid pPF310 on 0.7% agarose gel. The wells were loaded as follows : (1) 100ng BamH1/BglII double digest, (2) 100ng HindIII digest, (3) 100ng BglII digest, (4) 0.6µg uncut DNA, (5) 1µg uncut DNA (6) 200ng 1kb ladder.

LANES

1 2 3 4 5 6
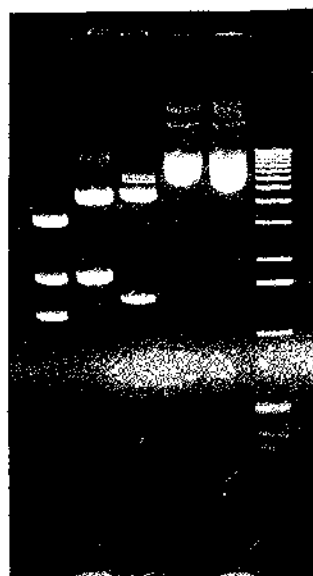
3.3.22 Radioactive Labelling of DNA

Both the entire pPF310 construct (figure 26) and the amplified *tra* III region (section 3.3.21) were radioactively labelled (section 2.14.2). Both probes containing greater than 60% radioactive incorporation. The entire pPF310 DNA probe contained approximately 2×10^8 counts, and the 300bp *tra* III fragment contained approximately 1.2×10^8 counts.

3.3.23 Southern Blots for Probing with Tra III DNA

Figure 32A shows a 0.7% agarose gel containing DNA isolated from *A.tumefaciens* C58 strain 2-17, *R.leguminosarum* bv trifolii strain 2163 and *R.leguminosarum* bv trifolii strain 2163::Tn5 (Table 1). All three strains had been digested with EcoR1 (section 2.8), and the gel was subsequently blotted onto a nylon (Hybond-N, Amersham) membrane (section 2.12.2). Confirmation of transfer of the DNA was provided by re-staining the gel (section 2.11) after blotting and observing that very little DNA was left in the gel.

Figure 33A shows a 1% agarose gel containing DNA from *A.tumefaciens* C58 strain 2-17 from four *R.leguminosarum bv trifolii* strains (Table 1). All the strains had been digested with EcoR1 (section 2.8), and the gel was subsequently blotted onto a nylon (Hybond-N, Amersham) membrane (section 2.12.2). Confirmation of transfer of the DNA was provided by re-staining the gel (section 2.11) after blotting and observing that very little DNA was left in the gel.

Figure 34A shows a 0.7% agarose gel containing DNA from *A.tumefaciens* C58 strain 2-17, *A.tumefaciens* strain LMG64, three *R.leguminosarum bv trifolii* strains, and a *R.loti* strain (Table 1). All the strains had been digested with EcoR1 (section 2.8), and the gel was subsequently blotted onto a nylon (Hybond-N, Amersham) membrane (section 2.12.2). Confirmation of transfer of the DNA was provided by re-staining the gel (section 2.11) after blotting and observing that very little DNA was left in the gel.

Figure 32 : DNA from *Agrobacterium and Rhizobium* strains separated by electrophoresis in 0.7% agarose, Southern blotted onto a nylon membrane and probed with pPF310. The wells were loaded as follows : (1) 200ng 1kb ladder, (2) no DNA, (3) 1μg *R.leguminosarum* bv trifolii strain ICMP2163::Tn5, (4) 1μg *R. leguminosarum* bv trifolii strain ICMP2163, (5) 0.8μg *A.tumefaciens* strain C58 2-17, (6) no DNA, (7) 150ng 1kb ladder

A : agarose gel. **B** : autoradiograph.
The final wash was carried out in 0.1 x SSC at 50°C.

A
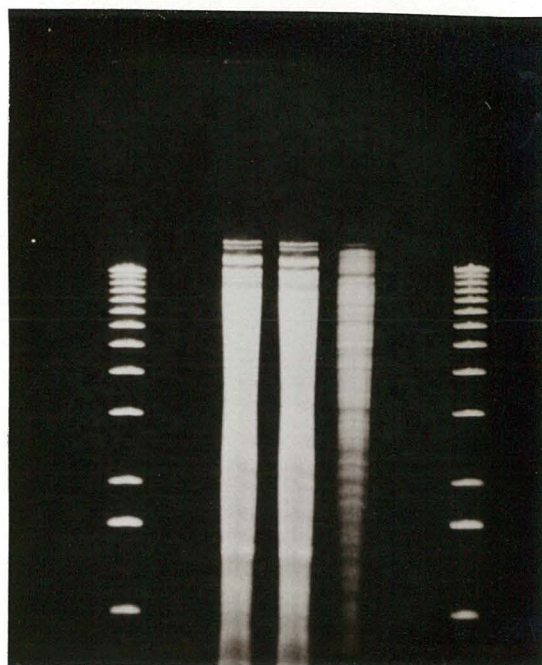
B

Figure 33 : Genomic DNA from *Agrobacterium and Rhizobium* strains, digested with EcoR1, separated by electrophoresis in 0.7% agarose, Southern blotted onto a nylon membrane and probed with the 350bp *traIII* polymerase chain reaction product. The wells were loaded as follows : (1) 0.8μg *R.leguminosarum* bv trifolii strain ICMP2163, (2) 1μg *R.loti* strain ATCC33669, (3) 0.8μg *R.leguminosarum* bv trifolii strain ATCC14480, (4) *A.tumefaciens* strain C58 2-17

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

LANE

1   2   3   4

A

LANE

1   2   3   4

B
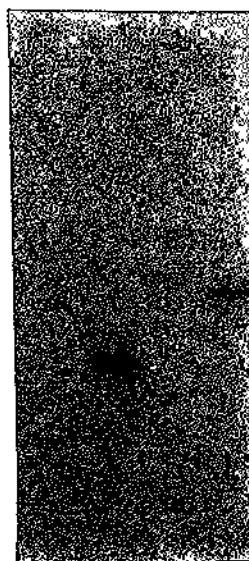
Figure 34 : Genomic DNA from *Agrobacterium* strains, digested with EcoR1, separated by electrophoresis in 0.7% agarose, Southern blotted onto a nylon membrane and probed with the 350bp *traIII* polymerase chain reaction product. The wells were loaded as follows : (1) 1µg *A.tumefaciens* strain LMG87, (2) 1µg *A.tumefaciens* strain LMG196, (3) 1µg *A.tumefaciens* strain LMG219, (4) 1µg *A.tumefaciens* strain LMG26, (5) 1µg *A.rhizogenes* strain ATCC15834, (6) 1µg *A.tumefaciens* strain LMG64, (7) 1µg *A. tumefaciens* strain C58 2-17

