IDENTIFICATION OF SOIL BACTERIA EXPRESSING A SYMBIOTIC PLASMID FROM *RHIZOBIUM LEGUMINOSARUM* BIOVAR TRIFOLII

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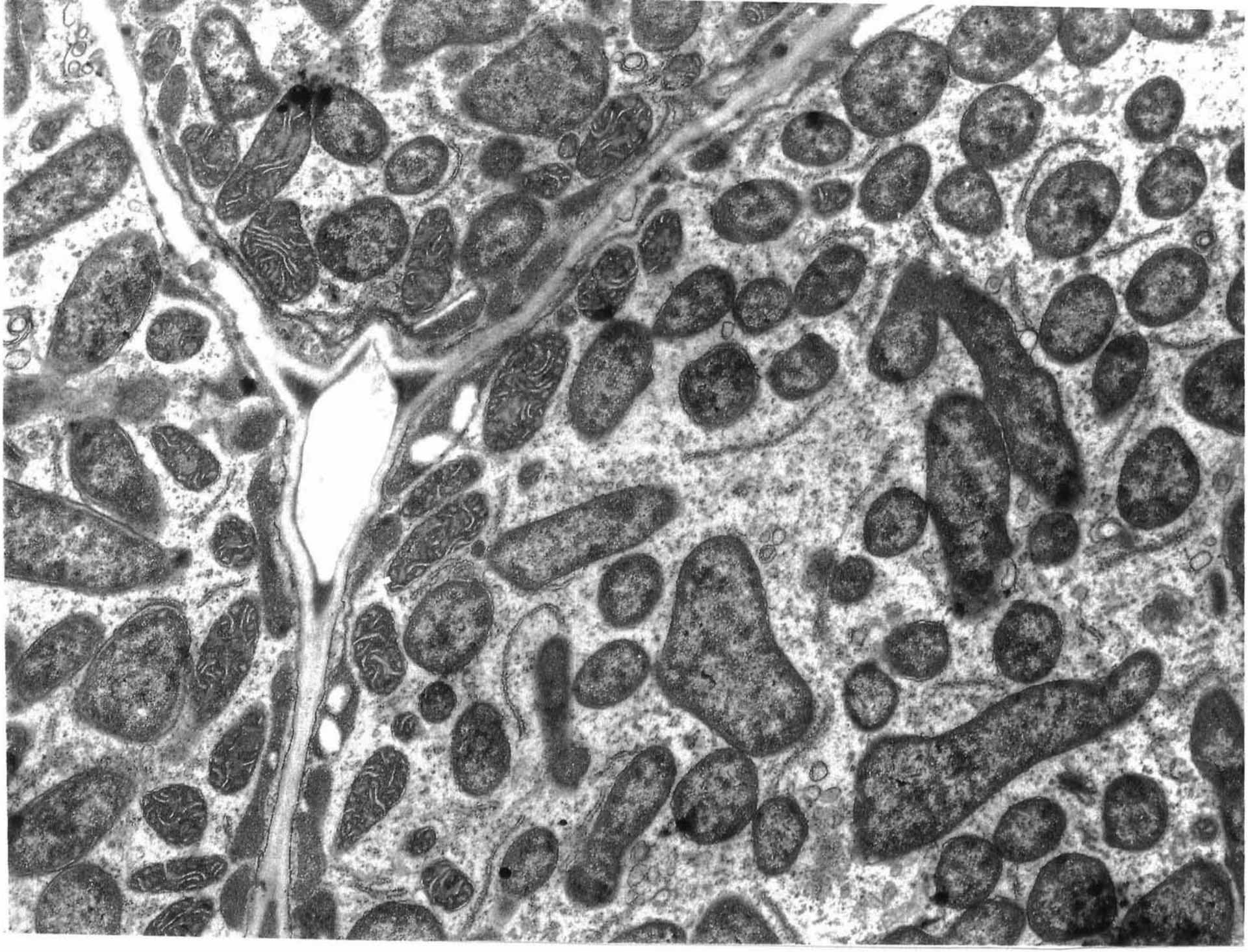
1994

FRONTISPIECE

Electron microscopic section across a nodule formed by the transconjugant soil bacterium KJ30 on white clover (*Trifolium repens*) cultivar Huia (20,900X).



IDENTIFICATION OF SOIL BACTERIA EXPRESSING A SYMBIOTIC PLASMID FROM *RHIZOBIUM LEGUMINOSARUM* BIOVAR TRIFOLII

A thesis presented in partial fulfilment
of the requirements for the degree of
Doctor of Phy losophy in Microbiology
at Massey University, Palmerston North, New Zealand

SIVALINGAM SIVAKUMARAN

1994

DEDICATION

.....to our parents for their love, care and encouragement.

...the sure and definite determination (of species of bacteria) requires so much time, so much acumen of eye and judgement, so much perseverance and patience that there is hardly anything else so difficult---Mueller

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Brion and Audrey Jarvis for helping me to settle in during my early stages of my Ph.D. studies and making me an independent and a mature person.

Finally thanks to all those I have missed out!.

ERRATUM

Page	Line	Incorrect text	Correct text
vi	30	plants inoculated	plants were inoculated
3	4	on fixed nitrogen	symbiotically fixed nitrogen
7	5	bacteroids lack	bacteroids which lack
7	11	represent	representing
9	18	possess	possesses
13	26	has	have
15	1	dendeogram	dendogram
16	25	example	examples
16	26	and will	and these will
18	27	competition of	competition with
24	6	to cured a	to a cured
32	31	is	are

ABSTRACT

The present study concerns the identification of soil bacteria, which could not in their isolated state nodulate white clover, but which could accept a transconjugant plasmid encoding the *nod* gene, and subsequently establish a symbiosis with white clover leading to nodulation. This follows earlier studies intended to characterize non-symbiotic *Rhizobium* strains from the soil. However, whilst these studies specifically examined the potential of non-symbiotic *Rhizobium* strains to nodulate, the present work was developed to examine the potential of any Gram negative soil bacteria to express a transconjugant *nod* plasmid.

A collection of soil bacteria from four different soil types namely (i) Ramiha silt loam, (ii) Tokomaru silt loam, (iii) Kairanga silt loam under white clover-ryegrass pastures and (iv) Manawatu sandy loam (a fallow land with shrubs of *Lupinus* sp.) were isolated and purified. A total of 100 strains of soil bacteria with varying colony morphology were isolated and maintained on media not selective for rhizobia. Each was checked for its ability to nodulate white clover (*Trifolium repens*) cultivar Grasslands Huia. Only four strains nodulated. Conjugation experiments were set up for non-nodulating strains using *Escherichia coli* strain PN200 which contained plasmid pPN1 (pRtr514a::R68.45). A total of 12 soil isolates out of 100 crosses made (12%) formed nodules on white clover, and one strain KJ1 formed transconjugants on a selective antibiotic plate but failed to nodulate white clover. The bacteria accepting and expressing pPN1 were from several soil types including leached, low phosphorous (P) and low pH soil such as Ramiha silt loam.

We showed that eight soil strains formed transconjugants with a mean frequency of transfer of 2.91×10^{-5}. Seven out of these eight strains nodulated white clover. We could not calculate the frequency of transfer for the remaining five isolates, as antibiotic resistant recipients could not be obtained but the transconjugant mixture was inoculated on clover seedlings and all the five strains nodulated white clover.

In our experiments nodulation by transconjugant soil bacteria was verified by plant tests in nitrogen-deficient medium. True nodules formed on white clover seedlings, and on the positive control *Rhizobium leguminosarum* biovar trifolii strain ICMP2163. The negative control plants inoculated with sterile water, *Escherichia coli* strains PN200 containing pPN1 or *E. coli* strain ATCC9637 and the recipient soil bacteria did

not nodulate. It was concluded that nodule formation was due to the transfer of pRtr514a by conjugation.

Eckhardt gels showed that the transconjugants contained different parts of the co-integrate. Strains KJ1 and KJ3 contained R68.45 only, strains KJ13, KJ19, KJ23, KJ26, KJ30 and KJ44 contained pPN1 and R68.45 whilst strains KJ5, KJ17, KJ27, KJ57, KJ203 and PN165 contained pPN1.

Microtome sections of nodule tissue were examined by light and electron microscopy to determine the distribution of infected plant cells and verify that these cells contained bacteroids enclosed in plant cell membranes. The nodule cells formed by the inoculant *R. leguminosarum* biovar trifolii strain ICMP2163 and most cells of all transconjugants were filled with bacteroids. A few nodule cells formed by the transconjugants were devoid of bacteroids.

Total genomic DNA was extracted from each of the transconjugants isolated from the nodules, and from a selective antibiotic plate for strain KJ1, and digested with restriction endonucleases. The fragments were separated by gel electrophoresis, transferred to nylon membrane and probed with an amplified 590 bp *nod*A sequence. Eleven strains of transconjugant soil bacteria gave a hybridization signal at 11.7 Kb with the *nod*A probe. However KJ1 and KJ3 failed to hybridize with the 590 bp *nod*A sequence. KJ1 did not nodulate but formed transconjugants on selective antibiotic plates whereas KJ3 nodulated white clover. The failure to detect *nod* genes in KJ3 may have been due to a loss of pPN1 during sub-culture. Overall the hybridization results confirmed that soil harbours non-nodulating soil bacteria which can maintain symbiotic genes and symbiotic plasmids.

Four methods were used for the identification of soil bacteria expressing pSym. These were (i) rRNA fingerprinting, (ii) 16S rRNA sequence analysis, (iii) DNA-DNA hybridization, and (iv) Total fatty acid analysis. Initially the transconjugants were characterized by rRNA fingerprinting. However this approach was insufficient to identify all isolates. 16S rRNA sequence analysis and DNA-DNA hybridization were subsequently used. These comparisons were more informative and all strains were identified as *Rhizobium* or *Agrobacterium* species. The fatty acid content of the strains was analyzed by gas-liquid chromatography. A comparison of the species names assigned by CFA with those assigned by DNA analyses showed only 50% agreement. These observations are discussed in relation to the phylogenetic distinctiveness of *Agrobacterium* and *Rhizobium*.

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1. INTRODUCTION

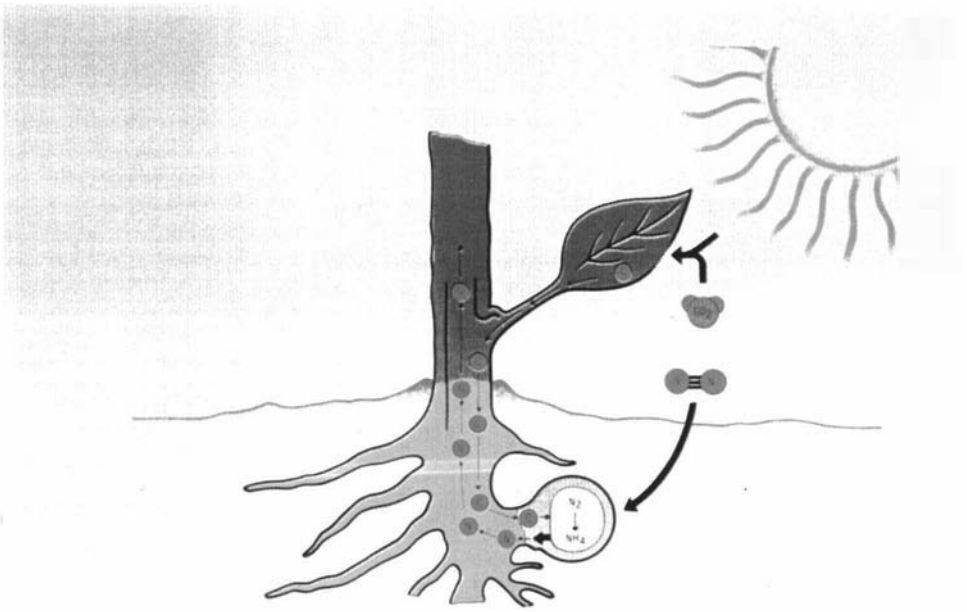
1. 1 Economic and environmental significance of biological nitrogen fixation

Soil is a complex habitat providing an environment for many different microorganisms. Among the different genera, the Gram negative soil bacteria belonging to the Genus *Rhizobium* can invade the root hairs of plants belonging to the family *Leguminosae*. The family *Leguminosae* contains approximately 700 genera in three subfamilies. About 1200 of the 12,000-14,000 known species have been tested for nodulation. 90% of those in the subfamilies *Mimosoideae* and *Papilionoideae* and 30% of those in the *Caesalpinioideae* have been found to nodulate (Beringer *et al.*, 1979). The *Mimosoideae* and the *Caesalpinioideae* consist mostly of trees and shrubs whereas the *Papilionoideae* is also called *Lotoideae* or bean subfamily because of its composition (Robertson and Farnden, 1980).

Nitrogen is a key element of living matter. In organic form it is predominantly found in amino acids and nucleotides. Like other elements, nitrogen is cycled between organic and inorganic forms. Bacterial nitrogen fixation can be achieved non-symbiotically and symbiotically, the latter being of more importance to this study. It is estimated that non-symbiotic nitrogen fixation may yield 1-3 kg N ha^{-1} $year^{-1}$, while symbiotic nitrogen fixation can yield 100-300 kg N ha^{-1} $year^{-1}$ (Schlegal, 1992). Non-symbiotic nitrogen fixation may be achieved by a large and diverse array of bacteria that are free-living or living as commensals in the rhizosphere of plants. Endosymbiotic, nodular nitrogen fixation is the association between legumes (*Leguminosae*) and rhizobia (*Rhizobiaceae*). Only one non-legume (*Parasponia* sp.) is found to nodulate with *Rhizobium*, while non-legumes (e. g. *Azolla*, *Alnus* and *Casuarina*) establish other endosymbioses with cyanobacteria (e. g. *Anabaena*) or actinomycetes (e. g. *Frankia*).

Research into symbiotic nitrogen fixation (Figure 1) in agriculture is important as it (1) promises to make nitrogen fertilizer application redundant in food-legume (grain or vegetable) cultivation thus saving financial and fossil energy resources, (2) can provide nitrogen-rich fodder for animal husbandry, (3) uses legumes in rotation with non-legumes to improve the nitrogen status of the soil and can be used for (4) intercropping with non-legumes for pasture improvement, agroforestry, understorey or shade legumes in plantation crops or in mixed cropping with cereals (Giller and Wilson, 1991).

Figure 1. Diagrammatic illustration of *Rhizobium*-legume symbiosis.



Rhizobium-legume symbiotic nitrogen fixation.

Symbiotic nitrogen fixation by agricultural legume crops is estimated to account for 35 x 10^6 tons $year^{-1}$, i.e approximately 20% of global biological nitrogen fixation. Such a process is crucial to food resources on a global scale (Grant and Long, 1981). In New Zealand the economy is closely linked with an agriculture dependent on fixed nitrogen. 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 (New Zealand official yearbook, 1993). This represents about 55% of total export earnings in New Zealand. Nitrogen fixation is not only vital to New Zealand agriculture but also plays a very important role in the financial welfare of the country.

In current agricultural practice nitrogen is commonly supplied to pastures by using expensive artificial man-made fertilizers such as urea and ammonium sulphate. A disadvantage of this approach is that these nitrogen rich chemicals readily leach from the soil and have to be replenished. Alternatively nitrogen may be added in organic fertilizers such as compost or by inoculating pasture legumes with a suitable *Rhizobium* strain. When white clover (*Trifolium repens*) is inoculated with a suitable strain of *Rhizobium leguminosarum* biovar trifolii on sowing, rhizobia form nodules on the roots of white clover plants and develop a symbiotic relationship which can be long lasting, depending on farm management, environmental conditions and the ability of the particular rhizobia strain to compete with native soil bacteria (Figure 2). The *Rhizobium* legume symbiosis is one of the most efficient nitrogen fixing relationships (Grant and Long, 1981). There are many important legumes crops such as soybeans, peas and alfalfa which efficiently fix nitrogen in symbiotic relationships with rhizobia. Of greatest significance to New Zealand agriculture are the pasture legumes such as red and white clover and lucerne.

1. 2 Nodule formation

A series of complex steps are involved in the formation of nodules (Djordjevic *et al.*, 1987; Newcomb, 1981; Vincent, 1974). *Rhizobium* are chemotactic towards plant roots probably due in part to specific plant attractants (Bergman *et al.*, 1988; Caetano-Anolles *et al.*, 1988). The presence of *Rhizobium* in the rhizosphere alters the growth of epidermal hairs on the surface of root, such that they grow deformed and curled, normally referred to as root hair curling (Dazzo and Gardiol, 1984; Yao and Vincent, 1969). At the same time, cells of the root cortex, under the epidermis begin dividing (Libbenga and Harkes, 1973; Newcomb, 1981).

Figure 2. The effect of inoculation and nitrogen fixation on the growth of white clover in a nitrogen-free medium.

Rhizobia trapped in a curled root hair, or between a hair and another cell, proliferate and begin to infect the outer plant cells, as they do, the invaded plant cell is stimulated to produce a cell wall sheath, "infection thread" (Callaham and Torrey, 1981). As cell divisions in the plant root establish the body of the nodule, infection threads ramify and penetrate individual target cells within the nodule. Bacteria are released into the plant cytoplasm itself, enveloped in plant cell membrane (Robertson *et al.*, 1978). The bacteria and plant cells differentiate and begin symbiotic nitrogen fixation and metabolite exchange (Sutton *et al.*, 1981; Verma and Long, 1983).

The process of nodulation can be divided into four main stages (Bauer, 1981; Brock and Madigan, 1991; Kondorosi and Kondorosi, 1986; Long, 1989; Vance, 1983) namely: (1) Recognition of host plant, (2) Infection of root hairs, (3) Bacteroid formation, and (4) Maturation of nodules.

1. 2. 1 Recognition of host plant

Legume roots secrete a range of organic compounds which aid growth of microorganisms in the rhizosphere (Long, 1989). The effect of some of these is not specific to rhizobia, and they can assist the growth of any bacteria within the rhizosphere. Flavanoids induce *nod* gene transcription only in certain species of rhizobia (Djordjevic *et al.*, 1987; Downie and Johnston, 1986; Long, 1989). In particular, *nod*D gene transcription is induced by flavones (Long, 1989). The *nod*D gene itself is a regulatory gene, and its protein product induces other *nod* genes (Downie and Johnston, 1986; Long, 1989). 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 *nod*D. Chemicals called isoflavones can inhibit induction of *nod*D gene transcription in different species, and so a form of plant-bacterium specificity (Djordjevic *et al.*, 1987; Downie and Johnston, 1986; Long, 1989) is obtained.