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

LANE

1 2 3 4 5 6 7

A
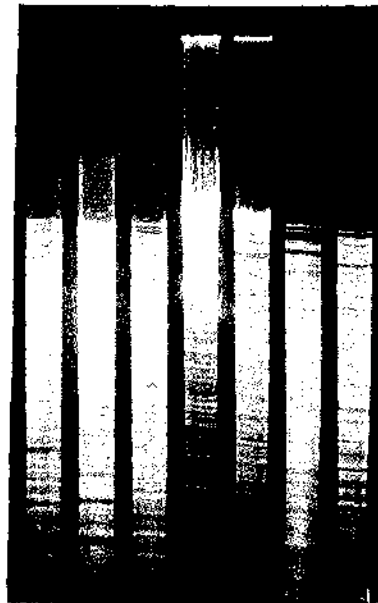
LANE

1 2 3 4 5 6 7

B
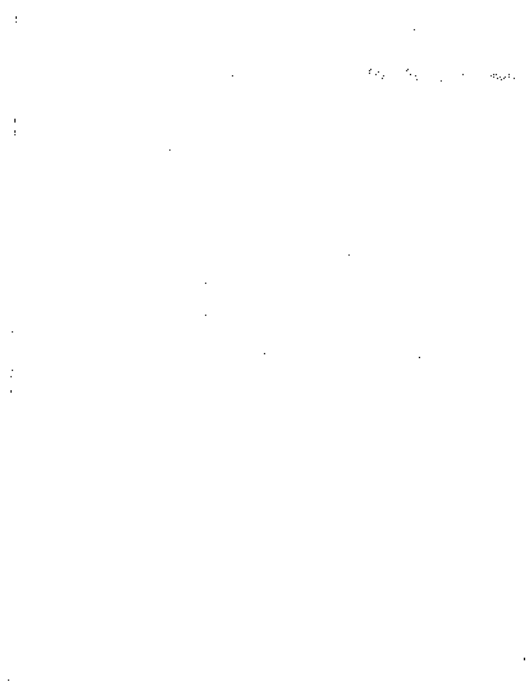
3.3.24 Hybridisation of the Probes to the Southern Blots

Figure 32B shows an autoradiograph of the gel in figure 32A. The gel was hybridised at high stringency using the entire pPF310 probe (section 3.3.22). The lane containing *R.leguminosarum* bv trifolii 2163 DNA showed no bands at a stringency 20°C below the predicted Tm (section 2.15). *A.tumefaciens* C58 strain 2-17 showed two bands as expected. The lower band showed hybridisation of the Tn5 within the *tra* I area of the EcoR1 fragment #21 (figure 10) to the Tn5 within the probe (figure 26). The upper band showed hybridisation of the *tra* III area of the EcoR1 fragment #2 (figure 25) to the 300bp *tra* III region of the probe (figure 26). The *tra* III band was much lighter in intensity then the Tn5 band, which was predicted because the probe pPF310 (figure 26) contained 2.7kb of Tn5 DNA and only 300bp of *tra* III DNA. Therefore the *tra* III area was amplified (section 3.3.21) and this new probe containing mostly *tra* III DNA was used as the probe in figures 33B and 34B.

Figure 33B shows an autoradiogram of the gel in figure 33A. None of the *R.leguminosarum* strains showed any hybridisation to the 300bp *tra* III (section 3.2.21) probe. The single band on the autoradiograph was the result of hybridisation of the probe to the EcoR1 #2 fragment of *A.tumefaciens* C58 strain 2-17. Hybridisation and the final wash were both carried out at 30°C below the predicted Tm.

Figure 34B shows an autoradiograph of the gel in figure 34A. The 300bp probe did not hybridise to the DNA of any of the *R.leguminosarum* strains, but did hybridise to the DNA of *A.tumefaciens* C58 strain 2-17 and to the *R.loti* strain. The hybridisation was carried out at 30°C which was calculated to be approximately 42°C below the predicted Tm (section 2.15).

Table 3 summarises the results of all the blots which were probed with *tra* DNA.

TABLE I : The Bacterial Strains Used In This Study

SPECIES	STRAIN	ANTIBIOTIC RESISTANCE	REFERENCE
R.leguminosarum bv trifolii	ICMP2163	Sp	Rao,1993
R.leguminosarum bv trifolii	2163::Tn5	Sp,Kan	Rao,1993
R.leguminosarum bv trifolii	PN165	Tc	Rao,1993
R.leguminosarum bv trifolii	ATCC14480	-	Skerman,1980
R.loti	ATCC33669	-	Jarvis,1986
Ag.tumefaciens	C58 (T37)	-	De Ley,1973
Ag.tumefaciens	C58 2-17	Kan	von Bodman,1989
Ag.tumefaciens	C58 15-26	Kan	von Bodman,1989
Ag.tumefaciens	C58 2-16	Kan	von Bodman,1989
Ag.tumefaciens	LMG 26	-	De Ley,1973
Ag.tumefaciens	LMG 64	-	De Ley,1973
Ag.tumefaciens	LMG 187	-	De Ley,1973
Ag.tumefaciens	LMG 196	-	De Ley,1973
Ag.tumefaciens	LMG 219	-	De Ley,1973
Ag.rhizogenes	ATCC15834	-	De Ley,1973
E.coli	HB101	Sm	Maniatis,1982
E.coli	DH-1	-	Maniatis,1982
E.coli	αDH-5	-	Sambrook,1989
E.coli	PF110	Kan	This Study
E.coli	PF210	Sm,Tc,Kan	This Study
E.coli	PF310	Amp	This Study

TABLE II : The Frequency of HB101 Transformants on Selective Plates after Electroporation

Plasmid DNA	Media Containing Antibiotics			
	Tc^a	Cam^b	Sm	$Tc/Kan/Sm^b$
Tube #1 (pBR328,100ng)	4,500	4,200	$>10^7$	0
Tube #2^c (ligation mix,63ng)	0	0	0	0
Tube #3^c (ligation mix,38ng)	197	84	$>10^7$	5
Tube #4^c (ligation mix,25ng)	148	70	$>10^7$	3

All plates consisted of Luria agar (section 2.1) and selected antibiotics (Appendix 1)

a The results shown in this column are the mean of three plates, and therefore only represent one third of the total number of colonies which grew on tetracycline plates.

b The colonies shown in these two columns had either been patched (section 2.21) from 200 Tc resistant colonies (tube #3), or 150 Tc resistant colonies (tube #4), or were replica plated (section 2.22) from 4,500 Tc resistant colonies (tube #1).

c The ligation mix contained several A.tumefaciens strain C58 genomic DNA bands which had been digested with EcoR1, were 8-10kb in size and had been ligated into pBR328. Tra II DNA was included in the mixture.

Tc = tetracycline
Cam = chloramphenicol
Sm = streptomycin
Kan = kanamycin

TABLE III : The Presence of *Rhizobium* and *Agrobacterium* DNA Homologous to pTiC58 *tra I, tra II,* and *tra III* Probes

GENOMIC DNA	CLUSTER[a]	GROUP[b]	PROBES		
			tra I	*tra II*	*tra III*
R.leg bv trifolii ICMP2163	NA	NA	-	-	-
R.leg bv trifolii ICMP2163 ::Tn5	NA	NA	+/-	+/-	+/-
R.leg bv trifolii PN165	NA	NA	-	-	-
R.leg bv trifolii ATCC14480	NA	NA	+	-	-
R.loti ATCC33669	NA	NA	-	-	+
Ag.tumefaciens C58 (T37)	1	TT9	NA	NA	NA
Ag.tumefaciens C58 2-17	1	TT9	+	NA	NA
Ag.tumefaciens C58 15-26	1	TT9	NA	+	NA
Ag.tumefaciens C58 2-16	1	TT9	NA	NA	+
Ag.tumefaciens LMG26 (0362)	1	0362	-	-	-
Ag.tumefaciens LMG64 (TT9)	1	TT9	+	-	+
Ag.tumefaciens LMG187 (B6-806) 1		B6	-	-	-
Ag.tumefaciens LMG196(TTIII) 1		TTIII	-	-	-
Ag.tumefaciens LMG219 (Apple 185)	2	NA	_	_	_
Ag.rhizogenes 15834	2	NA	-	-	-

NA = not applicable

- :- no bands were observed at any stringency on blots containing genomic DNA from the strain shown that row, when probed with strain C58 DNA shown in the corresponding "probes" column.

+ :- bands were observed on blots containing genomic DNA from the strain shown in that row, when probed with strain C58 DNA shown in the corresponding "probes" column.

+/- :- the only bands observed on blots containing genomic DNA from the strain shown in that row, whe probed with strain C58 DNA corresponded to the Tn5 portions of the probes.

a The cluster column refers only to the agrobacteria, and refers to the clusters which they were placed i after phylogenetic analysis of nearly 90 *Agrobacterium* strains (De Ley,1973)

b The group column refers to only cluster 1 agrobacteria, and refers to some of the 7 cluster 1 groups which they were placed in after phylogenetic analysis (De Ley,1973).

4 Discussion

The *tra* probes were used to identify DNA with significant homology (usually above 50%) among several *Agrobacterium* and *Rhizobium* strains to locate *tra* genes potentially present in these strains. The percent of homology between a probe and the DNA to which it was bound was estimated by using the equation in section 2.15 to calculate the Tm;- the temperature at which 50% of the DNA binds. If binding occurred at the Tm in 0.1 x SSC, the DNA of the probe and the DNA to which it had bound were considered to be completely (100%) homologous. For each degree Celsius below the Tm that the wash was carried out in, DNA with 1% difference to the probe could bind the probe DNA. Therefore, a washing stringency of 40°C below the calculated Tm would allow the binding of two strands of DNA with 60% homology or greater. However, this does not allow for the fact that DNA, once bound requires a higher stringency than this predicted value to break its inter-strand bonding. The stringency of hybridisation must also be noted, which was carried out at 65°C, 31°C or 36°C below the predicted Tm in the hybridisation buffer, which was 99°C for the *tra I* and *tra II* probes and 94°C for the *tra III* probe.

Two DNA strands with less than approximately 75% sequence homology are unlikely to bind even at low stringencies, therefore any bands seen on the autoradiographs were likely to indicate a minimum of 75% sequence homology.

Of particular interest were the results of the probing of *R.leguminosarum* bv trifolii strain ICMP2163 DNA, as the strain ICMP2163 contains a Sym plasmid which is responsible for nodulation of clover. The genes responsible for the transfer of this plasmid to other bacteria in the soil, if located could be targeted for genetic manipulation. Mutation of such genes would aid in the study of the environmental fate of the Sym plasmid in the rhizosphere (section 1).

4.1 The Construction of Probes Containing *Tra* DNA

All three *tra* DNA probes were cloned into plasmid DNA and maintained in *E.coli* so that the DNA could be easily extracted and radioactively labelled.

4.1.1 The *Tra I* DNA Probes

Excision of the 9kb *tra I* fragment (section 3.1.3) was required to ensure that none of the pSA152 (Tait et al,1983) DNA was present in the probe. This 9kb fragment contained the *traR* sequence which showed high sequence homology to a gene whose product regulates other *tra* genes (personal communication, Farrand). The *traR* gene was included in the probe in order that any *R.leguminosarum* bv trifolii strain ICMP2163 DNA with homology to the gene would be detected. If homologous DNA was detected in strain ICMP2163, the involvement of such a gene in the transfer of the Sym plasmid could be observed through manipulation of the gene product.

4.1.2 The *Tra II* DNA Probes

The plasmid pPF210 (figure 14) was constructed by ligation of a 9kb fragment containing Tn5 DNA (Jorgenson et al,1979) with flanking *tra II* DNA (von Bodman,1989) into the *E.coli* plasmid pBR328 (Bolivar et al,1977). Plasmid pPF210 was electroporated into, and maintained in *E.coli* HB101 cells (Table 1).

The *tra II* portion of the 9kb EcoR1 fragment (section 3.2) contained an *oriT* site (Cook et al,1992). This *oriT* site is recognised and cleaved by site-specific nicking complexes which linearise the plasmid DNA. Linearisation plays an important part in transconjugal plasmid transfer (Cook et al,1992). The *oriT* site is a 65bp stretch of DNA (Cook et al,1992) which does not produce a protein product, and therefore is not considered to be a gene. Because the site is on the plasmid itself, it's function as a target for nicking is specific to the plasmid on which it is located, and it is unlikely that the *oriT* site would directly affect the transfer of any other plasmids within the *A.tumefaciens* cell. Therefore the 1.1kb *tra II* DNA probe containing the *oriT* site (section 3.2.14) was used to detect the presence of homologous DNA on the Sym plasmid of *R.leguminosarum* bv trifolii strain ICMP2163 (Table 1). If such DNA was detected on the Sym plasmid, it would provide a promising target for study of *R.leguminosarum* bv trifolii Sym plasmid transfer.

Although the area containing *tra II* DNA was located on plasmid pTiC58 (von Bodman), the molecular size of the plasmid was greater than 150kb (von Bodman) and we considered it to be too large for easy isolation. Therefore a 9kb DNA band was selected from an EcoR1 digest of the entire *A.tumefaciens* strain C58 genome. Digestion of the genomic DNA (which includes plasmid DNA) with EcoR1 produced a fragment which was small enough to clone (9kb), and contained the complete Tn5 DNA. The Tn5 DNA contained a gene whose product conferred resistance to the antibiotic kanamycin (the neo^r gene in figure 16). Therefore, although the 9kb fragment of interest was ligated into pBR328 and electroporated into *E.coli* strain HB101 with a number of other EcoR1 fragments of similar size (sections 3.2.8 and 3.2.10), only the fragment containing the Tn5 DNA (with flanking *tra II* DNA) was able to provide resistance to kanamycin on LB Tc/Kan/Sm plates (section 3.2.11).

4.1.3 The *Tra III* DNA Probes

The tra III DNA contained genetic elements necessary for the construction of a mating bridge (personal communication, Farrand).

Because the restriction enzymes which did not digest Tn5 DNA produced bands of greater than 22kb in size in a genomic digest of *A.tumefaciens* strain C58 *tra*⁻ 2-16, BamH1 was used to separate the tra III region from the bulk of the genome. The isolation of the *tra III* BamH1 fragment from genomic DNA bands of similar size was difficult because BamH1 digestion separated the kanamycin resistance gene of Tn5 from the 3kb *tra III* fragment (section 3.3.14). The Tn5 DNA could have previously been inserted into the pTiC58 in one of two orientations. There was a Sma1 restriction site to the left of the BamH1 restriction site. The autoradiogram shown in figure 31B indicated that the right side of the Tn5 restriction map shown in figure 16 (without the kanamycin resistance gene) was adjacent to the 300bp *tra III* BamH1 fragment, because digestion with Sma1 did not reduce the size of the fragment by approximately 400bp. Consequently the construct could not be separated from other constructs containing non-*tra III* 3kb bands by antibiotic selection, as was the case with the *tra II* construct pPF210 (section 4.1.2).