The root hairs of legumes such as white clover also contain lectins on their surface (Bauer, 1981; Djordjevic *et al.*, 1987). Lectins are proteins, produced by the legume, and they are present before, during and after nodulation (Djordjevic *et al.*, 1987). Lectins bind specifically to exo- and capsular polysaccharides (Latchford *et al.*, 1991; Vance, 1983) 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 *et al.*, 1987). In many cases only a specific species 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* biovar 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 amount of nodulation (Vance, 1983). Once *Rhizobium* has bound to the root hair, infection can begin.

1. 2. 2. Infection of root hairs

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 (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 can now loosen the cell wall, thus allowing the release of the rhizobia into the cells of the plant cortex (Ljunggren and Fåhraeus, 1959; Ljunggren and Fåhraeus, 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).

Nodules are of two types; determinate and indeterminate. In indeterminate nodules (e.g. clover and alfalfa) infection threads continue to penetrate the cortical cells in the nodule meristem as it grows, and thus provide a continuous release of rhizobia into plant cells (Figure 3a). In determinate nodules infection threads are a transient feature of nodule development and an increase in nodule size occurs by the division of a few cortical cells containing rhizobia (Beringer *et al.*, 1979).

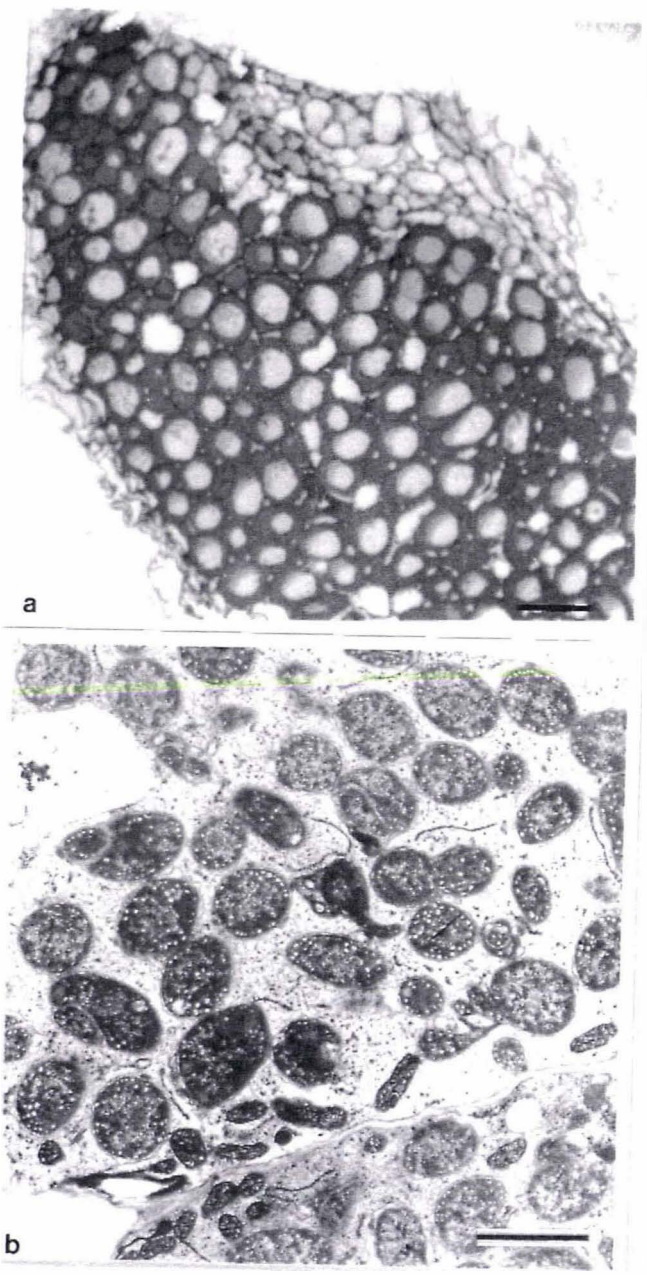
1. 2. 3 Bacteroid formation

Bacteroids (bacterial cells surrounded by plant membrane) now develop within the tetraploid cells. The plant membrane is referred to now as the peribacteroid membrane (Long, 1989). There are a number of differences between pre- and post-bacteroid rhizobia and when discussing bacteroids (Figure 3b), it is convenient to recognize three developmental stages; i) Immature bacteroids lack nitrogenase activity and are present in nodule tissue that has not yet produced significant quantities of leghaemoglobin. Immature bacteroids depend on the plant cytoplasm for energy and combined nitrogen. ii) Mature bacteroids characterized by high leghaemoglobin content. Mature bacteroids depend on the plant cytoplasm for energy, but they excrete substantial quantities of combined nitrogen in the form of ammonia. iii) Senescent bacteroids represent the terminal stage of nodule symbiosis when nitrogenase activity and leghaemoglobin content decline and the peribacteroid membrane disintegrates (Sutton *et al.*, 1981). Verma and Long, (1983) liken the bacteroids to chloroplasts and mitochondria. They suggest that bacteroids may be an early stage, in the evolution, of 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 an exchange of cell constituents, such as growth hormones and flavanoids, across both the bacterial and the peribacteroid membrane, allowing legume-*Rhizobium* communication.

1. 2. 4 Maturation of nodules

During nodule maturation the bacteroid experiences a different environment to 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. The nitrogenase complex requires energy to work and is inhibited by oxygen. The role of another protein, leghaemoglobin is significant in this context (Brock and Madigan, 1991; Downie and Johnston, 1986; Long, 1989; Verma and Long, 1983).

Figure 3. Sections from white clover (*Trifolium repens*) nodules infected by *Rhizobium leguminosarum* biovar trifolii strain ICMP2163: (a) nodule infected by the fully effective *Rhizobium* strain ICMP2163, scale bar = 100 µm; (b) electron micrograph of plant cells infected by *Rhizobium* strain ICMP2163, scale bar = 1 µm (Rao *et al.*, 1994).


a
b

Leghaemoglobin is a nodulin (nodulins are plant gene products which are expressed only in nodules, and their expression is regulated either directly or indirectly by *Rhizobium* inducers), present only in the legume and in the nodule itself (Long, 1989). Leghaemoglobin binds to oxygen thus reducing the amount of free oxygen available to inhibit nitrogenase activity (Brock and Madigan, 1991; Long, 1989). An interesting observation on 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 is 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. 3 Genetic requirements of *Rhizobium* for nodulation

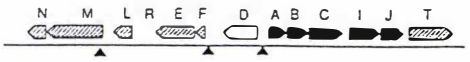
The genetics of *Rhizobium* has been greatly advanced by transposon mutagenesis, gene cloning, and plasmid transfer experiments (Denarie *et al.*, 1981; Kondorosi and Johnston, 1981; Kondorosi, 1991; Long, 1984; Long *et al.*, 1991). The fast growing *Rhizobium* species normally have one or more large plasmids. One of the plasmids carrying the symbiotic genes is designated pSym and possess the nodulation (*nod*) genes required for infection and nodule initiation (Banfalvi *et al.*, 1981; Beringer *et al.*, 1980; Brewin *et al.*, 1980; Djordjevic *et al.*, 1983; Rosenberg *et al.*, 1981). Sym plasmids also contain many genes required for the fixation of nitrogen (*fix* genes) and nitrogenase (*nif* genes) (Banfalvi *et al.*, 1981; Beringer *et al.*, 1980; Rosenberg *et al.*, 1981). In *Bradyrhizobium* and *R. loti* symbiotic genes are apparently not located on plasmids but on the chromosome (Lamb and Hennecke, 1986; Russell *et al.*, 1985; Scott, 1986; Stacey *et al.*, 1982). Several groups of symbiotic genes such as *nod, exo, nif,* and *fix* are discussed below and shown in Figure 4 a-c.

1. 3. 1 Nodulation genes

The nodulation genes of *Rhizobium* have been studied and defined by sequencing, transposon mutagenesis and in some cases protein analysis. Most gene definitions have emerged from the study of *R. meliloti, R. leguminosarum* biovar viciae and *R. leguminosarum* biovar trifolii.

Figure 4. (a) Genetic organization of *nod* genes in *R. leguminosarum* biovar trifolii. (a) The genes are presented as arrows which point according to the direction of their transcription. Common *nod* genes are indicated with black arrows, host-specific *nod* genes are indicated with shaded arrows, and the *nod*D genes are indicated with white arrows. Black triangles indicate the positions of *nod* boxes (Schlaman *et al.*, 1992), (b) Schematic representation (not in scale) of relative positions of *nod* and *nif* genes on Sym plasmids (Martinez *et al.*, 1990), (c) *Hind* III (H) and *Eco*RI (E) restriction enzyme map of the *nif* and *nod* gene region of pPN1 (Scott *et al.*, 1984).

a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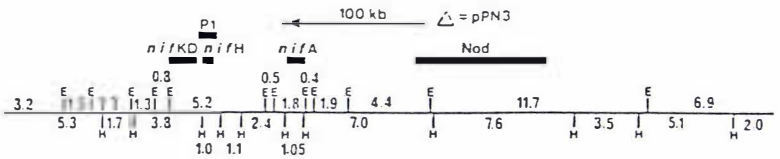
b


nod
nif
F E D A B C I J
D H

c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E
H

Genes for nodulation are defined by the effect on the correct host. These *nod* genes are required for bacteria to cause plant cell division (Dudely *et al.*, 1987) and deformation of root hairs (Djordevic *et al.*, 1985b: Kondorosi *et al.*, 1984; Rossen *et al.*, 1984). Mutants in *nod*A, *nod*B, or *nod*C are completely Nod$^-$ (no nodules form). The *nod*ABC genes appear to be functionally interchangeable among all *Rhizobium* species (Djordjevic *et al.*, 1985a; Fisher *et al.*, 1985; Kondorosi *et al.*, 1984; Marvel *et al.*, 1985). Bacteria carrying mutations in other genes, such as *nod*FE, *nod*G, *nod*H, and *nod*LMN, elicit abnormal root hair reactions on their usual host and sometimes elicit root hair deformation and curling on hosts they normally ignore (Debelle *et al.*, 1986; Djordjevic *et al.*, 1985b). These genes are not conserved, since alleles from different *Rhizobium* cannot substitute for each other on different host plants (Djordjevic *et al.*, 1985b; Kondorosi *et al.*, 1984). Based on the above observations, the *nod* genes are tentatively grouped as "common" and "host-specific" nodulation genes (Horvath *et al.*, 1986).

1. 3. 2 Regulation of nodulation genes

The functions of nodulation genes are discussed in brief with regard to *Rhizobium leguminosarum* biovar trifolii viz., *nod*ABC. Genes AB may produce a low molecular weight substance that induces cell division (John *et al.*, 1988). The gene C product is associated with membranes and may be a receptor (John *et al.*, 1988). The products of genes ABC, cause the thick and short root effect on some legumes mediated by *nod*D (Zaat *et al.*, 1987). The *nod*D gene product binds to the *nod-box* when activated by plant inducers (Downie and Johnston, 1988; Kondorosi *et al.*, 1988) and has domains with different inducer specificities (Djordjevic *et al.*, 1988; Horvath *et al.*, 1987; Spaink *et al.*, 1987; Spaink *et al.*, 1988). The gene F product resembles acyl carrier proteins (Debelle and Sharma, 1986; Shearman *et al.*, 1986). Gene E product resembles an enzyme involved in polyketide synthesis, that in turn is similar to a fatty acid synthetase (Johnston *et al.*, 1988). The EF gene product may be involved in lipopolysaccharide or fatty acid biosynthesis (Debelle and Sharma, 1986; Horvath *et al.*, 1986; Shearman *et al.*, 1986). The *nod*L product may be involved in dominant suppression of nodulation (*hsn* dominance) (Faucher *et al.*, 1988), *nod*M resembles an amidophosphoribosyl transferase, and the MN gene product may function to transfer amides for the chemical modification of EPS sugar residues (Dazzo *et al.*, 1988; Downie *et al.*, 1987; Weinman *et al.*, 1988).

Functions of *nod* genes

In the fast-growing *Rhizobium* species, the *nod* genes are located on a large plasmid, known as pSym. Specific falvones, flavanones, and chalcones are the inducers of *nod* genes in the fast-growing species. In the slow-growing *Bradyrhizobium* species, the *nod* genes are chromosomally borne (Györgypal *et al.*, 1991).

The common *nod* genes (*nod*ABCIJ) as well as nodulation genes involved in host specificity (*nod*FE, *nod*G, *nod*H and *nod*L) not only play a major role in root hair deformation (*Had*; *ha*ir *d*eformation) and shepherd's crook formation (*Hac*; *ha*ir *c*urling), but also in the initiation of *C*ortical *c*ell *d*ivisions (*Ccd*) which establish the nodule primordium (*Noi*; *no*dule *i*nitiation). If any one of the *nod*ABC genes is mutated, the ability of *Rhizobium* to deform root hairs and to initiate cortical cell divisions on its host is eliminated (Kondorosi *et al.*, 1991; Long, 1992). The common *nod* genes are so-called because they have been detected in all nodulating rhizobia species examined so far, for example *R. meliloti*, *nod* genes functionally complement comparable genes in other *Rhizobium* species.

Falvonoids act together with the product of the regulatory gene *nod*D, which is found in all nodulating rhizobia that have been analysed. Although *nod*D is constitiutively expressed, the genes of the *nod* operon are normally not expressed if host-derived molecules are absent. To some extent, *nod*D functions in host specificity. Chimeric genes have been constructed from *nod*D genes of rhizobia that nodulate different hosts. These studies have shown that the C-terminal end of the NodD protein determines flavonoid specificity, while the N-terminal region is involved in binding to regions of DNA known as *nod* boxes (Horvath *et al.*, 1987; Spaink *et al.*, 1987). The *nod* box is a highly conserved 47 bp long, *cis* regulatory region found in the promoters of *nod* operons (Rostas *et al.*, 1986). Although it is not exactly known how flavonoids interact with NodD, it is thought that the protein binds to the *nod* box more tightly after interaction with the correct flavonoid (Györgypal *et al.*, 1991). Other *nod* genes also mediate host specificity. Mutations in *nod*H genes enable *R. meliloti* to deform root hairs of white colver and vetch, species not normally compatible with that species (Faucher *et al.*, 1988a).

The primary signal for nodule morphogenesis comes from *Rhizobium*. Both the common and host specificity *nod* genes are involved in the production of a factor, identified as a lipo-oligosaccharide (glyco-lipid), that causes root hair deformation and cortical cell divisions in a compatible host. The chemical structure if the root hair

deformation factor of *R. meliloti* (designated NodRm-1) was the first to be identified (Lerouge *et al.*, 1990). NodRm-1 is a sulphated β-1, 4-tetra-D-glucosamine with three acetylated amino groups. A C16 unsaturated fatty acid occupies the non-reducing end of the molecule, while the reducing end contains a sulphate group.

A uniform method for naming Nod factors has been proposed (Hirsch, 1992). The naming is based on the various substitutions present on the glucosamine backbone. For example, NodRm-1 is now described as NodRm-IV (S) - Rm signifies *R. meliloti*, IV the four glucosamine residues, and S the sulphate on the reducing end of the molecule. *R. leguminoasrum* bv. viciae Nod factors, which are either tetra- or pentaglucosamines, are acetylated and lack a sulphate group. They are designated Rlv-IV (Ac) or Rlv-V (Ac).

The model proposed for the *nod* factor-receptor for rhizobial invasion: (1) the N-glucosamine residues of the Nod factor react with a sugar-binding site of a receptor, presumably a lectin; and (2) the strength of the interaction between Nod factor and its receptor regulates early events in nodulation. The strength of the interaction between Nod factor and receptor depends on several properties: the length of the glucosamine backbone, the presence or absence of various substituents like sulphate, and the composition of the lipid side chain. The extent of unsaturation, as well as the number of carbons in the fatty acid, are proposed to influence the mobility and orientation of the glucosamine residues. However, the lipid itself does not bind to the plant receptor (Hirsch, 1992).

Although Nod factor is secreted into the medium by rhizobia, the Nod factor is proposed to function *in situ* as part of the bacterial membrane with the lipid moiety inserted into the membrane. Two pieces of information support this proposal: (1) root hair curling factors added to Nod$^-$ bacteria do not restore the wild-type conditions (Banfalvi and Kondorosi, 1989) and (2) the molecular structure of the Nod factors suggests a membrane location. The failure of secreted Nod factor molecules to complement Nod$^-$ *Rhizobium* may indicate that either (1) a soluble form of the factor is insufficient by itself, or (2) an exact orientation of the Nod factor is essential for the full response of the plant.

Receptor molecules that bind the lipo-oligosaccharide are presumed to be present on the root hairs. The chemical nature of the receptor molecule is so far unknown, but it has been postulated to be a lectin (Lugtenberg *et al.*, 1991). Previously, lectins were thought to play a major role in specific attachment of *Rhizobium* to its host. Bohlool and Schmidt (1974); Dazzo and Hubbell (1975) proposed the lectin recognition hypothesis, which stated that lectins with unique sugar-binding properties would

interact with specific saccharides on the rhizobial surface. Dazzo *et al.*, (1984a) examined slide cultures by light and electron microscopy, the time course and orientation of attachment of *Rhizobium trifolii* 0403 to white clover root hairs. The interface between polarly attached bacteria and the root hair cell wall was shown to contain trifoliin A by immunofluorescence microscopy. Also, this interface was shown by transmission electron microscopy to contain electron-dense granules of host origin. Scanning electron microscopy revealed an accumulation of extracellular microfibrilis associated with the lateral and polar surfaces of the attached bacteria, detectable after 12 h of incubation with seedling roots. At this same time, there was a significant reduction in the effectiveness of 2-deoxy-D-glucose in dislodging bacteria already attached to root hairs and an increase in firm attachment of bacteria to the root hair surface, which withstood the hydrodynamic shear forces of high-speed vortexing. These reults are interpreted as a sequence of phases in attachment, beginning with specific reversible interactions between bacterial and plant surfaces (phase 1 attachment), followed by production of extracellular microfibrils which firmly anchor the bacterium to the root hair (phase 2 adhesion). Thus, attachment of *R. trifolii* to clover root hairs is a specific process requiring more than just the inherent adhesiveness of the bacteria to the plant cell wall. Root hair tips were found to be sites of early attachment and colonization of *R. trifolii* 0403 in soil. Lectins are attractive candidates for receptors also because some are located in the region of the root that is most susceptible to *Rhizobium* infection (Diaz *et al.*, 1986). There have been numerous studies on the role of lectins in attachment, but until now their role in host recognition remains elusive and controversial. Kijne *et al.*, (1986) proposed that lectins are more likely to be involved in invasion rather than attachment of rhizobia. The test of whether lectins are the receptors for Nod factors will occur if plant mutants with defective root lectin are found, and these have altered host specificity or are defective in nodulation.

Root hair curling does not take place, in peanut and *Stylosanthes* or in the nonlegume *Parasponia.* In these plants, rhizobia enter the roots between epidermal cells where lateral roots emerge (Dart, 1977: Torrey, 1986). The rhizobia that infect these plants carry the common *nod* genes, and it is assumed that the Nod factor produced following the induction of these genes will be structurally related to NodRm-1. The Nod factor of *Rhizobium* species NGR234, which nodulates at least 35 different genera of legumes as well as the nonlegume *Parasponia*, is chemically related to NodRm-1 (Broughton *et al.*, 1991). However, it is not known whether rhizobial *nod* gene products are required for non-root hair cell invasion or whether specific receptor molecules are localized on the root at the invasion sites. Too little information is available regarding these less typical *Rhizobium*-host plant symbiosis.

The relationship of *Rhizobium* cell surface components such as lipopolysaccharides (LPS), exopolysaccharides (EPS) and capsular polysaccharides (CPS), and Nod factor

remains undefined. *R. meliloti exo* mutants, which induce small, white nodules on alfalfa (Finan *et al.*, 1985; Leigh *et al.*, 1987) have functioning *nod* genes (Klein *et al.*, 1988). They elicit root hair cell deformation and can initiate infection thread formation. However, the threads abort in the peripheral cells of the bacteria-free, "empty" nodule (Finan *et al.*, 1985).

Thick and short roots (Tsr) factor

Several factors, exclusive of the lipo-oligosaccharides just described, influence root hair proliferation, branching or deformation, cortical cell divisions or cause a phenotype known as the thick, short root response (Tsr) (van Brussel *et al.*, 1986). Experiments in which plants and bacteria were grown separately in the sterile supernatant fluids of each other established the following sequence of events. (i) The plant produces a factor, designated as factor A. (ii) Factor A causes the sym plasmid-harboring bacteria to produce Tsr factor. (iii) Growth of young plants in the presence of Tsr factor results in the Tsr phenotype.

Two models have been proposed to explain these results. In the first, factor A directly or indirectly causes induction or derepression of the *tsr* genes leading to the synthesis of Tsr factor. These genes could either have a structural or a regulatory function. In the case of indirect induction it is regulatory function. Direct induction factor A could be a plant enzyme, acting on a bacterial excretion product or on the bacterial cell surface, whose product induces the synthesis of Tsr factor.

In the second model, plant factor A would be a precursor molecule which is converted into Tsr factor by a bacterial enzyme that requires the *nod*A, B, C, and D genes for activity. Alternatively, factor A might be a plant enzyme which converts a bacterial substrate, whose synthesis or modification depends on the *nod*A, B, C, and D genes into Tsr factor.

The chemical structure of some Tsr factors have been elucidated and a number of bacterial factors (BF) that are produced by *R. leguminosarum* bv. trifolii have been identified. Some promote root hair proliferation or work synergistically to elicit hair deformation and cortical cell divisions on clover (Hollingsworth *et al.*, 1990). One of these factors, BF-5, which is dependent on *nod* gene induction by flavonoids, has been identified as N-acetylglutamic acid (Philip-Hollingsworth *et al.*, 1991). BF-5, when added to clover roots, causes root hair branching and tip swelling. It also increases the number of foci of cortical cell divisions. BF-5 does not elicit these responses on alfalfa or *Lotus* (Philip-Hollingsworth *et al.*, 1991). However, it is not known whether the genes required for production of the factor are essential for the symbiosis.

1. 3. 3 Rhizobium *nif* and *fix* genes

Rhizobium genes involved in nitrogen fixation are generally divided into two groups: those with homologs in free-living nitrogen fixation systems, such as that found in *Klebsiella*, are referred to as *nif* genes; those that are required for symbiotic nitrogen fixation, but whose function is not known to be analogous to a free-living function, are referred to as *fix* genes. It is understood that both *nif* and *fix* gene mutants are able to cause nodule development, but the nodules do not fix nitrogen (Nod^+Fix^-). *Azorhizobium caulinodans* is a stem-nodulating bacterium that is unique in being able to grow by fixing nitrogen in the free living-state. This system has therefore been useful for study of *nif* genes, *nif* and *ntr* regulatory genes, and the metabolic infrastructure supporting *Rhizobium* nitrogen fixation (Long, 1989; Ludwig, 1986; Norel *et al.*, 1985; Pawlowski *et al.*, 1987). Another bacterium with interesting genetic features is *R. phaseoli*, which contains multiple copies of *nif* genes and complex patterns of symbiotic plasmids (Flores *et al.*, 1988; Quinto *et al.*, 1985; Soberón-Chávez *et al.*, 1986).

1. 3. 4 Functions of *nif* and *fix* genes

The *nif*A gene product binds a DNA sequence upstream of the majority of *nif* and *fix* genes activating their expression (Gussin *et al.*, 1986). The *nif*H product codes for the nitrogenase reductase (component II) and the *nif*D and K genes code for the *alpha* and *beta* subunits of nitrogenase (component I), respectively (Martinez *et al.*, 1990). In *Rhizobium leguminosarum*, no genes on plasmids other than pSym have as yet been reported to be essential for the formation of a nitrogen-fixing nodule but there are choromosomal genes involved in exopolysaccharide (EPS) and lipopolysaccharide (LPS) synthesis (Borthakur *et al.*, 1985; 1986; Cava *et al.*, 1989; Noel *et al.*, 1986), and genes involved in purine biosynthesis (Noel *et al.*, 1988) which have been shown to be necessary for nodule development.

1. 4 Taxonomy of *Rhizobium*

Family *Rhizobiaceae* comprises five different genera *Rhizobium, Bradyrhizobium, Agrobacterium, Phyllobacterium* (Jordan, 1984) and *Azorhizobium. Azorhizobium* is a new genus which induces nodules in *Sesbania* species (Dreyfus *et al.*, 1988). There is one species *A. caulinodans*. An additional genus *Sinorhizobium* has also been proposed by Chen *et al.*, (1988). However Jarvis *et al.*, (1992) found that the sequences of

Sinorhizobium fredii and *Sinorhizobium xinjiangensis* were both identical with those of *Rhizobium meliloti* and similar to those of *Rhizobium leguminosarum* and that therefore the strains should be classified as *Rhizobium fredii* (i. e. classified with the Genus *Rhizobium*). At present the family *Rhizobiaceae* contains 5 genera with about 22 named species as shown in Table 1. The discovery in the *Rhizobium* species of symbiotic plasmids carrying genes which can be transferred from one strain to another, changing specificity, or which may be lost due to environmental factors argues against a classification based on what might be a transient property (Martinez *et al.*, 1990). Classification of rhizobia urgently requires a basis, independent of plasmid-borne characters.

Rhizobia were originally described as gram negative soil bacteria which fix nitrogen in symbiotic association with plants of the family *Leguminosae* (Prakash and Atherly, 1986). However, this classical definition must be extended now to include nodulation and nitrogen fixation on a nonlegume, *Parasponia* sp. (*"Rhizobium parasponia"*) (Trinick, 1973).

From rRNA similarity maps and a $T_{m(e)}$ dendrogram the following conclusions were drawn: (Figure 5) (Jarvis *et al.*, 1985; Jarvis *et al.*, 1986) Group I includes *R. meliloti*; host plant *Medicago sativa* (Rosenberg *et al.*, 1981), *R. leguminosarum* biovar phaseoli; host plant *Phaseolus vulgaris* L. (Quinto *et al.*, 1982; Robert and Schmidt, 1985), biovar trifolii; host plant *Trifolium pratense* L. and *T. subterraneum* L., biovar viciae; host plant *Vicia faba* L., *Rhizobium fredii*; host plant *Glycine max* (Dowdle and Bohlool, 1985; Keyser *et al.*, 1982). Group II *: Rhizobium loti*; host plant *Lotus divaricatus* (Crow *et al.*, 1981; Jarvis *et al.*, 1982). Group III: *Rhizobium galegae*; host plant *Galega orientalis* and *G. officinalis* (Lindstörm, 1989; Wedlock and Jarvis, 1986).

In addition to these species Chen *et al.*, (1991) has isolated rhizobial strains from nodules of *Astragalus sincius*. These strains constitute a group that is quite different from previously described *Rhizobium, Bradyrhizobium and Agrobacterium* species. For this species Chen *et al.*, (1991) have proposed the name *Rhizobium huakuii* using data from gel electrophoresis of whole-cell proteins, DNA G+C content data, and DNA-DNA hybridization data.

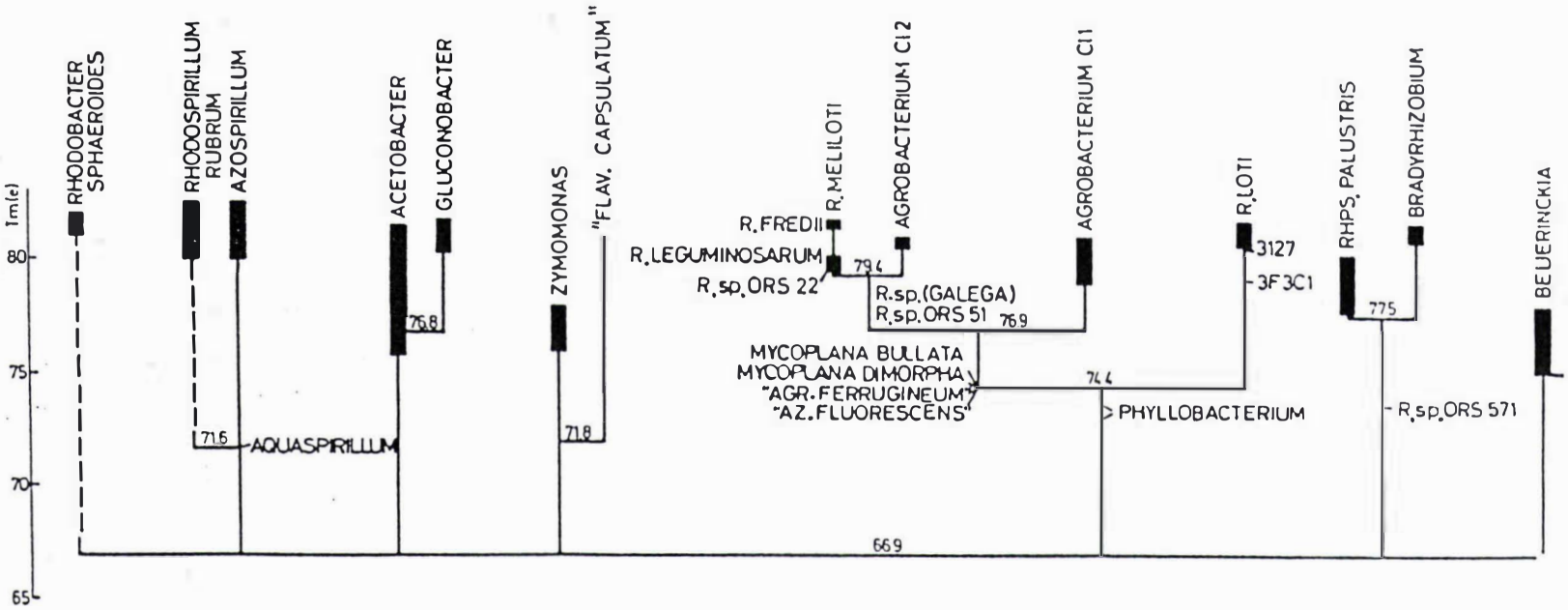
Rhizobium tropici a new species that nodulates *Phaseolus vulgaris* L. and *Leucaena* spp. is proposed on the basis of the results of multilocus enzyme electrophoresis,

Table 1. Named species of the family *Rhizobiaceae* and a typical leguminous plant host.

Genus	Species biovar	Host legume (examples) Latin name	Trivial name
Rhizobium	*leguminosarum* bv. viciae	*Vicia sativa*	Vetch
		Pisum sativum	Peas
		Lens esculenta	Lentil
	leguminosarum bv. trifolii	*Trifolium pratens*	Clover
		T. subterraneum	Clover
	leguminosarum bv. phaseoli	*Phaseolus vulgaris*	Bean
	etli[a]	*Phaseolus vulgaris*	Bean
	tropici[b]	*Phaseolus vulgaris*	Bean
	meliloti	*Medicago sativa*	Lucerne
	huakuii[c]	*Astragalus sinicus*	Astragalus
	loti	*Lotus corniculatus*	Trefoil
		Lupinus luteus	Lupin
	galegae[d]	*Galega orientalis*	Goatsrue
		Leucaena sp.	Leucaena
	fredii[e, f]	*Glycine max*	Soybean
	ciceri[g]	*Cicer arietinum*	Chickpea
Bradyrhizobium	*japonicum*	*Glycine max*	Soybean
	elkanii[h]	*Glycine max*	Soybean
	sp. (host genus)	*Arachis hypogaea*	Groundnut
		Vigna unguiculata	Cowpea
		Macroptilium atropurpureum	Siratro
		Acacia sp.	Acacia
		Lupinus luteus	Lupin
		Lotononis bainesii	
		Parasponia sp. (non-legume)	
Azorhizobium	*caulinodans*	*Sesbania rostrata*	Sesbania
Phyllobacterium	*rubiacearum*	*Zimmermaniana pavetta*	
	myrsinacearum	*Ardisia crispa*	
Agrobacterium	*tumefaciens*	*Nicotiana tabacum*	Tabacco
	vitis	*Vitis vinifera*	Grape
	rhizogenes	*Rosa* sp.	Rose
	rubi	*Rubus ursinus*	Blackberry
	radiobacter		(clinical)

[a], Segovia *et al.*, 1993; [b], Martínez *et al.*, 1991, [c], Chen *et al.*, 1991, [d], Lindström, 1989, [e], Chen *et al.*, 1988, [f], Jarvis *et al.*, 1992, [g], Nour *et al.*, 1994, [h], Kuykendall *et al.*, 1992.

Figure 5. Simplified rRNA cistron similarity dendeogram of part of rRNA superfamily IV, based on $T_{m(e)}S$ of DNA-rRNA hybrids. The vertical lines represent labeled rRNA from reference strains. The thick bars indicate the variation which we observed for the taxon indicated. The groups of strains were clustered by using the unweighted average pair group method (Sokal and Sneath, 1963). Abbreviations: FLAV., *Flavobacterium*; R., *Rhizobium*; Cl, cluster; AGR., *Agrobacterium*; AZ., *Azotomonas*; RHPS., *Rhodopseudomonas*. (Jarvis *et al.*, 1986).


Tm(c)
80
75
70
65
RHODOBACTER SPHAEROIDES
RHODOSPIRILLUM RUBRUM
AZOSPIRILLUM
ACETOBACTER
GLUCONOBACTER
ZYMOMONAS
"FLAV. CAPSULATUM"
R.MELILOTI
AGROBACTERIUM C12
AGROBACTERIUM C11
R.LOTI
RHPS. PALUSTRIS
BRADYRHIZOBIUM
BEIJERINCKIA
R.FREDII
R.LEGUMINOSARUM
R.sp.ORS 22
3127
3F3C1
R.sp.(GALEGA)
R.sp.ORS 51
MYCOPLANA BULLATA
MYCOPLANA DIMORPHA
"AGR.FERRUGINEUM"
"AZ.FLUORESCENS"
PHYLLOBACTERIUM
R.sp.ORS 571
AQUASPIRILLUM
71.6
76.8
71.8
79.4
76.9
74.4
77.5
66.9

DNA-DNA hybridization, an analysis of ribosomal DNA organization, a sequence analysis of 16S rDNA and an analysis phenotypic characters (Martinez *et al.*, 1991). This taxon *Rhizobium tropici* sp. nov., was previously named *Rhizobium leguminosarum* biovar phaseoli type II and was recognized by its host range (which includes *Leucaena* spp.) and *nif* gene organization. In contrast to *R. leguminosarum* biovar phaseoli, *R. tropici* strains tolerate high temperatures and high levels of acidity in culture and are symbiotically more stable.

A new *Rhizobium* species that nodulates *Phaseolus vulgaris* L. is proposed on the basis of a sequence analysis of 16S ribosomal DNA. This taxon, *Rhizobium etli* sp. nov., was previously named *Rhizobium leguminosarum* biovar phaseoli type I and is characterized by the capacity to establish an effective symbiosis with bean plants, the reiteration of the nitrogenase structural genes, the organization of the common nodulation genes into two separate transcriptional units bearing *nod*A and *nod*BC, the presence of a polysaccharide inhibition gene, *psi*, and the 16S ribosomal DNA sequence. An analysis of the sequence of a fragment of the 16S rRNA gene shows that this gene is quite different from the gene of *R. leguminosarum* (Segovia *et al.*, 1993).

Based on DNA homology, Guanine-plus-cytosine content, restriction fragment length polymorphism of the amplified 16S-intergenic spacer rRNA gene, partial 16S rRNA sequencing, and auxanographic tests performed with 147 carbon sources, Nour *et al.*, (1994) proposed a new species, *Rhizobium ciceri* sp. nov., consisting of strains that nodulate chickpeas (*Cicer arietinum* L.) and were previously determined to belong to two groups (groups A and B) when compared with reference strains belonging to different genera and species of the family *Rhizobiaceae.* However, other groups of *Rhizobium* strains which do not cluster with the existing species have been identified and some example are *R. "hedysari"* (Casella *et al.*, 1986) and *R. "giardini"* (Laguerre *et al.*, 1993b) and will be published shortly.