Colony hybridisation (section 2.13) was used to select for cells which had been electroporated with the pPF310 construct. A probe containing Tn5 DNA was used, but after probing 80 colonies it was decided that the 3kb BamH1 fragment of interest was contaminated with too many other 3kb BamH1 bands because less than 1 colony in 80 colonies contained Tn5 DNA, yet all 80 colonies had pUC118 constructs with inserts. A mixture containing 2.5-4kb DNA bands, which had been previously digested with BamH1 and excised from an agarose gel (section 2.9.3), was digested with EcoR1 and Sma1 neither of which digested the 3kb Tn5/*tra III* fragment. The *tra III* DNA mixture contained a much higher portion of bands containing the *tra III* DNA fragment because many of the contaminating bands were digested and could be separated from the *tra III* DNA by electrophoresis. The separation of the contaminating 3kb bands from tra III DNA with further digests was considered to greatly enhance the chances of successful selection of the required plasmid. The results confirmed this because plasmid profiles and digests of three of the twenty colonies selected (15%) confirmed the presence of the plasmid pPF310 (fig 26), whereas not even 1 colony out of 80 was shown to hybridise previously.

Because only 300bp (5%) of the 6kb probe contained *tra III* DNA, it was decided that a probe would be made from the DNA of the amplified *tra III* region (section 3.3.21). Approximately 85% of this amplified DNA contained *tra III* DNA.

4.2 The Presence of *Tra* Genes in *Rhizobium* and *Agrobacterium* Strains

The plasmid pTiC58 is a large plasmid present in the *Agrobacterium tumefaciens* strain C58 (von Bodman,1989). *A.tumefaciens* strain C58 is located in *Agrobacterium* cluster 1 (De Ley,1973) in the TT9 group and is also referred to as *A.tumefaciens* strain T37 (De Ley,1973). The phylogenetic relationship of cluster 1 agrobacteria to other agrobacteria and to rhizobia, obtained by sequencing 16S rRNA genes is shown in figure 2. A number of *Agrobacterium* strains from both cluster 1 and cluster 2 were selected for probing, as well as several *Rhizobium*

strains. Table 3 lists the *Agrobacterium* strains used in this study, the group and cluster to which they belong, and the results of probing their genomic DNA with the various *tra* probes. Each *Agrobacterium* strain described in Table 3 was selected as a representative of a different group related to *A.tumefaciens* strain C58 as indicated by the thermal stability of DNA:DNA hybrids (De Ley,1973).

A.tumefaciens strain TT9 (LMG64) had been placed in the same group as *A.tumefaciens* strain C58 (De Ley,1973), and was chosen as a strain with a high degree of rRNA gene homology to strain C58. The high degree of sequence homology among rRNA genes is partially due to regions which are conserved across genera regardless of their phylogenetic relationship. Other regions of rRNA genes are less conserved, and may imply a more useful degree of relatedness between strains. However, this relatedness is determined by a single chromosomal gene, which is a very small portion of the genome.

The rhizobia probed included *R.leguminosarum* bv trifolii strains ICMP2163, ICMP2163::Tn5, PN165 and ATCC14480, and the *R.loti* strain ATCC33669 (Table 1). The strain *R.leguminosarum* bv trifolii strain ICMP2163 was of particular interest as it is used commercially as an inoculant of white clover in New Zealand. Strain ATCC14480 was a type strain (Skerman,1980) used in this thesis because it was expected that it would have a high level of chromosomal relatedness with ICMP2163. PN165 was used because it was a Sym cured derivative of bv trifolii strain ICMP2163. If the *tra* DNA probes had indicated that DNA with significant homology to the *tra* DNA of *A.tumefaciens* strain C58 was present in bv trifolii strain ICMP2163, and not in bv trifolii strain PN165, it could be presumed that the homologous bands were on the Sym plasmid. PN165 also acted as a negative control. If strain PN165 DNA had shown homology with strain C58 *tra* DNA, and strain ICMP2163 DNA had not, a technical error must have occurred. If both PN165 and ICMP2163 had shown homology with strain C58 the homologous bands would have come from either the genome or one of the four plasmids common to both strains.

The DNA of *R.loti* strain ATCC33669 was included because the chromosomal DNA of *R.loti* was considered to be less homologous to

R.leguminosarum, and *A.tumefaciens* strain C58 chromosomal DNA based on phylogenetic techniques (fig 2) than any other *Rhizobium* species.

Although the chromosomal rRNA genes of all the strains used in this study indicated a high degree of similarity to *A.tumefaciens* strain C58, the *tra* DNA probes were isolated from a plasmid. DNA of plasmid origin can often contain DNA from a different evolutionary origin than the DNA in the chromosome, because of its ability to recombine, and to transfer within and across species (Broughton,1987;De Jong,1982) and even genus (Plazinski and Rolfe,1985) boundaries. Therefore, it was never assumed that two strains with similar rRNA gene sequences, or with similar chromosomal DNA would automatically share homologous plasmid genes, although a close phylogenetic relationship may be a useful guide since two strains could have evolved from a common ancestor which contained the original plasmid DNA.

Aside from the fact that plasmid genes may have come from highly divergent sources compared with chromosomal DNA, the use of *A.tumefaciens tra* genes as probes to find genes with significant homology in rhizobia was likely to succeed because *tra* genes of all species serve the same function ;- to aid in the transfer of plasmid DNA from one bacterial cell to another. Although there may be a number of different ways in which plasmid transfer occurs, enzymes which perform the same function might contain homologous domains. For example, four ORFs were found (personal communication, Farrand) to the right of the *oriT* site (Cook and Farrand,1992), all of which showed homology to *tra* genes from other plasmids. One open reading frame (ORF) was related to the plasmid RSF1010 nickase, two other ORFs were related to *traF* and a helicase of the plasmid RP4, and the *oriT* site itself showed homology to the transfer origins of the plasmids RSF1010, pTF1 and RP4 (Cook and Farrand,1992). RP4 was originally obtained from *Pseudomonas aeruginosa* (Datta et al,1971) and has since been transferred into a number of different species, such as *R.meliloti.* RP4 was integrated into the Sym plasmid of *R.meliloti* strain 2011 (Julliot et al,1984). This indicates that DNA with strong homology to the *oriT* site of pTiC58 is present in a genus as unrelated to *Agrobacterium* as *Pseudomonas.* It is possible that DNA

homologous with RP4 is present in some of the plasmids in the *Rhizobium* strains which were probed with the *tra II* probe.

The results of the probing of *Rhizobium* and *Agrobacterium* species with strain C58 *tra* DNA are summarised in Table 3.

4.2.1 *Tra* DNA in the *Agrobacterium* strains

Of all the *A.tumefaciens* strains which were probed with DNA from the three *tra* areas of pTiC58 (Table 3), only strain C58 (from which the probe DNA originated) and strain LMG64 showed homology to any of the probes. Bands were observed on Southern blots when genomic (and plasmid) DNA from strain LMG64 was probed with the *tra I* probe and with the *tra III* probe. Strain LMG64, or TT9, has the closest phylogenetic homology to strain C58 (De Ley,1973) of all the strains probed, and so it is not very surprising that it shared plasmid DNA homology with strain C58. None of the other *A.tumefaciens* strains showed any homology to any *tra* probe, even at stringencies as low as 42°C below the predicted Tm, which was unexpected because all the strains still contained their plasmids.

The *A.rhizogenes* strain 15834 (a Cluster 2 strain) also showed no DNA homology to the *tra* probes, even at low hybridisation and washing stringencies.

4.2.2 *Tra* DNA in the *Rhizobium* strains

Given that only strain LMG64, the closest *Agrobacterium* relative (De Ley,1973) to *A.tumefaciens* strain C58 showed any homology to pTiC58 *tra* DNA, it was not surprising that *R.leguminosarum* bv trifolii strains ICMP2163 and PN165 did not show homology to it. However, *R.loti* strain ATCC33669, the most distant relative to *A.tumefaciens* strain C58 (De Ley,1974) showed homology to the *tra III* probe at low stringency. And *R.leguminosarum* strain ATCC14480 showed homology to the *tra I* probe (section 3.1) at low stringency.