Following are two examples of symbiotic divergence in plant-*Rhizobium* interactions. *Rhizobium* strains isolated from some ineffective alfalfa nodules have the ability to nodulate beans (Eardly *et al.*, 1985) and *Rhizobium* strain NGR234 effectively nodulates *Arachis hypogea,* two species of *Desmodium,* one cultivar of *Glycine max, Lab-lab, Leucaena leucocephala, Macroptilium atropurpureum, Medicago sativa, Pachyrhius tuberosus, Vigna unguiculata,* non-legume *Parasponia* sp. and ineffectively nodulates *Sesbania* (Lewin *et al.,* 1987; Nayudu and Rolfe, 1987; Trinick, 1980; Trinick and Galbraith, 1980).

Claims have been made that the classification of *R. phaseoli, R. trifolii* and *R. leguminosarum* actually refers to a classification of their symbiotic plasmids and their pSym possess distinctive features (Watson and Schofield, 1985). Genetic exchange in *Rhizobium* bacteria in soil and recombination frequencies will have strong implications for the validity and usefulness of taxonomy. If the *Rhizobium* genome recombines infrequently, thus behaving clonally (Pinero *et al.*, 1988; Young, 1985), *Rhizobium* bacteria would be diverging lineages, accumulating differences and sharing only symbiotic capabilities. Thus, taxonomy would be faced with an unpredictable huge number of genomes, which in addition may be interchanging plasmids (Schofield *et al.*, 1987; Young and Wexler, 1988).

Several studies suggest that *R. leguminosarum* may not be a single species but rather a group of inter-related species capable of expressing the appropriate symbiotic genes (Rao *et al.*, 1994). Genomic heterogeneity among the French *Rhizobium* strains isolated from *Phaseolus vulgaris* L. and conformity and diversity among field isolates of *Rhizobium leguminosarum* bv. viciae, bv. trifolii, and bv. phaseoli by DNA hybridization using chromosome and plasmid probes was reported (Laguerre *et al.*, 1993b; 1993c). Jarvis, (1983) also reported genetic diversity of *Rhizobium* strains which nodulate *Leucaena leucocephala.* This indicates that within field populations of *R. leguminosarum* isolated from root nodules, a variety of genotypes are able to nodulate the same host plant (Brockman and Bezdicek, 1989; Engvild *et al.*, 1990; Harrison *et al.*, 1987, 1989; Hynes and O'Connell, 1990; Laguerre *et al.*, 1992, 1993c); Schofield *et al.*, 1987; Young, 1985; Young *et al.*, 1987; Young and Wexler 1988). However, within biovars, the degree of diversity of strains, the genotypes of predominant strains in nodules, and the level of dominance of strains are dependent on the host plant species (Hynes and O'Connell, 1990; Laguerre *et al.*, 1992, 1993c; Mazurier, 1989).

1. 5 Seed inoculation

Seed inoculation with appropriate strains of *Rhizobium* is well established agricultural practice which can improve legume productivity (Rao *et al.*, 1994). Seed-producing companies ensure the presence of nitrogen-fixing bacteria near clover seeds by inoculating the seeds with commercial strains of *R. leguminosarum* biovar trifolii such as ICMP strains 2163, 2663 and 2668. The bacteria are added to the seeds in a slurry 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). A detailed understanding of interactions between the symbiotic genes and other microorganisms in the soil environment and their identity may contribute to the development of improved strains for use as inoculants.

Population density, effectiveness, and competitive ability are the primary characteristics of indigenous rhizobial populations that affect inoculation responses. In greenhouse studies, Singleton and Tavares, (1986) demonstrated that statistically significant inoculation responses can be eliminated when there are as few as 20 indigenous rhizobia g of soil^{-1} as long as the population contains some effective strains. Strains within populations of rhizobia differ significantly in their ability to supply the host plant with fixed N (effectiveness) under greenhouse conditions (Singleton *et al.*, 1985; Singleton and Stockinger, 1983; Singleton and Tavares, 1986). Differences in the effectiveness of inoculant strains can also be demonstrated under field conditions as long as the soil is free of indigenous rhizobia (Ham, 1980). In the presence of an indigenous population, however, improved crop yield through inoculation with more effective inoculant strains is difficult to demonstrate (Ham *et al.*,1971; Meade *et al.*, 1985). Successful competition for nodule sites by indigenous rhizobia is one reason for the failure to achieve a response to inoculation with elite rhizobial strains (Meade *et al.*, 1985; Weaver and Frederick, 1974).

1. 6 Factors affecting *Rhizobium* survival in Soil

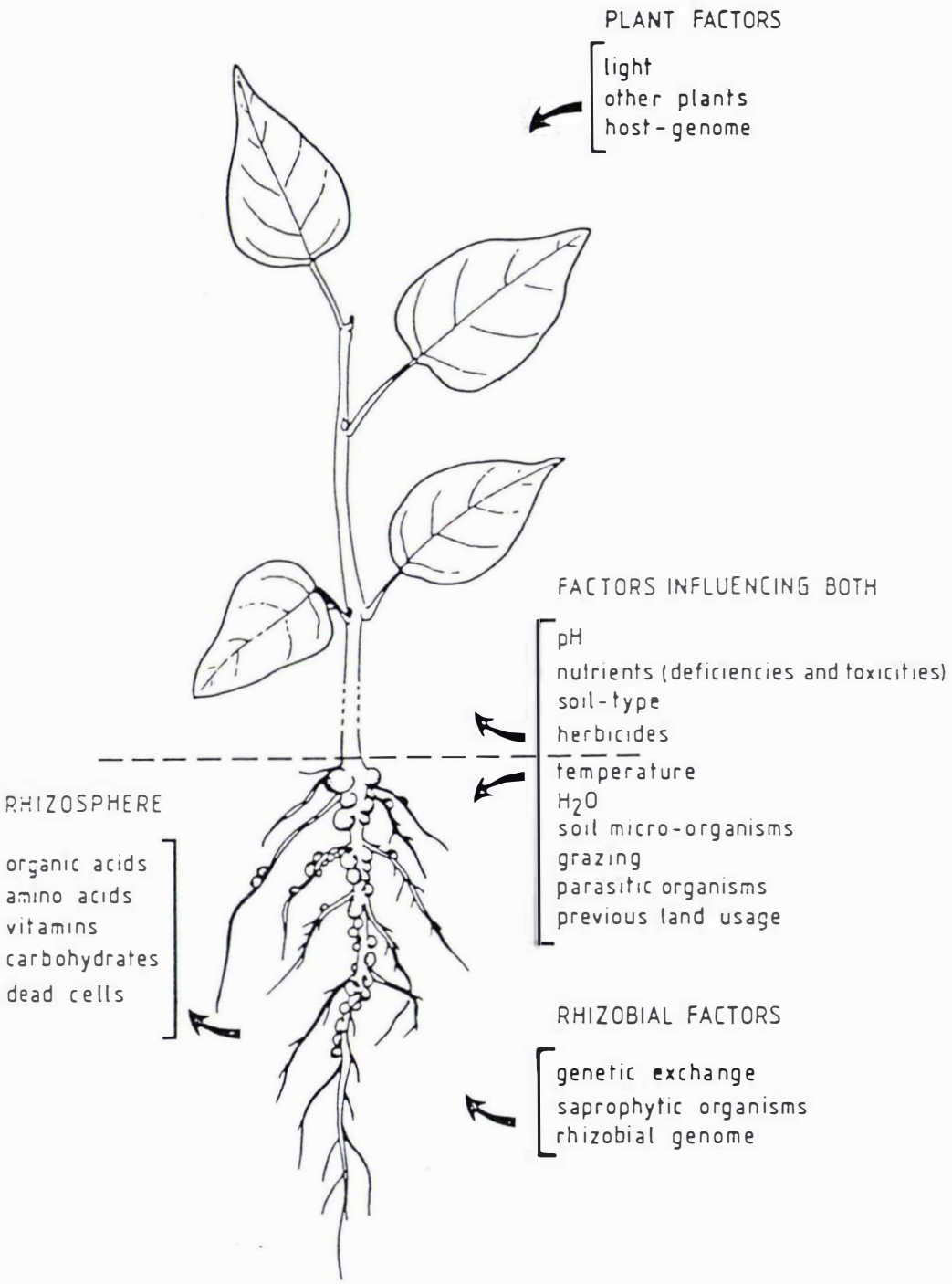
Soil is a reservoir of bacteria. The intrinsic makeup of which can effect survival of *Rhizobium*. Combined nitrogen especially nitrate delays nodule formation (Streeter, 1988). This may (McNeil, 1982) or may not (Kosslak and Bohool, 1985) effect the outcome of competition of *Rhizobium*. The number of rhizobia in the rhizosphere decreases 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, temperature, pesticides, herbicides, moisture and the size of the pores within the soil (Brewin *et al.*, 1983; Dowling and Broughton, 1986; Postma and van Veen, 1990; Rao *et al.*, 1994). Abiotic features differ widely from soil to soil; each location with its unique set of abiotic parameters must be considered separately.

In addition to abiotic factors, biotic factors affect survival in the rhizosphere. These include the effect of bacteriophage, epiphytic bacteria, protozoa, *Bdellovibrio,* bacteriocins and competition for nutrients within the rhizophere (Bauer, 1981; Brewin *et al.*, 1983; Broughton *et al.*, 1987; Djordjevic *et al.*, 1982; Dowling and Broughton, 1986; Rao *et al.*, 1994; 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) *Erwinia herbicola*, which blocks *Rhizobium* attachment sites on root hairs (Dowling and Broughton, 1986).

Transfer of symbiotic plasmids 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 in the recipient bacteria. This evidence and previous reports of self-transmissible pSym plasmids in *R. leguminosarum* (Brewin *et al.*, 1980) suggest that interstrain plasmid transfer is a common phenomenon in natural populations of this species. Chromosomally related *R. leguminosarum* biovar trifolii isolates have been shown to have unrelated plasmid profiles, whereas less chromosomally related *R. leguminosarm* biovar trifolii isolates may have very similar plasmid contents (Schofield *et al.*, 1987). Some strains which show very little chromosomal homology to any *Rhizobium* type strain have been isolated from nodules taken directly from a field (Laguerre *et al.*, 1993b). A non-symbiotic soil isolate which was shown to be a *R. leguminosarum* biovar phaseoli strain without a Sym plasmid was complemented with a pSym from another *R. leguminosarum* biovar phaseoli strain, then placed back in the soil. The Sym plasmid recipient was able to compete with indigenous rhizobia, could form nodules and even fix nitrogen (Soberón-Chávez and Nájera, 1989). Similarly a transconjugant soil bacterium, strain JR101, was also found to be inherently more competitive than the initial *Rhizobium* inoculant strain. In this case the ability to form nodules was also found to be dependent on the relative numbers of the two strains in the vicinity of the root and upon access to the root surface (Rao *et al.*, 1994).

It could be postulated that there is a tendency for rhizobia to get rid of their large, cumbersome Sym plasmids when the plasmids are not being used and soil bacteria could act as reservoirs. Such a theory requires further investigation, and the production of a non-transferable Sym plasmid, may theoretically result in reduced survivability of rhizobia in the soil under certain conditions. Figure 6 summarizes some of the interactions which affect *Rhizobium* survival in soil.

Figure 6. Factors that may influence the outcome of competition among *Rhizobium* strains for nodulation of legumes (Dowling and Broughton, 1986).


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

1. 7 *Rhizobium* symbiotic genes and indigenous soil bacteria

Population studies on soil strains of *R. leguminosarum* indicate that the same symbiotic plasmid occurs in different strains of bacteria and different symbiotic plasmids occur in otherwise similar strains of host bacteria. This suggests that genetic exchange may have occurred in soil (Schofield *et al.*, 1987; Young, 1985).There is also evidence to show that soil harbours non-nodulating bacteria (Jarvis *et al.*, 1989; Laguerre *et al.*, 1993a; Rao *et al.*, 1994; Segovia *et al.*, 1991; Soberón-Chávez and Nájera, 1989) able to express symbiotic plasmids from *Rhizobium* species. The expression of symbiotic genes by soil bacteria raises the possibility that symbiotic plasmids may be transferred, maintained and expressed in soil by a population which includes rhizobia and an unknown number of other bacterial species. Information on the diversity of the population expressing the pSym is scarce.

Jarvis *et al.*, (1989) reported the isolation of non-rhizobial soil bacteria from native soils and their ability to express *nod* genes. Rao *et al.*, (1994) showed the self transmission of Tn5 marked symbiotic plasmids from inoculant strains of *Rhizobium leguminosarum* biovar trifolii ATCC10004, ICMP2163, ICMP2668 and ICMP2666 to plasmid cured rhizobia and to some native soil bacteria. Similarly when a *Rhizobium leguminosarum* biovar phaseoli Sym plasmid was transferred to a soil isolate, the recipient was able to nodulate and fix nitrogen in bean roots, and could also compete affectively with other indigenous *Rhizobium* strains in the soil (Soberón-Chávez, 1989). Laguerre *et al.*, (1993a) also observed *R. leguminosarum* soil isolates which lacked symbiotic information but were able to gain nodulation capacity with the acquisition of a conjugative symbiotic plasmid. They were thus be considered as non-symbiotic *R. leguminosarum.*

1. 7. 1 Expression of symbiotic plasmid (pSym) in soil bacteria

The study of symbiotic nitrogen fixation in *Rhizobium* has been limited to *Rhizobium* plasmids. Up to 25% of the DNA in *Rhizobium* is in the form of large plasmids and it is of interest to know what genes are carried on these large plasmids (Prakash and Atherly, 1986). Some early genetic evidence indicated that in *Rhizobium* the symbiotic nitrogen fixation genes might be on the plasmid DNA (Higashi, 1967). Several genetic and physical studies have now clearly established that, at least in fast growing *Rhizobium* species, the genes for symbiotic nitrogen fixation are usually on a large plasmid.

Symbiotic (Sym) plasmids bear the genes required for nodulation. Sym plasmids range in size from 130 kb (kilobases) to greater than 290 kb in *R. leguminosarum* and greater than 1200 kb 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; Iismaa *et al.*, 1989), as well as genes whose products provide antibiotic resistance (Brewin *et al.*, 1982), hydrogenase activity (De Jong *et al.*, 1982), bacteriocinic properties (De Jong *et al.*,, 1982; Hirsch *et al.*, 1980), or affect polysaccharide production (Borthakur *et al.*, 1985; Latchford *et al.*, 1991) and carbon metabolism (Djordjevic *et al.*, 1982). *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.

Hooykaas *et al.*, (1981) and Zurkowski and Lorkiewicz, (1976, 1978, 1979) showed that non-nodulating mutants of *R. trifolli*, resulting from treatment at high temperature, had lost plasmid DNA. These *nod*$^-$ mutants lost their ability to attach to the root hair. Surface attachment and nodulation were restored upon the reintroduction of the plasmid. Furthermore, Johnston *et al.*, (1978) demonstrated that the transfer of a Tn5 marked plasmid into a *fix*$^-$ strain of *R. leguminosarum* restored its normal symbiotic functions, strongly suggesting that the symbiotic genes were located on the plasmid.

Ronson and Scott, (1983) obtained a co-integrate of pRtr514a and R68.45, which totalled about 219 Mda and transferred it to a variety of different bacterial strains at frequencies as high as 10^{-3}. Similarly, co-integrates of Sym plasmid from *R. fredii* strain USDA191 and PRL180 (Hooykaas *et al.*, 1982) were transferred to *E. coli* and *Agrobacterium tumefaciens* (Engwall and Atherly, 1986). But, co-integrates were very unstable in *rec*A$^+$ *E. coli* strains yielding only pRL180 upon conjugation. In *rec*A$^-$ *E. coli* hosts the Sym plasmids suffered random deletions and it was thus possible to create a family of deletions encompassing the entire plasmid. pPN1 was stable in recA$^-$ strains of *Escherichia coli* and *Pseudomonas aeruginosa* and in most fast-growing *Rhizobium* strains, suggesting that most *Rhizobium* strains may be naturally inefficient in genetic recombination (Ronson and Scott, 1983). Jarvis *et al.*, (1989) transferred by conjugation, plasmid pPN1, to representative strains of non-nodulating, gram negative, rod-shaped soil bacteria. Transconjugants which formed nodules were obtained from 6 of 18 (33%) strains whose DNA hybridized with that of PN165 and 1 of 9 (11%) strains containing DNA which did not hybridize with that of PN165. The presence and location of R68.45 and *nod* genes was confirmed in transconjugants from three of the strains which formed nodules.

Sym plasmid transfer within *Rhizobiaceae* often results in variable expression and most plasmids on which genetic markers have been found have been shown to be self-transmissible (Denarie *et al.*, 1981; Gussin *et al.*, 1986; Prakash and Atherly, 1986). Hooykaas *et al.*, (1981, 1982) observed that the Sym plasmids of *R. leguminosarum* and *R. trifolii* expressed symbiotic nitrogen fixation properties completely when transferred between these two strains. However when these Sym plasmids were transferred to *A. tumefaciens* or *R. meliloti* they induced root nodules but did not fix nitrogen. Djordjevic *et al.*, (1983) and Rolfe *et al.*, (1983) found that transfer of plasmid pJB5JI or pBRIAN, which encode pea and clover specificity, respectively, to various *R. meliloti* plasmid-cured strains did not confer the ability to nodulate peas and clover. Transfer of pBRIAN to an *A. tumefaciens* strain conferred clover-specific nodulation but pJB5JI could not induce this *Agrobacterium* strain to nodulate peas. The transfer of pBRIAN to various Sym plasmid-cured or deleted *R. leguminosarum* or *R. trifolii* strains resulted in the ability of these strains to nodulate clover, whereas the reverse was true when pJB5JI was transferred to these strains. When plasmids pJB5JI and pBRIAN were introduced into a fast growing *"R. parasponium"* strain, the resulting transconjugants showed a change in the spectrum of plants that could be nodulated. The plasmids have also been transferred to slow-growing *Rhizobium* species, but the bacteria had an ineffective phenotype. The transfer of a host-range plasmid pJB5JI from *R. leguminosarum* to *R. fredii* elicited only early stages of nodule development on peas (Ruiz-Sainz *et al.*, 1984). On the other hand the *R. fredii* Sym plasmid from strain USDA191 is Fix^+ in ANU265 genetic background (Appelbaum *et al.*, 1985). ANU265 is a pSym-cured derivative of NGR234, a broad-host-range fast-growing strain. This implies that the chromosomal genetic background of NGR234 is very similar to *R. fredii*. Incompatibility and instability of the Sym plasmids could also be attributed to variable expression of the Sym plasmid in different hosts (Christensen and Schubert, 1983; Djordjevic *et al.*, 1982). A large Sym plasmid from fast-growing cowpea *Rhizobium* species was mobilizable by co-integration with plasmid pSUP1011, which carries the *ori*T region of RP4. This mobilizable Sym plasmid was transferred to a number of *Rhizobium* strains, in which nodulation and nitrogen fixation functions for symbiosis with plants of the cowpea group were expressed (Morrison *et al.*, 1984).