It is concluded that none of the pTiC58 probes showed significant homology to *R.leguminosarum* bv trifolii strain ICMP2163, but

homology was seen in *A.tumefaciens* strain LMG64, *R.leguminosarum* bv trifolii strain ATCC14480 and *R.loti* strain ATCC33669. Thus *Agrobacterium tra* DNA is present in some *Rhizobium* species, even though it was not observed among closer *Agrobacterium* relatives, and so it seems that large plasmids have the ability to mix among the species and genera to a much higher degree than chromosomal DNA. This is due to the mobility of plasmid DNA and suggests that Ti and Sym plasmids may be exchanged within the same bacterial population.

4.3 Conclusion

Three pTiC58 *tra* DNA probes were successfully constructed by cloning appropriate fragments into *E.coli* plasmids. The probes were subsequently radioactively labelled.

The use of the DNA from three regions of the *A.tumefaciens* strain C58 plasmid pTiC58 which contained *tra* genes (genes responsible for transfer of the plasmid through transconjugation), or the *oriT* (origin of plasmid transfer) site, as probes to locate DNA with significant homology to themselves in *R.leguminosarum* bv trifolii strain ICMP2163 was unsuccessful. However, DNA with homology to the *tra III* probe was observed in the *R.loti* strain ATCC33669. This result indicated that somewhere within either the plasmid, or the chromosomal DNA of *R.loti* strain ATCC33669 there was DNA with homology to a pTiC58 DNA fragment, which is responsible for mating bridge assembly during plasmid transfer. Also, DNA with significant homology to the *tra I* probe was observed in *R.leguminosarum* bv trifolii strain ATCC14480. Because the *tra I* probe contained 9kb of DNA, within which *traR* (a gene responsible for regulation of other *tra* genes) only took up approximately 1kb, it was not absolutely certain that this part of the probe was the DNA which was binding to the bv trifolii strain ATCC14480 DNA. Within the *tra I* probe were other genes, but these genes were remnants of an opine catabolism operon (personal communication, Farrand) which is a feature of *Agrobacterium* species, and not expected to be present in *Rhizobium* species.

The presence of pTiC58 *tra* DNA in *R.loti,* and in bv trifolii strain ATCC14480 was interesting because the same *tra* DNA was not

observed in some of the *Agrobacterium* strains which had closer phylogenetic similarity to strain C58. This result was easily explained by the fact that Ti plasmid DNA is capable of moving and recombining across species, and even genus boundaries. This has been shown to occur in laboratories with the Sym plasmids from a range of *Rhizobium* species (Plazinski and Rolfe,1985;Espuny et al,1987). Verma and Brisson (1987) reported the transfer of a *R.leguminosarum* bv trifolii Sym plasmid into *A.tumefaciens,* therefore it is not surprising that *A.tumefaciens* Ti plasmid *tra* genes were found in *R.loti* and *R.leguminosarum* strains.

One implication of this mobility is that Ti and Sym plasmids may move freely between agrobacteria and rhizobia, and consequently, classification of bacteria based on plasmid traits would provide false results.

If *Agrobacterium* Ti plasmid genes are found in rhizobia, it would be likely that Sym plasmid genes may be found in agrobacteria. The discovery of such genes in soil isolates would support the hypothesis that the Sym plasmid transfers to other bacteria in the soil.

Now that bands with significant homology to *Agrobacterium* genes have been located in *Rhizobium* species, isolation, characterisation and inactivation of the DNA must follow before the DNA can be recombined into a wild type *Rhizobium.* This sequence of events would produce a *Rhizobium tra* mutant. Reduction of Sym plasmid transfer will have to be confirmed before such a mutant was used because the *tra* genes may be present on any of the plasmids in the rhizobia, and may not affect transfer of the Sym plasmid.

If a *Rhizobium* strain with a *tra⁻* Sym plasmid is produced, the strain, and the wild type can be inoculated into soil and their plasmid flow, rate of survival, efficiency of nodulation and efficiency of nitrogen fixation compared. With information from these comparisons we can determine whether or not to inoculate seed with a *Rhizobium* strain containing a *tra⁻* Sym plasmid.

APPENDIX 1 : Antibiotic Solutions

Antibiotic	Stock solution concentration	storage	Working concentration
Ampicillin	60mg/ml in H_2O	-20°C	60µg/ml
Chloramphenicol	35mg/ml in ethanol	-20°C	150µg/ml
Kanamycin	10mg/ml in H_2O	-20°C	50µg/ml
Streptomycin	10mg/ml in H_2O	-20°C	50µg/ml
Tetracycline	10mg/ml in ethanol	-20°C	50µg/ml

APPENDIX 2 : Primers Used In Polymerase Chain Reactions

1) Tn5 17-mer (Schofield and Watson,1986)

   5'- CGT TCA GGA CGC TAC TT - 3'

2a) 16SrRNA 24-mer Y1 (Young et al,1981)

   5' - TGG CTC AGA ACG AAC GCT GGC GGC - 3'

2b) 16SrRNA 24-mer Y2 (Young et al,1981)

   5' - CCC ACT GCT GCC TCC CGT AGG AGT - 3'

3) Chloramphenicol/EcoR1 (CAT-E1) 20-mer (Bolivar et al,1977)

   5' - TTA TTC ACA TTC TTG CCC GC - 3'

4) pUC Forward 17-mer (Yannisch-Perron,1985)

   5' - GTT TGC CCA GTC ACG AC - 3'

Bibliography

Ausabel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Smith,J.A., Seidman,J.G., Struhl,K.(Ed.). 1991. Current Protocols in Molecular Biology. Publ.Wiley Interscience and Greene Publishing Associates. New York.

Barea,J.M., Azcon-Aguilar,C. 1983. Mycorrhizas and their significance in nodulating nitrogen-fixing plants.J.Adv.Agron. 36:1-54.

Bauer,W.D. 1981. Infection of legumes by rhizobia. Annu.Rev.Plant Physiology. 32:407-449.

Beringer,J., Beynon,J.L., Buchanan-Wollaston,A.V., Johnston,A.W.B. 1978. High frequency transfer of nodulating ability between strains and species of *Rhizobium*. Nature. 276:634-636.

Beringer,J.E. 1974. R factor transfer in *Rhizobium leguminosarum*. J.Gen.Micro. 84:188-198.

Beynon,J.L., Beringer,J.E., Johnston,A.W.B. 1980. Plasmids and host-range in *Rhizobium leguminosarum* and *Rhizobium phaseoli*. J.Gen.Micro. 120:421-429.

Bohlool,B.B., Schmidt,E.L. 1974. Lectins: a possible basis for specificity in the *Rhizobium*-legume root nodule symbiosis. Science. 185:269-271.

Bolivar,F., Rodriguez,R., Green,P.J., Betlach,M., Heyneker,H.L., Boyer,H.W., Crosa,J., Falkow,S. 1977. Construction and characterisation of new cloning vehicles. II. A multipurpose cloning system. Gene 2:95

Borthakur,D., Downie,J.A., Johnston,A.W.B., Lamb,J.W. 1985. *psi*, a plasmid-linked *Rhizobium phaseoli* gene that inhibits

exopolysaccharide production and which is required for symbiotic nitrogen fixation. Mol.Gen.Genet. **200**:278-282.