It is concluded that symbiotic plasmid transfer occurs between *Rhizobium* strains and other bacteria in soil but the nature and diversity of the recipient remains unclear.

Bacteria resembling *Rhizobium leguminosarum*, but lacking symbiotic information, have been isolated from soil of two different geographical origins, thus giving evidence for persistence in soil of *Rhizobium* lacking symbiotic information (Soberón-Chávez and Nájera, 1989). When complemented with an *R. leguminosarum* bv.

phaseoli symbiotic plasmid p42d, the non-symbiotic isolates were able to fix nitrogen in symbiosis with bean roots at levels similar to the parental strain. The symbiotic isolates were found at a relative frequency of 1 in 40 non-symbiotic *R. leguminosarum* (Segovia *et al.*, 1991). Rao *et al.*, (1994) demonstrated *in vitro* self-transmission of symbiotic plasmids from three *Rhizobium leguminosarum* biovar trifolii strains used as clover seed inoculants and from two other strains to cured a derivative of *R. leguminosarum* biovar trifolii. Tn5-marked symbiotic plasmids from strains ICMP2163 and ICMP2668 were transferred to three strains of native soil bacteria at frequencies of 10^{-4}. Plasmid transfer to one of the native soil bacterial isolates was also demonstrated in a soil microcosom containing white clover seedlings. Laguerre *et al.*, (1993a) isolated from soil symbiotic and nonsymbiotic *Rhizobium leguminosarum* by DNA hybridization. The *R. leguminosarum* soil isolates lacked symbiotic information but were able to gain nodulation capacity with the acquisition of a conjugative symbiotic plasmid pMA1. Transfer has been reported of the pea symbiotic plasmid pJB5JI between strains of rhizobia in sterile and in non-sterile soil (Kinkle and Schmit, 1991). A detailed molecular approach is needed to identify soil bacteria expressing a pSym from *Rhizobium leguminosarum* biovar trifolii.

1. 8 Identification of bacteria

As more knowledge is acquired and new isolates are studied, new species are discovered and the former species are split. There is an increasing need for reliable methods which can enable rhizobiologists, and especially ecologists, to identify the bacteria they are working with. Recent approaches include DNA hybridization, characterization of rRNA genes and their hybridization patterns as well as fatty acid analysis.

Studies have shown that *Legionella* species can be identified by their rRNA patterns (Grimont *et al.*, 1987; Saunders *et al.*, 1988). Laguerre *et al.*, (1994) reported rapid identification of rhizobia by restriction fragment length polymorphism analysis of PCR-amplified 16S rRNA genes. This PCR-RFLP method has also been used successfully used in the differentiation of *Leptospira* species, (Ralph *et al.*, 1993). Ramadass *et al.*, (1992) reported genetic characterization of pathogenic *Leptospira* species by DNA hybridization. Spierings *et al.*, (1992) reported identification of *Klebsiella pneumoniae* by DNA hybridization and fatty acid analysis. Jarvis and Tighe, (1994) reported rapid identification of *Rhizobium* species based on cellular fatty acid analysis.

In the present study we employed four methods to characterize the soil bacteria expressing symbiotic genes. Background information on such methodology is described in the following subsections.

1. 8. 1 Ribosomal hybridization

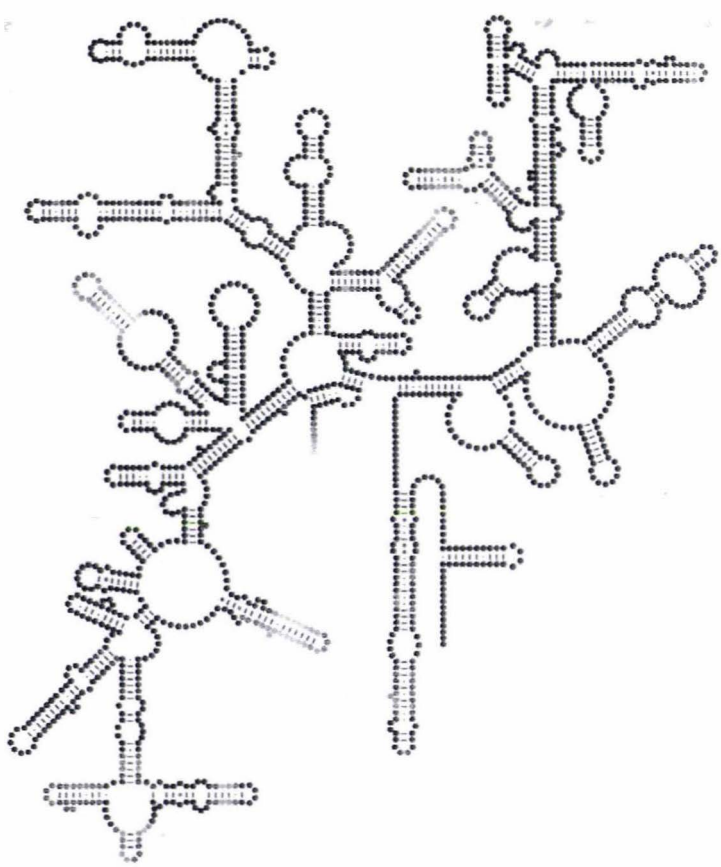
This method generally involves the use of ribosomal RNA (rRNA) (or the corresponding genes) as universal probes (Grimont and Grimont, 1986). Since rDNA loci contain both extremely conserved regions and variable regions, typing bacteria is achieved by the examination of restriction endonuclease patterns of DNA fragments containing rRNA genes (Grimont and Grimont, 1986).

Segovia *et al.*, (1991) used this method to identify eight non-symbiotic isolates (strains CFN402, CFN415, CFN426, CFN439, CFN449, CFN460, CFN469, and CFN478. He found the hybridization patterns of the non-symbiotic isolates were the same as those of *R. leguminosarum* biovar phaseoli type I strains. However this method has not been used extensively in identifying soil bacteria and studies on the natural variability of species are required.

1. 8. 2 16S rRNA sequence analysis

In the recent years the 16S rRNA (Figure 7) molecule has assumed a pivotal role in ascertaining the phylogentic relationships of bacteria (Young *et al.*, 1991). rRNAs are at present the most commonly used of the molecular chronometers (Woese, 1987). They show a high degree of structural and functional constancy, which assures relatively good clockwise behavior (Woese, 1982). They occur in all organisms, and different positions in their sequences change at very different rates, allowing most phylogenetic relationships to be measured. Their sizes are large and they consist of many domains. There are about 50 helical stalks in the 16S rRNA secondary structure and roughly twice that number in the 23S rRNA (Gutell *et al.*, 1985; Noller, 1984). Earlier phylogenetic conclusions with rRNA were based on hybridization (De Smedt and De Ley, 1977) or oligonucleotide catalogs (Woese *et al.*, 1983), but primary sequence information has now replaced such approaches (Lane *et al.*, 1985; Yang *et al.*, 1985).

Figure 7. Positional conservation representation of the 16S rRNA secondary structure derived from the comparison of 27 diverse eubacterial species. Shading intensity varies according to the relative conservation of each homologous nucleotide. Invariant positions are black (Stahl and Amann, 1991).



With the advent of the polymerase chain reaction (PCR) and techniques for the direct sequencing of the amplified DNA (Innes *et al.*, 1988; Winship, 1989), reliable sequences can be obtained rapidly (Böttger, 1989). PCR quickly and efficiently produces many copies of specific DNA regions. Standard DNA cloning methods achieve similar results, but with much greater effort. The main limitations of PCR are the size of the region that can be amplified, and the requirement for some knowledge of the sequences flanking the "target" DNA.

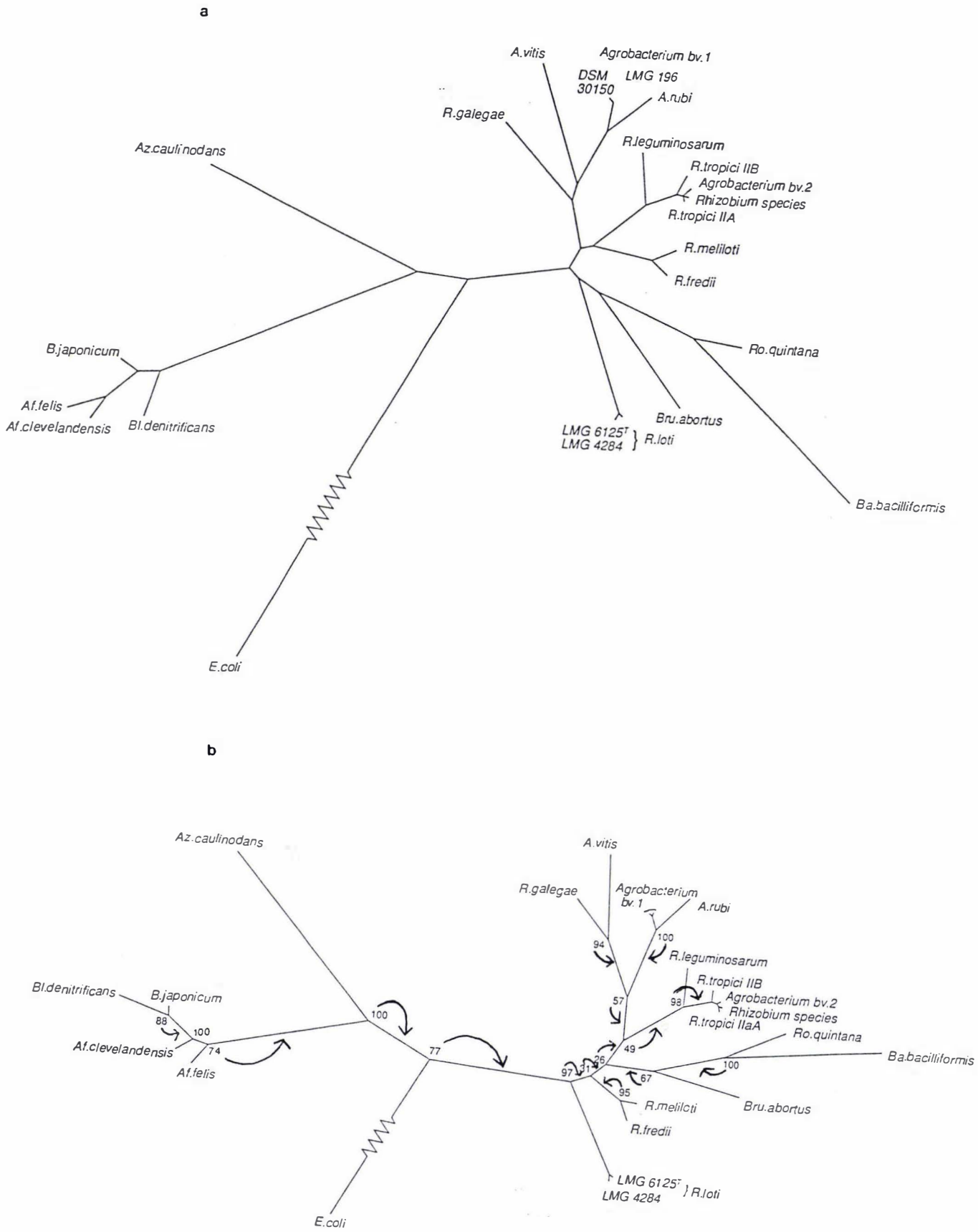
The requirement for sequence information about the target means that PCR is most useful for investigating DNA domains about which there is already partial knowledge, for instance, alleles of known genes. Much of the molecular systematics relies upon sequence comparisons among known genes, it is evident that the PCR is ideally suited for this purpose. In addition methods in nucleic acid sequencing have developed so rapidly in recent years that comparative sequencing of homologous genes is now a standard technique in molecular systematics and phylogenetic studies, in fact this is the most powerful method applied in bacterial systematics, (Ludwig, 1991). Nucleic acid sequence data are regularly compiled in several databases: GenBank (U. S. National Institutes of Health) is perhaps the best known and most widely used. Another well known data base is compiled by the European Molecular Biology Laboratory, (EMBL) (Hillis *et al.*, 1990).

Relationships between members of the *Rhizobiaceae* have been successfully delineated (Figure 8 a-d) using complete (Willems and Collins, 1993; Yanagi and Yamasato, 1993) and partial (Young *et al.*, 1991) 16S rRNA gene (rDNA) sequences. Sawada *et al.*, (1993) showed that the 15 strains of *Agrobacterium* and *Rhizobium* species formed a compact phylogenetic cluster clearly separated from the other members of the alpha subclass of the *Proteobacteria* (Figure 8d) based on using complete 16S rRNA sequences. This study aimed to identify soil bacteria expressing a symbiotic plasmid from partial 16S rRNA gene sequences by comparing the unknown sequences to known sequences in the databases and then constructing a phylogenetic tree.

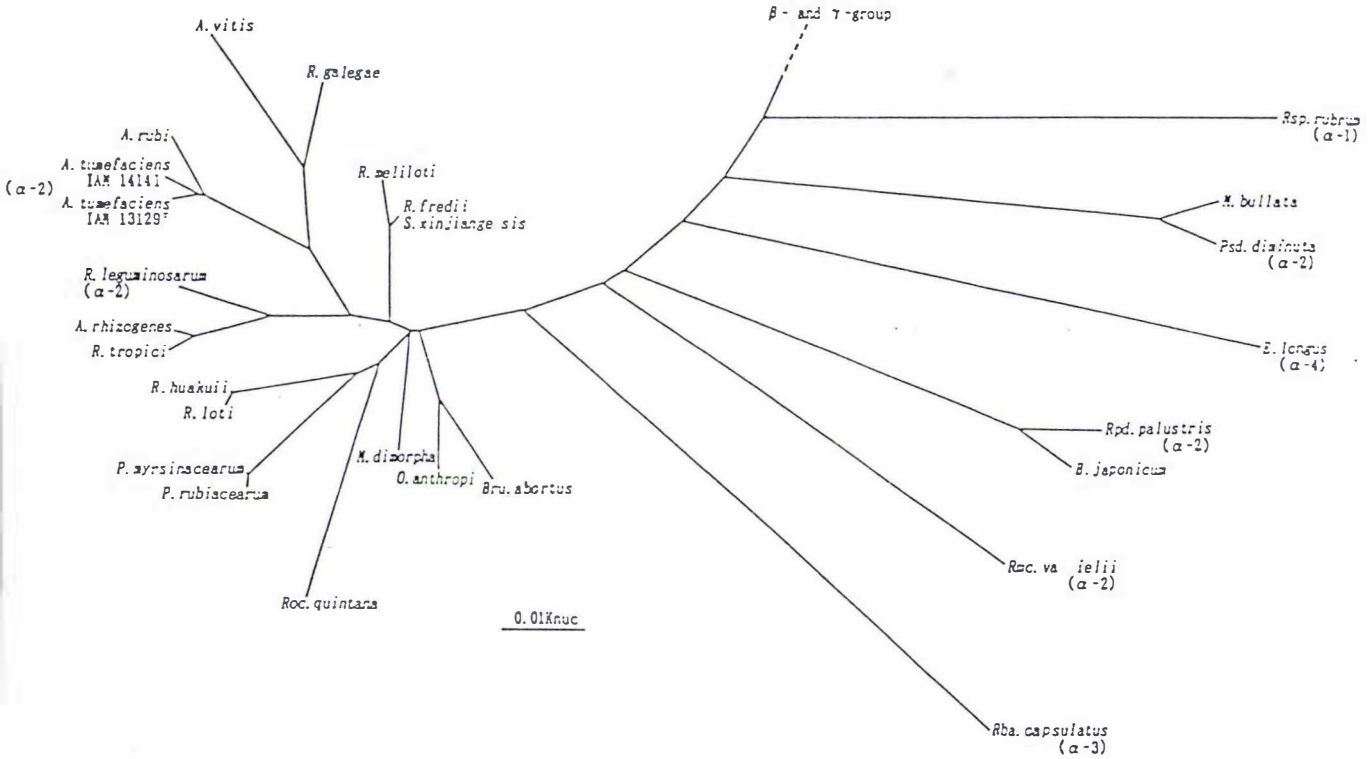
1. 8. 3 DNA-DNA hybridization

DNA-DNA relatedness based on DNA-DNA hybridization has been considered the standard method for the designation of bacterial species (Graham *et al.*, 1991; Wayne *et al.*, 1987).

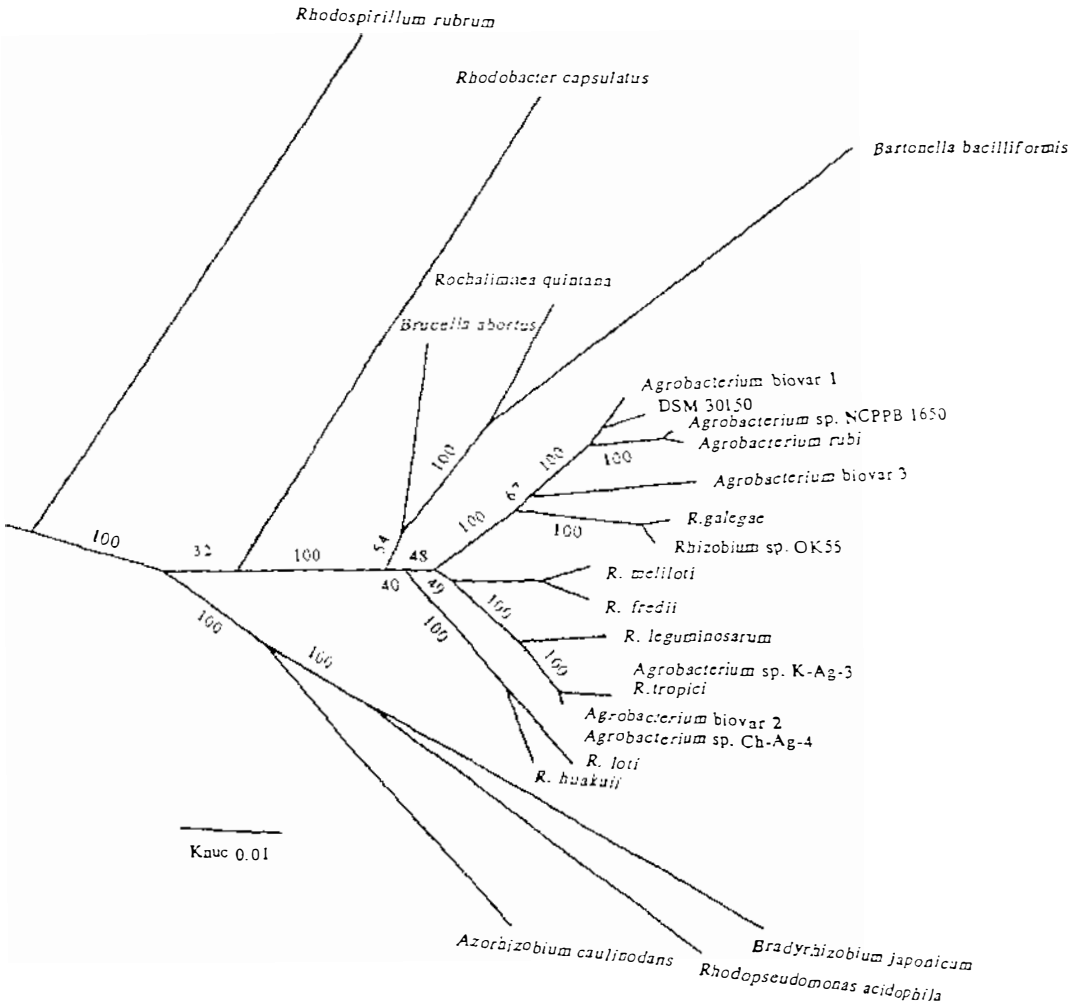
Figure 8. Phylogenetic tree derived from the whole 16S rRNA sequences. (a) Unrooted phylogenetic tree, obtained by Fitch and Margoliash analysis, showing the relationships of *Rhizobium* and *Agrobacterium* species and several related taxa from the alpha-2 subgroup of the *Proteobacteria*. *Af. clevelandensis*, *Afipia clevelandensis; Af. felis, Afipia felis; Ba. bacilliformis, Bartonella bacilliformis; B. japonicum, Bradyrhizobium japonicum; Bru. abortus, Brucella abortus; Ro. quintana, Rochalimaea quintana* (Willems and Collins, 1993). (b) Unrooted phylogenetic tree, obtained by parsimony analysis, showing the relationships of *Rhizobium* and *Agrobacterium* species and several taxa from the alpha-2 subgroup of the *Proteobacteria*. Bootstrap probabilities are inidicated at the branching points (Willems and Collins, 1993). (c) Phylogenetic tree of the members of the *Rhizobiaceae* and other bacteria. Genus abbrevations are as follows: *A., Agrobacterium; B., Bradyrhizobium*; *Bru., Brucella; E., Erythrobacter; M., Mycoplana; O., Ochrobactrum; P., Phyllobacterium; Psd., Pseudomonas; R., Rhizobium; Rba., Rhodobacter; Rmc., Rhodomicrobium; Roc., Rochalimaea; Rpd., Rhodopseudomonas; Rsp., Rhodospirillum; S., Sinorhizobium* (Yanagi and Yamasato, 1993). (d) Phylogenetic tree derived from the whole 16S rRNA sequences for members of the genera *Agrobacterium* and *Rhizobium* and their relatives (Sawada *et al.*, 1993). The value on each edge indicates the relative support in the data for that edge. Large values approaching 100 indicate there are largely uncontradicted patterns in the data supporting a particular grouping (Lockhart *et al.*, 1994).


a
A.vitis
Agrobacterium bv.1
DSM 30150
LMG 196
A.rubi
R.galegae
R.leguminosarum
R.tropici IIB
Agrobacterium bv.2
Rhizobium species
R.tropici IIA
Az.caulinodans
R.meliloti
R.fredii
B.japonicum
Ro.quintana
Af.felis
Af.clevelandensis
Bl.denitrificans
Bru.abortus
LMG 6125T
LMG 4284
R.loti
Ba.bacilliformis
E.coli
b
Az.caulinodans
A.vitis
R.galegae
Agrobacterium bv.1
A.rubi
100
94
R.leguminosarum
R.tropici IIB
Agrobacterium bv.2
Rhizobium species
R.tropici IIaA
57
98
Bl.denitrificans
B.japonicum
88
100
Af.clevelandensis
74
Af.felis
100
77
49
26
97
71
Ro.quintana
Ba.bacilliformis
100
67
95
R.meliloti
Bru.abortus
R.fredii
LMG 6125T
LMG 4284
R.loti
E.coli

c


A. vitis
R. galegae
A. rubi
A. tumefaciens IAM 14141
(α-2)
A. tumefaciens IAM 13129T
R. meliloti
R. fredii
S. xinjiange sis
R. leguminosarum (α-2)
A. rhizogenes
R. tropici
R. huakuii
R. loti
P. myrsinacearum
P. rubiscearum
M. diazotrophicus
O. anthropi
Bru. abortus
Roc. quintana
0.01Knuc
β- and γ-group
Rsp. rubrum (α-1)
M. bullata
Psd. diminuta (α-2)
E. longus (α-4)
Rpd. palustris (α-2)
B. japonicum
Rmc. va ielii (α-2)
Rba. capsulatus (α-3)

d


Rhodospirillum rubrum
Rhodobacter capsulatus
Bartonella bacilliformis
Rochalimaea quintana
Brucella abortus
Agrobacterium biovar 1
DSM 30150
Agrobacterium sp. NCPPB 1650
Agrobacterium rubi
Agrobacterium biovar 3
R.galegae
Rhizobium sp. OK55
R. meliloti
R. fredii
R. leguminosarum
Agrobacterium sp. K-Ag-3
R.tropici
Agrobacterium biovar 2
Agrobacterium sp. Ch-Ag-4
R. loti
R. huakuii
Azorhizobium caulinodans
Bradyrhizobium japonicum
Rhodopseudomonas acidophila
Knuc 0.01

Jarvis *et al.*, 1980 studied DNA relatedness among 27 strains of *Rhizobium trifolii*, 4 strains of *Rhizobium leguminosarum*, and 4 strains of *Rhizobium phaseoli* and indicated that DNA relatedness correlated with serological relationships and that the ability to form nodules on legume roots can be lost without a detectable change in relatedness with an independent reference strain.

Crow *et al.*, (1981) studied 122 strains of *Rhizobium* by DNA-DNA hybridization and identified four genetic groups of fast-growing, acid-producing rhizobia. Group 1 included strains of *Rhizobium trifolii* (except strains obtained from *Trifolium lupinaster*), *Rhizobium leguminosarum*, *Rhizobium phaseoli* (obtained from *Phaseolus vulgaris*), and two strains obtained from *Neptunia gracilis*. Group 2 comprised six American strains obtained from crown vetch (*Coronilla varia*), sainfoin (*Onobrychis vicifolia*), and *Sophora* spp. Group 3 corresponded with *Rhizobium meliloti*. Group 4 included fast-growing *Lotus* rhizobia, two strains of obtained from *T. lupinaster*, and a wide variety of previously unclassified strains. Nine fast-growing strains could not be included in any of these groups. In the present study a DNA reassociation technique was used to determine the identity of soil isolates expressing symbiotic genes and their agreement with the 16S rRNA sequence analysis.

1. 8. 4 Total cellular fatty acids

Within the past decade, cellular fatty acid profiles have been increasingly used to identify bacterial species (Alcorn *et al.*, 1991; Bobbie and White, 1980; Jarvis and Tighe, 1994; Kuykendall *et al.*, 1988; Lechevalier, 1977). Since the fatty acid profile of a bacterial species, analyzed as fatty acid methyl esters (FAMEs), is unique to that species, FAME analysis is an important identification tool (Foster and Fogleman, 1993). This is evidenced by the fact that the major fatty acids are included in many of the species descriptions in the ninth edition (1984) of *Bergey's Manual of Systematic Bacteriology* (Kreig and Holt, 1984). However this technique has not been widely used to characterize soil bacteria from naturally occurring environments.

1. 9 Summary: the nature of rhizobia

The legume-*Rhizobium* combination is the most significant in terms of global nitrogen fixation when compared to other nitrogen-fixing symbiosis between bacteria and plants. It is the best understood of all plant-microbe interactions (Palacios and Verma, 1988). Biologically, the two partners engage themselves in this extraordinary and agronomically important interaction is that both contain genes that are expressed only

in the presence of each other (Johnston, 1989). This leads to nodulation being regarded as the characteristic identifier of *Rhizobium* species and nitrogen fixation as a normal but not essential consequence of nodulation (Jordan, 1984).

However defining the genus *Rhizobium* by its capacity to nodulate is unsatisfactory because it usually depends on the presence of the Sym plasmids which can be lost in strains maintained on laboratory media (Jordan, 1984). It would be preferable to define rhizobia in terms of non-transmissible characteristics. In the present study independent means have been carried out to do just this.

Normally the primary isolation of *Rhizobium* strains is from nodulated legumes (Mozzo *et al.,* 1988; Schofield *et al.,* 1987; Vincent, 1970; Woomer *et al.,* 1988; Young, 1985) and this has tended to obscure relationships with other bacteria in the soil. Strains isolated from nodules on red (*Trifolium pratense*) and white (*Trifolium repens*) clover are all classified as *Rhizobium leguminosarum* biovar trifolii (Jordan, 1984), but they do exhibit considerable genotypic (Crow *et al.,* 1981; Jarvis *et al.,* 1980;) and phenotypic (Young, 1985; Young *et al.,* 1987) diversity. Recognized inoculum strains as sources of reference DNA showed optimal DNA homology and divergence in related sequences consistent with the presence of subspecies or perhaps multiple species by accepted criteria (Brenner *et al.,* 1982; Crow *et al.,* 1981; Jarvis *et al.,* 1980; Johnson, 1984). Young, (1985) and Young *et al.,* (1987) used enzyme polymorphism and showed a number of different lineages among *R. leguminosarum* biovars viceae, trifolii and phaseoli in soil and the distinct lineages could carry any of several different host-range determinants. They postulated that soil might contain bacteria which were electrophoretically indistinguishable from rhizobia but did not contain the symbiotic (Sym) plasmid and were therefore unrecognizable as rhizobia by conventional methods (Jarvis *et al.,* 1989; Young, 1985). The design of the present project was intended to characterize any such species.

1. 10 Aims of the study

It was hypothesized that soil contains a diverse group of bacteria not necessarily rhizobia which can express pSym. To test this hypothesis different soil types with varying flora and fauna were examined and non-nodulating bacteria which could express a Sym plasmid were characterized. The approaches taken to do this is listed below, detailed in the materials and methods section and summarised in Figure 9.

1) Isolation of Gram negative bacteria unable to nodulate *Trifolium repens* (white clover) cultivar Grasslands Huia from different soil types,

2) Transfer symbiotic genes from *Rhizobium leguminosarum* biovar trifolii by conjugation to soil bacteria,

3) Obtain expression of the symbiotic plasmid in a plant test,

4) Examine any root nodules formed by light and electron microscopy,

5) Examine the plasmid composition of those bacteria which could express pSym

6) Verify the transfer of pSym using a (α-^{32}P) labelled probe and

7) characterize and identify soil bacteria which could express pSym by the following methods:
i) Ribosomal hybridization,
ii) 16S rRNA sequence analysis,
iii) DNA-DNA hybridization and
iv) Total cellular fatty acids.

Figure 9. Summary of the project.

How diverse is the population of soil bacteria which can express a symbiotic plasmid from *Rhizobium leguminosarum* biovar trifolii ?

samples from four soil types

↓ ↓

↓ ↓

↓ ↓

discard Gram negative bacteria which nodulate white clovers ←←←← select for non-nodulating Gram negative soil bacteria

↓

↓

↓

discard strains which do not express the symbiotic plasmid pSym ←←←← select transconjugants which accept a symbiotic plasmid pSym by conjugation

↓

↓

↓

confirm the presence of symbiotic genes in the transconjugants which express pSym

↓

↓

↓

characterization of transconjugants

↓

↓

RNA fingerprinting
DNA-DNA hybridization
16S rRNA sequencing
Complete fatty acid analysis

↓

↓

Conclusions

ERRATUM

Page	Line	Incorrect text	Correct text
43	14	Mercury	Mercuric
44	1	transconjugant	transconjugation
64	11 & 32	gel is	gel was
64	26	gel becomes	gel became
64	29	as it is	as it was
64	34	reflect	reflected
67	23	fatty acids separated	fatty acids were separated

2. MATERIALS AND METHODS

2. 1 Bacterial strains and plasmids used in this study

The bacteria and plasmids used in the investigation are listed in Table 2.

2. 2 Growth of bacteria

Soil isolates were grown at 28°C in YMG (section 2. 3. 1) or SE (section 2. 3. 3) or TY (section 2. 3. 5) supplemented where necessary with Kanamycin (Km; 50 μg/ml) or Rifampicin (Rif; 50 μg/ml) or Kan; 50 μg/ml + Rif; 50 μg/ml. *Rhizobium* cultures were grown at 28°C in YMG (section 2. 3. 1) or TY (section 2. 3. 5). *Escherichia coli* cultures were grown at 28°C in LB (section 2. 3. 4) or TY (section 2. 3. 5) supplemented where necessary with Km; 50 μg/ml or Rif; 50 μg/ml or Km; 50 μg/ml + Rif; 50 μg/ml.

2. 3 Media used in the investigation

All media used in this study were sterilized at 121°C (15 psi) for 15 minutes unless otherwise indicated. Solid media were cooled to 55°C before pouring.

2. 3. 1 Yeast-mannitol-glucose (YMG) agar (Vincent, 1970) contained (g/l): Mannitol (Difco), 10.0; Yeast extract (Difco), 0.5; Dipotassium phosphate (K_2HPO_4), 0.5; Magnesium sulphate ($MgSO_4.7H_2O$), 0.2; Sodium chloride, 0.1; Glucose, 1.0. The pH was adjusted to 7.0. Solid media was obtained by adding agar (15 g/l, Davis).

2. 3. 2 Soil extract (Pramer and Schmidt, 1965) was prepared from 1000 g of fertile soil which was added to 1000 ml of tap water and autoclaved at 121°C (15 psi) for 20 min. Calcium carbonate (0.5 g) was added to flocculate colloidal material and the suspension filtered to clarify it.

2. 3. 3 Soil extract (SE) agar (Pramer and Schmidt, 1965) contained (g/l): Glucose, 1.0; Dipotassium phosphate (K_2HPO_4), 0.5; Potassium nitrate (KNO_3), 0.1; Soil extract (section 2. 3. 2), 100 ml; Water, 900 ml (Milli-Q Reagent water System, Millipore continental water systems); The pH was adjusted to 6.5-7.0. Solid media was obtained by adding agar (15 g/l, Davis).

Table 2. Bacterial strains and plasmids used in this study

Bacterial strains and plasmids	Relevant characteristics	Source or reference
Rhizobium leguminosarum biovar trifolii ICMP2163 (NZP561)	Nod^+Fix^+	DSIR culture collection (Palmerston North, New Zealand)
NZP514	Nod^+Fix^+	DSIR culture collection
ATCC10004	Nod^+Fix^+	ATCC
ATCC14480	Nod^+Fix^+	ATCC
PN165	Sym plasmid cured derivative of NZP 561	Ronson and Scott, 1983
Rhizobium meliloti NZP2011	effective nodules on *Medicago sativa*	E.A.Terzaghi Massey University culture collection, Palmerston North, New Zealand
Rhizobium loti ATCC33669 (NZP2213)	effective nodules on *Lotus corniculatus*	D B Scott
Rhizobium tropici CFN299	effective nodules on *Phaseolus vulgaris*	Martinez *et al.*, 1987
Escherichia coli ATCC9637	Source of non-rhizobial DNA	ATCC
PN200	HB101 (pPN1)	Ronson and Scott, 1983
PN291	RR1 (pRt572)	Schofield *et al.*, 1983 and Scott *et al.*, 1982
PN600	HB101 (pPN26)	D.B.Scott

Bacterial strains and plasmids	Relevant characteristics	Source or reference
PN298	HB101 (R68.45)	D.B.Scott
Agrobacterium tumefaciens C58		C.Kado
Soil bacteria		
NR40	Gram-negative rod	Rao *et al.*, 1994
NR41	Gram-negative rod from *Rhizobium*-like colony on YMG	Jarvis *et al.*, 1989
NR42	as for NR41	Jarvis *et al.*, 1989
NR64	as for NR41	Jarvis *et al.*, 1989
OR168	Gram-negative rod	Jarvis *et al.*, 1989
KJ1	Gram-negative rod	This work
KJ3	Gram-negative rod	This work
KJ5	Gram-negative rod	This work
KJ13	Gram-negative rod	This work
KJ17	Gram-negative rod	This work
KJ19	Gram-negative rod	This work
KJ23	Gram-negative rod	This work
KJ26	Gram-negative rod	This work
KJ27	Gram-negative rod	This work
KJ30	Gram-negative rod	This work
KJ44	Gram-negative rod	This work
KJ57	Gram-negative rod	This work
KJ203	Gram-negative rod	This work

Bacterial strains and plasmids	Relevant characteristics	Source or reference
Plasmids		
pBR328	$Ap^rTc^rCm^r$	Bolivar *et al.*, 1977
R68.45	$Km^rTc^rCb^rIncP$	Haas and Holloway, 1976
pRtr514a	Nod^+ symbiotic plasmid from *Rhizobium leguminosarum* biovar trifolii NZP514	Skot *et al.*, 1986
pPN1	pRtr514a::R68.45	Scott and Ronson 1982
pKK3535	rRNA operon	Brosius *et al.*, 1981
pRt572	*R. leguminosarum* biovar trifolii ANU843 7.2 Kb *Eco*R I *nod* fragment cloned in pBR328	Schofield *et al.*, 1983, Scott *et al.*, 1985
pLAFR1	lambda *cos* derivative of pRK290	Friedman *et al.*, 1982
pPN26	pLAFR1 cosmid containing *nod* region of PN100	Scott *et al.*, 1985

Ap, ampicillin, Cm, chloramphenicol, Km, kanamycin, Tc, tetracycline; r : resistant; Nod^+Fix^+: effective (nitrogen-fixing) nodule.