Brewin,N.J., Wood,E.A., Johnston,A.W.B., Dibb,N.J., Hombrecher,G. 1982. Recombinant nodulation plasmids in *Rhizobium leguminosarum*. J.Gen.Micro. **128**:1817-1827.

Brewin,N.J.,Wood,E.A.,Young,J.P.W. 1983. Contribution of the Sym plasmid to the competitiveness of *Rhizobium leguminosarum*. J.Gen.Micro. **129**:2973-2977.

Brock,T.D., Madigan,M.T.(Ed.). 1991. Nitrogen fixation (chap 16). In *Biology of Micro-organisms*, 6th edition. Prentice-Hall International Editions. p562-609.

Brock,T.D., Madigan,M.T.(Ed.). 1991. Microbial Ecology (chap 17). In *Biology of Micro-organisms*, 6th edition. Prentice-Hall International Editions. p614-675.

Brosius,J., Dull,T.J., Sleeter,D.D., Noller,H.F. 1981. Gene organisation and primary structure of a ribosomal RNA operon for *E.coli*. J.Mol.Biol. **148**:107-127.

Broughton,W.J.,Bohlool,B.B.,Shaw,C.H.,Bohnert,H.J.,Pankhu rst,C.E. 1985. Conserved plasmid/chromosome sequences in fast- and slow-growing rhizobia that nodulate the same plant. Arch.Micro. **141**:14-21.

Broughton,W.J., Samrey,U., Stanley,J. 1987. Ecological genetics of *Rhizobium meliloti* : symbiotic plasmid transfer in the *Medicago sativa* rhizosphere. Federation of European Microbiological Society (FEMS) Microbiological Reviews. **40:** 251-255.

Casse,F., Boucher,C., Julliot,J.S., Michel,M., Denarie,J. 1979. Identification and characterization of large plasmids in *Rhizobium meliloti* using agarose gel electrophoresis. J.Gen.Micro. **113**:229-242.

Cook,D.M., Farrand,S.K. 1992. The *ori T* region of the *Agrobacterium tumefaciens* Ti plasmid pTiC58 shares DNA sequence identity with the transfer origins of RSF1010 and RK2/RP4, and with the T-region borders. J.Bacteriology. **174**:6238-6246.

Datta,N., Hedges,R.W., Shaw,E.J., Sykes,R.B., Richmond,M.H. 1971. Properties of an R factor from *Psuedomonas aeruginosa*. J.Bacteriol. **108**:1244-1249.

David,C., Tempe,J. 1988. Genetic transformation of cauliflower (*Brassica oleracea L.var.Botrytis*) by *Agrobacterium rhizogenes*. Plant Cell Reports. **7**:88-91.

Dazzo,F.B., Brill,W.J. 1977. Receptor site on clover and alfalfa roots for *Rhizobium*. Appl.and Env.Micro. **33**:132-136.

Dazzo,F.B., Hubbell,D.H. 1975. Cross-reactive antigens and lectin as determinants of symbiotic specificity in the *Rhizobium*-clover symbiosis. Appl.Micro. **30**:1017-1033.

De Jong,T.M., Brewin,N.J., Johnston,A.W.B., Phillips,D.A. 1982. Improvement of symbiotic properties in *Rhizobium leguminosarum* by plasmid transfer. J.Gen.Micro. **128**:1829-1838.

De Ley,J., Tijtgat,R., De Smedt,J., Michiels,M. 1973. Thermal stability of DNA:DNA hybrids within the genus *Agrobacterium*. J.Gen.Micro. **78**:241-252.

De Ley,J. 1974. Phylogeny of prokaryotes. Taxon. **23**:291-300.

De Smedt,J., De Ley,J. 1977. Intra- and intergeneric similarities of *Agrobacterium* ribosomal RNA cistrons. Int.J.Syst.Bact.**27**:222-240.

Ditta,G., Stanfield,S., Corbin,D., Helinski,D.R. 1980. Broad host range DNA cloning system for gram-negative bacteria :

Construction of a gene bank of *Rhizobium meliloti*. Proceedings of the National Academy of Sciences (P.N.A.S.) U.S.A. **77**:7347-7351.

Ditta,G., Virts,E., Palomares,A., Kim,C. 1987. The *nif A* gene of *Rhizobium meliloti* is oxygen regulated. J.Bacteriol. **169**:3217-3223.

Djordjevic,M.A., Zurkowski,W., Rolfe,B.G. 1982. Plasmids and stability of symbiotic properties of *Rhizobium trifolii*. J.Bacteriol. **162**:560-568.

Djordjevic,M.A., Gabriel,D.W., Rolfe,B.G. 1987. *Rhizobium* :- the refined parasite of legumes. Ann.Rev.Phytopathol. **25**:145-168.

Dower,W.J., Miller,J.F., Ragsdale,C.W. 1988. High efficiency transformation of *E.coli* by high voltage electroporation. Nucl. Acids Res. **16**:6127-6145.

Dowling,D.N., Broughton,W.J. 1986. Competition for nodulation of legumes. Annu.Rev.Microbiol. **40**:131-157.

Downie,J.A., Johnston,A.W.B. 1986. Nodulation of legumes by *Rhizobium* : the recognized root? Cell. **47**:153-154.

Eardly,B.D., Materon,L.A., Smith,N.H., Johnson,D.A., Rumbaugh,M.D., Selander,R.K. 1990. Genetic structure of natural populations of the nitrogen-fixing bacterium *Rhizobium meliloti*. J.Appl.and Env.Micro. **56**:187-194.

Eckhardt,T. 1978. A rapid method for the identification of plasmid DNA in bacteria. Plasmid. **1**:584-588.

Espuny,M.R., Ollero,F.J., Bellogin,R.A., Ruiz-Sainz,J.E. 1987. Transfer of the *Rhizobium leguminosarum bv trifolii* symbiotic plasmid pRtr5a to a strain of *Rhizobium* sp. that nodulates on *Hedysarum coronarium*. J.Appl.Bact. **63**:13-20.

Feinberg,A.P. and Vogelstein,B. 1983. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal.Biochem. **132**:6.

Feinberg,A.P. and Vogelstein,B. 1984. Addendum : A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal.Biochem. **137**:266.

Fisher,R.F., Tu,J.K., Long,S.R. 1985. Conserved nodulation genes in *Rhizobium meliloti* and *Rhizobium leguminosarum*. Appl.and Env.Micro. **50**:1432-1435.

Grant,W.D., Long,P.E.(Ed.). 1981. Environmental Microbiology. Publ.Blackie. (chap 1,4 and 6).

Grunstein, M., Hogness, D. 1975. Colony Hybridisation : a method for isolation of cloned DNAs that contain a specific gene. Proceedings of the National Academy of Sciences (P.N.A.S.) U.S.A. **72**:3961-3965.

Gussin,G.N., Ronson,C.W., Ausabel,F.M. 1986. Regulation of nitrogen fixation genes. Annu.Rev.Genet. **20**:567-591.

Hatterman,D.R., Stacey,G. 1990. Efficient DNA transformation of *Bradyrhizobium japonicum* by electroporation. Appl. and Env.Micro. **56**:833-836.

Heberlein,G.T., DeLey,J., Tijtgat,R. 1967. DNA homology and taxonomy of *Agrobacterium, Rhizobium* and *Chromobacterium*. J.Bacteriol. **94**:116-124.