2. 3. 4 Luria broth (LB) (Miller, 1972) contained (g/l): Tryptone (Difco), 10.0; Yeast extract (Difco), 5.0; Sodium chloride, 0.5. The pH was adjusted to 7.0. Solid media was prepared by adding agar (15 g/l, Davis).

2. 3. 5 Tryptone-yeast extract (TY) agar (Beringer, 1974) contained (g/l): Tryptone (Difco), 5.0; Yeast extract (Difco), 3.0; Calcium chloride, 1.3. The pH was adjusted to 7.0. Solid media was obtained by adding agar (15 g/l, Davis).

2. 3. 6 Hogland's trace element solution (Hoagland and Arnon, 1938) contained (g/l) H_3BO_3, 2.86; $MnCl_2.4H_2O$, 1.81; $ZnSO_4.H_2O$, 0.22; $CuSO_4.H_2O$, 0.08; $CoSO_4.7H_2O$, 0.095; $Na_2MoO_4.2H_2O$, 0.054.

2. 3. 7 Seedling agar (Thornton, 1930) contained (g/l): Calcium phosphate (Tribasic) ($Ca_3(PO_4)_2$), 2.0; Dipotassium phosphate, 0.5; Magnesium sulphate ($MgSO_4.7H_20$), 0.2; Sodium chloride (NaCl), 0.1; Ferric chloride anhydrous ($FeCl_3$), 0.017; Hogland's trace element solution, 1 ml (section 2. 3. 6); Agar, 15.0. Ten ml of molten seedling agar was dispensed into test tubes (20 x 150 mm), sterilized and inclined to solidify in order to present a 5-10 cm long agar face for seedling growth.

2. 3. 8 Tryptone-yeast extract agar (TYEA) (Jarvis and Tighe, 1994) contained (g/l) Bacto-tryptone yeast extract ISP medium 1, 8.0; $CaCl_2.2H_20$, 0.87; d-mannitol, 1.0; Agar, 15.

2. 4 Isolation of soil bacteria

Soil cores 70 to 100 mm long were collected from four different locations and soil types namely Rahima silt loam, Tokomaru silt loam, Kairanga silt loam and Manawatu sandy loam. The soils were placed in different plastic bags and stored at 4°C until they were cultured. Samples were diluted in 0.05% tryptone broth and immediately placed on yeast mannitol glucose (YMG) agar (section 2. 3. 1) or on soil extract (SE) agar (section 2. 3. 3). Cultures were incubated for five to seven days at 28°C. Isolated colonies were restreaked on YMG or SE agar and reincubated.

2. 4. 1 Preservation of bacterial cultures

Single colonies were picked and cultures were maintained on YMG or SE at 4°C or as cell suspension in TY broth (section 2. 3. 5) containing 50% glycerol at -20°C. Most frequently the "Protect" (Technical Service Consultants Ltd.) system was used. Single colonies were picked from solid media using a sterile loop and a thick suspension was made in the cryopreservative fluid provided. In the case of liquid media, cultures

were lightly centrifuged to obtain a cellular deposit and this was used to make a thick suspension. The tube ("Protect") was capped and inverted briskly at least ten times and using sterile pipette as much cryopreservative fluid was withdrawn, identified and stored at -20°C or -70°C.

2. 4. 1. 1 Recovery of bacterial cultures

A sterile inoculating needle was used to recover the cultures from the test tubes (50% glycerol in TY (section 2. 3. 5) broth. Fine sterile forceps were used to remove a single bead from the "Protect" and rub it over a portion of a suitable solid medium (section 2. 3). The portion was streaked with a sterile loop to obtain discrete colonies and the plate incubated at the appropriate temperature (section 2. 2). For some organisms the bead was placed in a suitable liquid medium (section 2. 3) and incubated before subculture.

2. 5 Plasmid isolation by Eckhardt method

The detection of plasmid pPN1 was carried out by the Eckhardt procedure (Eckhardt, 1978) with minor modifications described below.

Materials

Tris-borate electrophoresis (TBE) buffer pH 8.2: 89 mM Tris (hydroxymethyl) methylamine; 89 mM boric acid; 2.5 mM Na_2EDTA (Ethylenediamintetra-acetic acid; disodium salt), a 10 times concentrated stock solution was prepared by dissolving 107.8 g of Tris, 55 g of boric acid and 9.3 g of Na_2EDTA in one liter of deionized water (Milli-Q water). 1 x TBE: 100 ml of 10 x TBE was added to 900 ml of deionized water and adjusted to pH 8.2 with diluted HCl. TE Buffer pH 8.0: 10 mM Tris, 1.21 g, EDTA 1 mM, 0.372 g; Milli-Q water, 1 l. RNAse: A 100 mg/ml RNAse solution was made up in sterilized 0.4 M Sodium acetate pH 4.0 and placed in a boiling hot water bath for two minutes, dispensed 200 µl aliquots into Eppendorf tubes and stored at -20°C until use. Sarkosyl solution pH 8.0: 10 mM Tris, 1.21 g, EDTA 1 mM, 0.372 g, N-Lauryl sarcosine, 1.0 g; Milli-Q water, 1 l. Solution 1 was freshly prepared before each experiment: 10% Ficoll 400,000 (Sigma), 2.0 g; 0.05% Bromophenol blue, 0.01 g; Lysozyme powder, 20.0 mg (1 mg/ml); RNAse (1 mg/ml), 200 µl; 1 x TBE pH 8.2, 20.00 ml. Solution 2: 0.2% SDS, 0.02 g; 10% Ficoll 400,000; Proteinase K 50.00 mg (5 mg/ml) 1 x TBE pH 8.2, 10.00 ml, dispensed 500 µl aliquots into Eppendorf tubes and stored at -20°C until required. Solution 3: 0.2% SDS, 0.04 g; 5% Ficoll 400,000; 1 x TBE pH 8.2 20.00 ml, stored at 4°C until required. 0.8% (w/v) Agarose in 1 x TBE pH 8.0. Ethidium bromide solution, 0.5 µg/ml. 0.1% Magnesium sulphate. Horizontal

gel electrophoresis system (Horizon 11.14), BRL. BIO-RAD model 500/200 power supply. U.V.Transilluminator. Polaroid type 667, 665 or Kodak Tri-X pan professional film.

Method

A single colony isolate was picked from a plate, inoculated into 5 ml of TY (section 2. 3. 5) or LB broth (section 2. 3. 4) and incubated in a Controlled Environment Incubater Shaker (CEIS) (New Brunswick Scientific); 28°C; 200 rpm for 48 hour. Fifty µl of a 48 hour culture was inoculated into 5 ml TY (section 2. 3. 5) or LB broth (section 2. 3. 4) and incubated under same conditions for 18 to 24 hours. Cells were grown to the log phase (OD_{600} = 0.4 = 18 to 24 hours). Then 100 µl of cells were aliquoted into an Eppendorf tube and harvested by centrifugation (Eppendorf centrifuge 5414S; 15,600 x G; 5 min). The cell pellet was washed once with Sarkosyl solution (Schwinghamer, 1980), and once in 1 ml of TE and drained well. The cell pellet was resuspended in 20 µl of Solution 1 and immediately loaded into the well of 0.8% agarose gel (Horizon 11.14) and incubated for 15 minutes at room temperature. 20 µl of Solution 2 was layered over the Solution 1 in the same well, the two layers were gently mixed with a fine blunt glass rod by stirring once, and the well sealed with agarose. Plasmid DNA was separated by electrophoresis at 20 volts for 1 hour and 80 volts for 18 hours in the cold room (4°C). After electrophoresis the gel was stained for 15 minutes in ethidium bromide and washed with 0.1% magnesium chloride. The gel was photographed using an UV transilluminator. The molecular weight of pPN1 was determined by comparing its relative mobility with that of the two plasmids present in *Agrobacterium tumefaciens*, strain C58, pTi-C58, 130 Mdal (Holsters *et al.*, 1978) and pAt-C58, 275 Mdal (Denarie *et al.*, 1981).

2. 5. 2 Bacterial conjugation with pPN1

The nodulation genes from *Rhizobium leguminosarum* biovar trifolii to soil bacteria were transferred by conjugation using the methods of Beringer *et al.*, (1978) and Buchanan-Wollaston *et al.*, (1980) with slight modifications mentioned below.

Materials

95% Ethanol (for surface sterilization of Laminar flow). Sterile TY: section 2. 3. 5 and LB: section 2. 3. 4 agar medium. Sterile antibiotic plates: section 2. 2. Sterile 0.05% tryptone broth. Membrane filters cellulose nitrate (pore size 0.2 µm and diameter 25

mm). Spectronic 20 (Bausch and Lomb). Airpure (Laminar flow)-biological safety cabinet class II (EMAIL).

Method

Single colony isolates of an overnight culture of *Escherichia coli*, strain PN200 containing pPN1 and a 1-2 day culture of a soil bacterium were inoculated in 10 ml TY broth (section 2. 3. 5) or LB broth (section 2. 3. 4) in Kimax tubes and incubated in a CEIS (Controlled Environment Incubater Shaker) at 28°C, at 200 rpm. The OD_{600} of the cultures was measured and adjusted so that the OD of the donor and recipient was between 0.6 and 1.0. A Membrane filter was placed on the middle of a TY agar plate and 50 µl of recipient cell suspension spotted on it and allowed to dry in a laminar flow cabinet. Care was taken not to over dry the spot. Using a Pipetman (Gilson) or Battery-operated motorized pipette, (EDP2, Rainin) 50 µl of donor PN200 was spotted on to the recipient and allowed to dry in the cabinet. The TY plate was incubated overnight at 28°C. At the same time control plates were set up by plating 100 µl of recipient and donor separately on TY with Rif; 50 µg/ml, Kan; 50 µg/ml, and Rif; 50 µg/ml + Kan; 50 µg/ml.

2. 5. 2. 1 Selection of soil bacteria expressing nodulation genes

Selection of soil bacteria expressing nodulation genes was carried out by two methods. In the first method the crosses were plated on suitable antibiotic media where only recipient soil bacteria which had received the co-integrate plasmid could grow on the antibiotic media. Presence of the symbiotic plasmid was confirmed by the recipients ability to nodulate white clover (*Trifolium repens*) cultivar Grasslands Huia. In the second method the tranconjugant mixture was directly inoculated on to white clover seedlings to confirm the expression of the co-integrate Sym plasmid.

2. 5. 2. 1. 1 Plating crosses to select for transconjugants on antibiotic media

The membrane filter was removed from TY agar and the growth resuspended in 10 ml of sterile deionized water. The undiluted suspension (100 µl) was plated separately on five plates containing: TY with Kan; 50 µg/ml (donor resistant), Rif; 50 µg/ml (recipient resistant) and Kan; 50 µg/ml + Rif; 50 µg/ml (selective antibiotic for transconjugants) respectively. 10 fold dilutions of the cell suspension were made in 0.05% tryptone water between 10^{-1} and 10^{-6}. From the 10^{-1} and 10^{-2} dilutions 100 µl was plated on TY medium containing the selective antibiotics (Kan; 50 µg/ml + Rif; 50 µg/ml and from the 10^{-3} to 10^{-6} dilutions 100 µl were plated on the selective

antibiotic to which the recipient was resistant (TY medium with Rif; 50 μg/ml). All plates were incubated at 28°C for 2-6 days. Transconjugants were picked by observing the plates daily and maintained (section 2. 4. 1) for further study. Frequency of transfer of the antibiotic marker (pRtr514a::R68.45) was calculated as the number of transconjugants expressing Kanamycin and Rifampicin resistance or nodulation genes as a proportion of the total recipients.

2. 5. 2. 1. 2 Nodulation test to confirm the expression of nodulation genes

Plant tests were conducted to confirm the expression of nodulation genes in the recipient soil bacteria by using the method of Vincent, (1970) with slight modifications described below. Clover seeds from *Trifolium repens* (white clover) were sterilized and allowed to grow in the dark for two days on Thornton's seedling agar in order to get a healthy root system for inoculation.

Materials

Methanol. 95% Ethanol. Mercury chloride: 0.1% in deionized water. Sterile water: deionized by Milli-Q sytem (Millipore) and autoclaved. Water agar: 1%. TY plates: section 2. 3. 5. Seeds of *Trifolium repens* (white clover) cultivar Grasslands Huia. Thornton's seedling agar slopes: section 2. 3. 7. Glass tube 20 x 150 mm. Temperature-controlled room: set to 22°C, artificial lighting 550 mmol photons $m^{-2}s^{-1}$ (Einsteins) with a 12 hour photoperiod. Wooden blocks.

Method

White clover seeds in a 25 ml bijou bottle were immersed in methanol for 10 sec. The methanol was removed with a sterile autopipette and 15 ml of 0.1% mercuric chloride added for 10 min. The mercuric chloride was removed and the surface sterilized seeds thoroughly washed in successive changes (ten) of sterile water. To check the affect of this surface sterilization technique, a seed was rolled on a TY plate. No growth around the sterilized seed showed that the method was affective in eliminating or greatly reducing contamination on the seed surface. The sterile clover seeds were transferred to water agar plates with a sterile cooled loop and incubated in the dark for about 24 hours to allow the seeds to germinate and form a small healthy root. Germinated seeds were placed on slopes of the seedling agar, inserted in wooden blocks, kept in the dark for two days and used for the following procedures. In initial screening experiments, three seedlings were inoculated with each soil bacterium or with *Rhizobium leguminosarum* biovar trifolii strain ICMP2163 or *Escherichia coli* (strains PN200 or

ATCC9637) as positive and negative controls, respectively. In transconjugant experiments (section 2. 5. 2. 1) ten seedlings were used for each transconjugant or tranconjugation mixture (section 2. 5. 2. 1) and for each donor and recipient strain. *Rhizobium leguminosarum* biovar trifolii strain ICMP2163 was used as a positive control. All bacterial cultures used in the plant tests were diluted to 10^{-6} dilution and 1 ml was inoculated on the roots of two day old clover seedlings on seedling agar slants (section 2. 3. 7). All tubes were inserted in wooden blocks and grown under controlled environment conditions at 22°C under artificial light 550 mmol photons $m^{-2}s^{-1}$ (Einsteins) with a 12 hour photoperiod. Plants were examined for nodule number, shoot length and colour at regular intervals. Once the nodules were mature enough (usually after 4-6 weeks of incubation), bacteria were isolated from within the nodule for further study.

2. 6 Light and electron microscopy of nodules (Pankhurst *et al.*, 1979)

Light and electron microscopy of nodules was used to examine the extent and nature of bacteriod colonization of nodule tissues. Root nodules and nodule-like structures removed from the roots of plants with a scalpel blade were sectioned, stained and examined using the methods of Pankhurst *et al.*, (1979). Sections were prepared at the HortResearch Electron Microscope Unit, Batchelar Research Centre, Palmerston North, New Zealand. In light microscopy the sections were photographed using a Reichert "Zetopan" research microscope fitted with a Reichert "photo-automatic" camera and for electron microscopy the sections examined in a Phillips EM200 electron microscope at 80 KV.

2. 7 Isolation of bacteria from the nodules

Bacteria from the nodules were isolated when the nodules were about 4-6 weeks old by the method of Vincent, (1970) with modifications described below.

Materials

Clover seedling bearing nodules: section 2. 5. 2. 1. TY broth and TY plates: section 2. 3. 5). 95% Ethanol. Mercuric chloride: 0.1% in deionized water. Sterile scalpel blade. Petri dishes. Sterile blunt-ended glass rod. Sterile inoculating needle. "Protect" bacterial preservers: section 2. 4. 1.

Method

The nodules were isolated from the plant root by excision with a sterile scalpel blade leaving behind a small piece of root attached to the nodule for easy handling. Fifteen petri dishes were prepared in a laminar flow cabinet, as follows: dish one, contained 95% ethanol; dish 2, 0.1% mercuric chloride; dishes 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 and 15 sterile water. The nodules were surface sterilized by immersing the nodule in 95% ethanol for 10 seconds and then immersing it in 0.1% mercuric chloride for 10 minutes. The nodules were thoroughly washed in successive changes (13) of sterile water and aseptically transferred to a sterile petri dish which had 50 µl drops of sterile TY on it. Each nodule was placed in a drop and crushed with a sterile blunt-ended glass rod, thus releasing the nodule bacteria into the TY medium. Each drop of TY (with it's cell suspension) was transferred to a TY plate with a sterile loop, and streaked for single colony growth. The plates were incubated (section 2. 2) and the cultures preserved (section 2. 4. 1) for further study.

2. 8 Genomic DNA preparation

Two methods were used to isolate genomic DNA. The first was the modified method of Fisher and Lerman, (1979) as described below for restriction endonuclease digests, DNA-DNA hybridization, rRNA Fingerprinting and α-^{32}P labelling of DNA. The second was simplified procedure of Jarvis *et al.*, (1992) for DNA amplification.

2. 8. 1 Total genomic isolation by the modified methods of Fisher and Lerman, (1979)

Materials

TE buffer: 10 mM Tris (pH to 8.0 by adding conc HCl); 1 mM EDTA. 1.0 M NaCl. TE_{25} buffer: 10 mM Tris (pH to 8.0 by adding conc HCl); 25 mM EDTA. TES buffer: 10 mM Tris (pH to 8.0 by adding conc HCl); 25 mM EDTA; 150 mM NaCl. Sarkosyl (Sodium-n-lauroylsarcosine)/protease: 10% Sarkosyl and 5 mg/ml Protease (Type XIV from *Streptomyces griseus*) in TE_{25}, was freshly prepared and incubated for an hour at 37°C to get rid of nucleases which would interfere with the DNA isolation. Lysozyme 2 mg/ml in TE_{25}, was freshly prepared before adding. RNAse stock solution: 10 mg/ml DNAse-free RNAse in deionized sterile water, freshly prepared and boiled for 5 minutes to get rid of DNAse. Phenol: 500 ml of good quality solid phenol was taken, to which 300 ml of warm 25 mM NaCl was added, the NaCl had been previously heated to boiling before addition to the phenol, 10 g of Tris-base (Sigma), and 0.9 g of 8-hydroxyquinoline was added, the tris-equilibrated phenol was stored at 4°C, pH 7.8. Chloroform. Sodium acetate: 3 M sodium acetate (pH 5.2). Isopropanol. 70% Ethanol.