Hirsch,P.R. 1979. Plasmid-determined baceriocin produced by *Rhizobium leguminosarum*. J.Gen.Micro. **113**:219-228.

Hirsch,P.R., van Montagu,M., Johnston,A.W.B., Brewin,N.J., Schell,J. 1980. Physical identification of bacteriocinic, nodulation and other plasmids in strains of *Rhizobium leguminosarum*. J.Gen.Micro. **120**:403-412.

Hooykaas,P.J.J., van Brussel,A.A.N., den Dulk-RAS,H., van Slogteren, G.M.S., Schilperoort,R.A. 1981. Sym plasmid of *Rhizobium trifolii* expressed in different rhizobial species and *Agrobacterium tumefaciens*. Nature. 291:351-353.

Hooykaas,P.J.J., den Dulk-Ras,H., Schilperoort,R.A. 1982. Method for transfer of the large cryptic, non-self-transmissible plasmids: ex planta transfer of the virulence plasmid of *Agrobacterium rhizogenes*. Plasmid. 8:94-96.

Horwitz,M.A. 1964. Substrates for cytochemical demonstration of enzyme activity. Some substituted 3-indolyl-β-D-glycopyranosides. J.Med.Chem. 7:574

Iismaa,S.E., Ealing,P.M., Scott,K.F., Watson,J.M. 1989. Molecular linkage of the *nif/fix* and *nod* regions in *Rhizobium leguminosrum bv trifolii*. Molec.Micro. 3(12):1753-1764.

Irigoyen,J.J., Sanchez-Diaz,M., Emerich,D.W. 1990. Carbon metabolism enzymes of *Rhizobium meliloti* cultures and bacteroids and their distribution within alfalfa nodules. J.Appl.and Env.Micro. 56:2587-2589.

Ish-Horowitz,D., Burke, J.F. 1981. Rapid and efficient cosmid cloning. Nucl.Acids.Res. 9 p2989-2998.

Jarvis,B.D.W., Gillis,M., De Ley,J. 1986. Intra- and intergeneric similarities between the rRNA cistrons of *Rhizobium* and *Bradyrhizobium* species and some related bacteria. Int.J.Syst.Bacteriol. 36:129-138.

Jarvis,B.D.W., Ward,L.J.H., Slade,E.A. 1989. Expression by soil bacteria of nodulation genes from *Rhizobium leguminosarum* bv trifolii. Appl.and Env. Micro. June. p1426-1434.

Johnston,A.W.B., Hombrecher,G., Brewin,N.J., Cooper,M.C. 1982. Two transmissible plasmids in *Rhizobium leguminosarum* strain 300. J.Gen.Micro. 128:85-93.

Jorgensen,R.A., Rothstein,S.J., Reznikoff,W.S. 1979. A restriction enzyme cleavage map of Tn5 and location of a region encoding neomycin resistance. Mol.Gen.Genet. **177**:65-72.

Julliot,J.S., Dusha,I., Renalier,M.H., Terzarghi,B., Garneronr,A.M., Boistard,P. 1984. An RP4-Prime Containing a 285kb Fragment of *Rhizobium meliloti* pSym Megaplasmid : Structural Characterisation and Utilisation for Genetic Studies of Symbiotic Functions Controlled by pSym. J.Mol.Gen.Genet. **193**:17-26.

Kerr,A., Panagopoulos,C.G. 1977. Biotypes of *Agrobacterium radiobacter var. tumefaciens* and their biological control. Phytopath.Z. **90**:172-179.

Kokotek,W., Lotz,W. 1991. Construction of a mobilizing cloning vector for site-directed mutagenesis of gram-negative bacteria: application to *Rhizobium leguminosarum*. Gene. **98**:7-13.

Kondorosi,E., Kondorosi,A. 1986. Nodule induction on plant roots by *Rhizobium*. Trends in Biol.Sci. **11**:296-299.

Jordan,D.C. 1984. Family III. *Rhizobiaceae*. In *Bergey's Manual of Systematic Bacteriology*. Krieg,N.R., Holt,J.G.(Ed.) **Vol.1.** Publ.Williams and Wilkins, London and Baltimore.

Krol,A.J.M., Hontelez,J.G.J., van Kammen,A. 1982. Only one of the large plasmids in the *Rhizobium leguminosarum* strain PRE is strongely expressed in the endosymbiotic state. J.Gen.Micro. **128**:1839-1847.

Laguerre,G., Fernandez,M.P., Edel,V., Normand,P., Amarger,N. 1993. Genomic heterogeneity among French *Rhizobium* strains isolated from *Phaseolus vulgaris* L. Int.J.Syst.Bacteriol. **43**:761-767.

Latchford,J.W., Borthakur,D. Johnston,A.W.B. 1991. The products of *Rhizobium* genes *psi* and *pss*, which affect

exopolysaccharide production, are associated with the bacterial cell surface. Mol.Micro. **5(9):**2107-2114.

Lea,P.J., Awonaike,K.O., Cullimore,J.V., Miflin,B.J. 1982. The role of ammonia assimilatory enzymes during nitrogen fixation in root nodules. Israel.J.Botany. **31:**140-154.

Ljunggren,H., Fahraeus,G. The effect of *Rhizobium* polysaccharides on the formation of polygalacturonase in lucerne and clover. 1959. Nature. **184:**1578-1579.

Ljunggren,H., Fahraeus,G. 1961. The role of polygalacturonase in root-hair invasion by nodule bacteria. J.Gen.Micro. **26:**521-528.

Long,S.R. 1989. *Rhizobium*-legume nodulation: life together in the underground. Cell. **56:**203-214.

Maniatis,T., Fritsch,E.F., Sambrook,J.(Ed.). 1982. Molecular Cloning-a laboratory manual. Publ.Cold Spring Harbor Laboratories, Cold Spring Harbor, .N.Y.

Mathis,J.N., Barbour,W.M., Elkan,G.H. 1985. Effect of Sym plasmid curing on symbiotic effectiveness of *Rhizobium fredii*. Appl.and Env.Micro. **50:**1385-1388.

Martinez,.E., Romero,D., Palacios,R. 1990. The *Rhizobium* genome. Critical reviews in Plant Sciences. **9:**59-93.

Maxam,M., Gilbert,W. 1977. A new method for sequencing DNA. P.N.A.S. USA. **74:**560-564.

Meyers,J.A., Sanchez,D., Elwell,L.P., Falkow,S. 1976. Simple agarose gel electrophoretic method for identification and characterization of plasmid DNA. J.Bacteriol. **127:**1529-1537.

Mezei,L. 1991. Direct Purification of PCR Product From Amplification Reactions. Promega Notes **34**, Promega Corporation.

Miller,J.H.(Ed.) 1972. Experiments in Molecular Genetics. Cold Spring Harbour Laboratory, Cold Spring Harbour, New York.

Mullis,K.B. and Faloona,F.A. 1987. In *Methods of Enzymology* vol 155. Acad.Press, California. p335-350.

Napoli,C.A., Hubbell,D.H. 1975. Ultrastructure of *Rhizobium*-induced infection threads in clover root hairs. J.Appl.Micro. **30**:1003-1009.

New Zealand Official Yearbook. 1992. 95th Editon. Publ.Dept. of Statistics.p324 and 530.

Plazinski,J., Rolfe,B.G. 1985. Sym plasmid genes of *Rhizobium trifolii* expressed in *Lignobacter* and *Psuedomonas* strains. J.Bacteriol. **162**:1261-1269.

Ponce,M.R., Nicol,J.L. 1992. PCR amplification of long DNA fragments. Nucl.Acids.Res. **20**:623.

Postma,J., van Veen,J.A. 1990. Habitable pore space and survival of *Rhizobium leguminosarum bv trifolii* introduced into soil. Microbial Ecology. **19**:149-161.

Prakash,R.K., Hooykaas,P.J.J., Ledeboer,A.M., Kijne,J.W., Schilperoort, R.A., Nuti,M.P., Lepidi,A.A., Casse,F., Boucher,C., Julliot,J.S., Dénarié, J. 1980. Detection, isolation and characterization of large plasmids in *Rhizobium*. Int.Rev.Cytol. **104**:1-24.

Rao,J.R., Fenton,M., Jarvis,B.D.W. 1994. Symbiotic plasmid transfer in *Rhizobium leguminosarum bv trifolii* and competition between the inoculant strain ICMP2163 and transconjugant soil bacteria. Soil Biol.and Biochem. In press.

Reuber,T.L., Long,S., Walker,G.C. 1991. Regulation of *Rhizobium meliloti exo* genes in free-living cells and in planta by using Tn*pho*A fusions. J.Bacteriol. **173**:426-434.

Rolfe,B.G., Gresshoff,P.M., Shine,J., Vincent,J.M. 1980. Interaction between a non-nodulating and an ineffective mutant of *Rhizobium trifolii* resulting in ineffective (nitrogen-fixing) nodulation. Appl.and Env.Micro. **39**:449-452.

Roughley,R.J., Blowes,W.M., Herridge,D.F. 1976. Nodulation of *Trifolium subterraneum* by introduced rhizobia in competition with naturalized strains. Soil Biol.and Biochem. **8**:403-407.

Ruiz-Sainz,J.E., Chandler,M.R., Jiminez-Diez,R., Beringer,J.E. 1984. Transfer of a host-range plasmid from *Rhizobium leguminosarum* to fast-growing bacteria that nodulate soybeans. J.Appl.Bacteriol. **57**:309-315.

Sambrook, J., Maniatis,T., Fritsch,E.F. 1989. Molecular Cloning : A Laboratory Manual. (2nd Ed.) Cold Spring Laboratoy, Cold Spring Harbor, New York.

Sawada,H., Ieki,H., Oyaizu,H., Matsumoto,S. 1993. Proposal for the rejection of *Agrobacterium tumefaciens* and revised descriptions for the genus *Agrobacterium* and for *Agrobacterium radiobacter* and *Agrobacterium rhizogenes*. Int.J.Syst.Bacteriol. **43**:694-702.

Sayre,P., Miller.R.V. 1991. Bacterial mobile genetic elements: importance in assessing the environmental fate of genetically engineered sequences. Plasmid **26**:151-171.

Schofield,P.R., Watson,J.M. 1986. DNA Sequencing of *R.trifolii* nodulation genes reveals a reiterated and potentially regulatory sequence preceding *nodABC* and *nodFE*. Nucl.Acids.Res. **14**:2891-2903.

Schofield,P.R., Gibson,A.H., Dudman,W.F., Watson,J.M. 1987. Evidence for genetic exchange and recombination in *Rhizobium* symbiotic plasmids in a soil population. Appl.and Env.Micro. **53**:2942-2947.

Scott,K.F., Huges,J.E., Gresshoff,P.M., Beringer,J.E., Rolfe,B.G., Shine,J. 1982. Molecular cloning of *Rhizobium trifolii* genes involved in symbiotic nitrogen fixation. J.Mol.Appl.Genet. 1:315-326.

Segovia,L., Pinero,D., Palacios,R., Martinez-Romero,E. 1991. Genetic structure of a soil population of nonsymbiotic *Rhizobium leguminosarum*. J.Appl.and Env.Micro. 57:426-433.

Skerman,V.B.D., McGowan,V., Sneath,P.H.A.(Ed.). 1980. Approved lists of bacterial names. Int.J.Syst.Bact 30:225-420.

Skot,L., Hirsch,P.R., Witty,J.F. 1986. Genetic factors in *Rhizobium* affecting the symbiotic carbon costs of nitrogen fixation and host plant biomass production. J.Appl.Bacteriol. 61:239-246.

Soberon-Chavez,G., Najera,R. 1989. Isolation from soil of *Rhizobium leguminosarum* lacking symbiotic information. Can.J.Microbiol. 35:464-468.

Southern,E.M. 1975. Detection of specific sequences among DNA fragments separated by electrophoresis. J.Mol.Bio. 98:503-517.

Tait,R.C., Close,T.J., Lundquist,R.C., Hagiya,M., Rodriguez,R.L., Kado, C.I. 1983. Construction and characterization of a versatile broad host range DNA cloning system for gram negative bacteria. Biotechnology. 1:269-275.

Thornton,H.G. 1930. The early development of the root nodule of lucerne (*Medicago sativa* L.) Ann.Bot. 44:385-392

Thuring,R.W.J., Sanders,J.P.M., Borst,P. 1975. A freeze-squeeze method for recovering long DNA from agarose gels. Anal.Biochem. 66:213-220.

Vance,C.P., Johnson,L.E.B., Halvorsen,A.M., Heichel,G.H., Barnes,D.K. 1980. Histological and ultrastructural observations of *Medico sativa* root nodule senescence after foliage removal. Can.J.Bot. **60**:505-518.

Vance,C.P. 1983. *Rhizobium* infection and nodulation: a beneficial plant disease? Annu.Rev.Micro. **37**:399-424.

Verma,D.P.S., Long,S. 1983. The molecular biology of *Rhizobium*-legume symbiosis. Int.Rev.Cytol. Suppl **14**:211-245.

Verma,D.P.S., Brisson,N. 1987. Tumorigenesis and root nodulation by *Agrobacterium tumefaciens* carrying *Rhizobium* Sym plasmids. In *Molecular Genetics of Plant-microbe Interactions*. Verma,D.P.S., Brisson,N.(Ed.).Publ.Martinus-Nighoff.The Netherlands. p35-40.

Vincent,J.M. 1970. A Manual for the Practical Study of the Root Nodule Bacteria. Blackwell Scientific Publications, Oxford, England. p75.

von Bodman,S.B., McCutchan,J.E., Farrand,S.K. 1989. Characterization of conjugal transfer functions of *Agrobacterium tumefaciens* Ti plasmid pTiC58. J.Bacter. **171**:5281-5289.

Wang,C.L., Beringer,J.E., Hirsch,P.R. 1986. Host plant effects on hybrids of *Rhizobium* biovars *viceae* and *trifolii*. J.Gen.Micro. **132**:2063-2070.

Willems,A., Collins,M.D. 1993. Phylogenetic analysis of rhizobia and agrobacteria based on 16S rRNA sequences. Int J.Syst.Bacteriol. **43**:305-313.

Yanisch-Perron,C., Vieira,J., Messing,J. 1985. Improved M13 phage cloning vectors and host strains : nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103-119

Young,J.P.W., Downer,H.L., Eardly,B.D. 1991. Phylogeny of the phototrophic strain BTAi1 by polymerase chain reaction-based

sequencing of a 16S rRNA gene segment. J.Bacteriol. **173**:2271-2277.

Zurkowski,W., Lorkiewick,Z. 1979. Plasmid-mediated control of nodulation in *Rhizobium trifolii*. Arch.Micro. **123**:195-201.