TY broth: section 2. 3. 5 or LB broth: section 2. 3. 4. Controlled Environment Incubator Shaker (CEIS): 28°C; 200 rpm. Dessicator and vacuum system (Lab-Line Duo-Vac Oven, Lab-Line Instrument Inc.). Water bath (Smith-Biolab Ltd.). Bench top centrifuge (Megafuge 1.0, Heraeus Sepatech). Fume hood. Sterile Falcon tubes. Sterile pipettes.

Method

A volume of 40 ml of freshly grown cells (18-24 hours) in LB (section 2. 3. 4) or TY (section 2. 3. 5) was centrifuged at 6,240 x G for 10 min in a Falcon tube. The cell pellet was resuspended in 1.0 M NaCl and put on a shaker in the cold for 30 min. The cells were centrifuged at 6,240 x G for 10 min and the cell pellet was resuspended and washed in 25 ml TES. The cells were centrifuged again at 6,240 x G for 10 min. The cell pellet was resuspended in 5 ml TE buffer, 0.5 ml of freshly prepared lysozyme solution were added and the cells incubated at 37°C for 15 min for cell lysis. DNAse-free RNAse was also added to a final concentration of 10 μg/ml to get rid of RNA. Sarkosyl/protease (0.6 ml) was added and the solution incubated for 1 hour at 37°C to remove proteins. The viscous lysate was extracted by adding an equal volume of phenol and inverting the tube several times with gentle shaking to mix the phases. The lysate/phenol solution was centrifuged at 6,240 x G to separate the two phases. The top (aqueous) phase was removed with a 1-5 ml pipette, and transferred to a clean Falcon tube. A minimum of interphase material was transferred into the clean tube, as this was contaminating material to be avoided. The 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 with chloroform to remove traces of phenol, and the lysate/chloroform mixture was centrifuged at 6,240 x G for 10 min to separate the phases. The aqueous phase was transferred into a sterile 25 ml bijou bottle and the volume estimated. One ninth of that volume of 3 M Na-acetate was added and mixed well. To this mixture 0.6 volume of isopropanol was added and swirled gently to precipitate the DNA. The DNA was wound onto a hooked glass rod, washed in 1 ml of 70% ethanol and dried for 5-10 minutes. The DNA was dissolved in 1 ml of TE in a sterile Eppendorf and stored it at 4°C or -20°C (temporarily) or -70°C (long-term). The DNA concentration was measured using Unicam SP1800 Ultraviolet spectrophotometer, (Pye Unicam) (section 2. 9).

2. 8. 2 Rapid method for genomic DNA isolation (Jarvis *et al.*, 1992)

Materials

TY slope: section 2. 3. 5. TEL buffer: 50 mM Tris; 20 mM EDTA. TE buffer: 10 mM Tris; 1 mM EDTA. Phenol: to 1 kg phenol added 110 ml deionized water; 140 ml m-Cresol; 1 g 8-hydroxyquinoline, mixed overnight on a magnetic stirrer and saturated overnight with 0.1 M Tris pH 8.0 (stored in brown bottle). Diethyl ether. 100% Ethanol. Protease Type XIV: 5 mg/ml in TE buffer. 10% SDS. 3 M Sodium Acetate: pH 4.85. 70% Ethanol. Sterile Falcon tubes, bijous and pipettes. Fume cupboard. Centrifuge.

Method

Bacterial cells grown on a TY slope in a Universal bottle (25 ml) were washed off with 3.0 ml of TEL buffer and the suspension was collected in a sterile Falcon tube. Protease (375 µl) and 10% SDS (375 µl) were added to the suspension and left on ice for 30 min and on the bench for 30 min to lyse the cells and to remove proteins. Phenol 10-15 ml was added to the tube which was capped tightly, inverted 25 times and centrifuged at 6,240 x G to separate the two phases. The aqueous phase (upper phase) was transferred to a clean sterile Universal bottle (25 ml), extracted twice with 3 ml diethyl ether and allowed to stand to separate the two phases. The aqueous phase (lower phase) was transferred to a clean sterile baby bijou and 200 µl of 3 M Sodium acetate was added and mixed well gently. The DNA was precipitated by adding 100% Ethanol (4°C). The DNA was recovered with a disposable Pasteur pipette, washed in 70% Ethanol and dried to remove traces of Ethanol which would interfere with *Taq* polymerase. The DNA was dissolved in 200 µl of TE buffer and stored at 4°C, -20°C or -70°C. The DNA concentration was measured using UV Spectrophotometer (section 2. 9).

2. 9 Determination of DNA purity and concentration

DNA purity and concentration were measured spectrophotometrically. Dilute solutions were measured undiluted in 0.5 ml quartz cuvettes with 1 cm path length. More concentrated solutions were diluted 1:20 and measured in a 1 cm quartz cuvette. The purity was determined at 230 nm, 258 nm and 280 nm. The following spectral ratios were regarded as satisfactory A_{258}/A_{230} (indication of protein contamination), 1.8-2.3 and A_{258}/A_{280} (indication of phenol or protein contamination), 1.8-2.0 (Jarvis *et al.*, 1986). DNA concentration was determined at 258 nm and 300 nm using extinction coefficients of 20 for unsheared DNA and 24 for sheared DNA for 1 mg/ml DNA solution (Brenner and Falkow, 1971). The final DNA concentration was calculated using formula: DNA concentration (mg/ml) = $A_{258} - A_{300}/20$ x dilution factor.

2. 10 Restriction endonuclease digests (Maniatis *et al.*, 1982)

DNA can be cleaved at specific sites with restriction enzymes. Fragments can be separated on an agarose gel by electrophoresis and latter transferred to a nylon membrane for probing. DNA is also cleaved for the preparation of template in solution or for recovery from low-melting-point-agarose and labelling with α-^{32}P for use as a specific probe.

Materials

1-2 µg DNA. Restriction endonucleases: *Eco*R I (Gibco, BRL) or *Hind* III (Gibco, BRL; Promega; BioLabs; Boehringer Mannheim). 10X concentrate assay buffer (Gibco, BRL; Promega; BioLabs; Boehringer Mannheim). Microcentrifuge. Water bath set at 37°C.

Method

Genomic (1-2 µg) DNA and 2 µl of 10X concentrate assay buffer (Final concentration: 50 mM Tris-HCl (pH 8.0); 10 mM $MgCl_2$; 100 mM NaCl) were made up to 19 µl with H_2O and 1 µl (10 U/ µl: One unit is the amount of enzyme required to cleave 1 µg of lambda DNA in one hour at 37°C in the appropriate buffer) of restriction endonuclease was added and the tube was pulsed for 2-3 sec in a microcentrifuge and incubated at 37°C water bath for 1-5 hours.

2. 11 Preparing horizontal agarose gel for electrophoresis

DNA fragments of different sizes can be separated by electrophoresis in an agarose gel. Smaller fragments travel faster than large ones. Gels containing 0.6-0.7% agarose were generally used for DNA fingerprinting or plasmid identification. 2% agarose gels were generally used for amplified DNA products (Polymerase chain reaction).

Materials

Agarose (Ultra pure DNA Grade agarose; BioRad). TBE buffer: 89 mM Tris; 2.5 mM EDTA; 89 mM Boric acid; or Tris-Acetate Electrophoresis (TAE) buffer: 40 mM Tris; 5 mM Sodium acetate (pH 7.8 using glacial acetic acid). Loading buffer: (10X) 20% Ficoll; 0.1 M EDTA; 1% Sodium dodecyl sulphate; 0.2% Bromophenol blue; or 50% sucrose/dye marker: 50% sucrose; 0.1 M EDTA; 0.05% Bromophenol blue. DNA molecular weight marker. Ethidium bromide 0.5 µg/ml. 1% Magnesium sulphate.

Power supply, Model 500/200, BioRad. UV Transilluminator. Polaroid film. Horizontal Gel Electrophoresis System; Horizon 11.14 or Horizon 58 (BRL). The gel platforms were cleaned by soaking the platform in 1% sodium hypochlorite solution for 30 min and then soaking it in dish washing liquid for 30 minutes. The platform was washed with six subsequent changes of deionized water.

Method (Sambrook *et al.*, 1989)

Concentrations of agarose used were based on the size of the DNA. Usually 0.7% agarose gels were used. When small bands of 600 bp or less had to be detected, 2% agarose was used. Low melting temperature gels were allowed to run at 4°C. Agarose was melted in TBE or TAE buffer, cooled to 55°C, poured into a gel platform (Horizon 11.14 or Horizon 58) and allowed to set for an hour with a gel comb near one end. After the gel was set the comb was removed carefully and gel flooded with buffer. DNA was mixed with 2 µl of loading dye and loaded in to the wells. Usually 1-2 µg of DNA was added for genomic restriction digests and a DNA ladder was included in a separate well so that the size of DNA fragments could be determined. 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 20 Volts for 15 minutes. The voltage was increased to 90 Volts and run for one and a half hours with the mini-gel (Horizon 58), or 40 Volts and run for 15 hours with the Horizon 11.14. The gel was removed from the gel platform and stained in ethidium bromide for 20 minutes, destained and washed in 1% Magnesium sulphate. The gel was photographed using a Transilluminator (Ultra-Violet products Inc.), Polaroid MP-4 land camera and polariod type 667, 665 or Kodak Tri-X pan professional film.

2. 12 Purification of plasmid DNA on CsCl gradient

Plasmid DNA was amplified by the method of Clewell, (1972) and isolated by the methods of Ish-Horowicz and Burke, (1981) and Banfalvi *et al.*, (1983). *Nod* probes were prepared from pRt572 or pPN26 by digestion with restriction enzyme *Eco*R I, separation by agarose gel electrophoresis, and extraction of the appropriate fragment for use as a specific probe.

Materials

Luria Broth: section 2. 3. 4 or TY broth: section 2. 3. 5. Solution I: 50 mM glucose; 25 mM Tris-HCl; 10 mM EDTA (Solution 1 was brought to pH 8.0 by adding conc HCl). Solution II: 0.2 M NaOH; 1% SDS. Solution III: 60 ml of 5 M K-acetate; 11.5 ml of glacial acetic acid. Lysozyme: 50 mg/ml in solution I. Na-acetate: 3 M.

Chloramphenicol 150 mg/l. Ethidium bromide stock (EtBr): 10 mg/ml. Cesium chloride. Isopropanol. 95% Ethanol. Cheese-cloth: approx. 15 cm x 15 cm. Refractometer. Sorvall RC-5B refrigerated super speed centrifuge with GSA rotor (DuPont instruments). Crimper (DuPont). Syringes and spare needles: 1 ml capacity. Combi Sorvall Ultra Centrifuge with TV-865 rotor (DuPont). Hand held UV (Model UVGL-58; mineral light lamp).

Method

A culture of *E. coli* harbouring the required plasmid was inoculated in 10 ml of Luria broth or TY and incubated overnight at 37°C. The overnight culture (5 ml) was used to inoculate Luria broth or TY (1 l), and 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, 150 mg of chloramphenicol was added. This increased the copy number of the targeted plasmids greatly. Overnight cultures were harvested by centrifugation, at 10,410 x G for 10 min at 4°C using a Sorvall GSA rotor and resuspended in 30 ml of Solution I. Lysozyme solution (3 ml) was added and the cells were allowed to stand for 5 min at room temperature. Solution II (60 ml) was added, mixed by inversion and the mixture allowed to stand on ice for 5 min. Solution III (45 ml) was added, mixed by vortexing and the solution allowed to stand on ice for 5 min. The mixture was centrifuged using a GSA rotor at 9,154 x G 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 allowed to stand at room temperature for 5-10 min. To avoid large fragments of pelleted debris entering the clean GSA bottle the supernatant was decanted through a cheese-cloth. The supernatant was then centrifuged at 10,410 x G 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 30 ml of TE, 30.7 g of CsCl added, and the solution allowed to dissolve at room temperature. Ethidium bromide (1.9 ml of 10 mg/ml) was added to the solution after 20 min. The succeeding steps were carried out in subdued light. The solution was centrifuged in a GSA rotor at 16,270 x G for 20 min at 15-20°C. The refractive index was checked by using an Abbe refractometer (section 2. 12. 1) and the concentrations of CsCl adjusted to give a reading of 1.3885. Then the supernatant was divided into, 4.5 ml quantities, balanced to 2 decimal places in Sorvall ultracentrifuge tubes, and placed in a Sorvall vertical TV865 rotor. Using a torque wrench, the tubes were secured within the rotor, and centrifuged at 400,700 x G o8vernight and the brake was applied at 12,330 x G to slow the rotor, then the brake was 8switched off to allow the rotor to coast to a stop. The tubes were transferred to the dark room, and DNA bands were visualized with long wavelength UV light. 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, so that liquid could be drawn off from lower portion. To avoid leaking ethidium bromide over the bench area, the tube was put into a clamp, suspended over a beaker. A second needle was inserted, with a syringe attached directly under the plasmid band and the band drawn off. About 0.2 ml of the solution was drawn from each tube. Ethidium bromide was extracted by topping each Eppendorf tube up with isopropanol saturated with CsCl and TE buffer. The isopropanol separated out as an upper layer, and it contained most of the ethidium bromide. This upper layer was discarded and the procedure repeated two more times. Further steps were undertaken in normal light. The solution was dialyzed against three changes of 500 ml TE buffer over a period of 24 hours and the OD readings were taken to calculate the purity and concentration of the plasmid DNA (section 2. 9).

2. 12. 1 Operation of a Abbe refractometer

The two halves of the cell were opened and 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 and a lower knob was used to line up the two rectangles with the bisecting cross hair. The refractive index was read from a scale visible through the eyepiece. The required refractive index is 1.3885. The refractometer was washed and dried thoroughly between readings.

2. 13 Extraction of DNA from agarose

DNA was extracted from agarose by three methods viz., 1. Filtration through glass wool. 2. Freeze-squeeze method. 3. Promega's Magic PCR preps DNA purification system (Mezei, 1991; Sambrook *et al.*, 1989).

2. 13. 1 Filtration through glass wool

Materials

Low melting temperature agarose (SeaPlaque) dissolved in TAE buffer (section 2. 11). 750 µl and 1.5 ml Eppendorf tubes. Siliconised glass wool. Sterile scalpel blade. Long wave UV source.

Method

Small slices of agarose gel (≤ 200 µl) were used in this method. An area around the DNA bands of interest was excised, using a sterile scalpel blade, under long wave UV light. This was done as quickly as possible, as even the longer wave UV light will damage the DNA. Using a hot wire, a hole was made in the bottom of a small (750 µl) Eppendorf tube, the Eppendorf tube was packed with siliconised glass wool (enough to provide a filter which did not allow agarose to pass through under centrifugation at 15,600 x G for 30 sec). The agarose was placed in the small Eppendorf tube, and this tube was inserted inside a large 1.5 ml Eppendorf tube. The tubes were centrifuged at 15,600 x G for 30 sec. The gel slice should collapse after centrifugation at this speed, and liquid should collect in the large Eppendorf tube. If not, the slice was centrifuged for a further 20 sec at the same speed. The purified DNA was checked by gel electrophoresis (section 2. 11) and stored at 4°C, -20°C or -70°C for further use.

2. 13. 2 Freeze-squeeze method of DNA purification

Materials

Low gelling temperature agarose dissolved in TAE buffer (section 2. 11). Sterile scalpel blade. Long wave UV source. Syringe: 3 ml capacity.

Method

This method was used for larger slices of agarose gel. The agarose was placed in a 3 ml syringe barrel, and stored overnight at -70°C. The frozen gel was warmed slightly and the syringe plunger used to express all the liquid into an Eppendorf tube. The purified DNA was checked by gel electrophoresis (section 2. 11) and stored at 4°C, -20°C or -70°C.

2. 13. 3 Magic PCR preps (Promega) DNA purification system

Materials

Magic PCR Preps kit (Promega: A7170): Magic^{TM} PCR Preps DNA purification resin. Purification buffer: 50 mM KCl; 10 mM Tris-HCl (pH 8.8); 1.5 mM $MgCl_2$; Magic^{TM} Mini-columns. Isopropanol: 80%. Deionised water: sterilized by autoclaving. Syringe: 3 ml capacity. Centrifuge. TE buffer (sterile): section 2. 8. 2.

Method

DNA solution (amplified products: 30-300 µl) was added to a 1.5 ml Eppendorf tube, 100 µl of purification buffer was added and vortexed briefly. PCR preps DNA purification resin (1 ml) was added and the resulting slurry vortexed briefly three times over a one minute period. The slurry was added to a 3 ml syringe attached to a mini-column. The mini-column itself was attached to a 1.5 ml Eppendorf tube. 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 column washed with 80% isopropanol by adding 2 ml of the isopropanol to the syringe and pushing into another 1.5 ml Eppendorf tube attached to the column. The column was removed from the Eppendorf tube and the syringe barrel, attached to another 1.5 ml Eppendorf tube, and centrifuged at 15,600 x G for 20 sec. The mini-column was left on the bench for 15 min. Sterile TE buffer (20-50 µl) was added to the column for 1 min and then the column was attached to a clean, sterile 1.5 ml Eppendorf tube and centrifuged for 20 sec to elute the DNA. The purified PCR product was checked by agarose gel electrophoresis (section 2. 11) and stored at 4°C, -20°C or -70°C for further study.

2. 14 Southern blotting (Southern, 1975)

DNA fragments that have been separated according to size by electrophoresis through an agarose gel are depurinated, denatured, neutralized, transferred to a nylon membrane, and immobilized. The relative positions of the DNA fragments in the gel are preserved during transfer to the membrane (Maniatis *et al.*, 1982). The DNA fragments attached to the nylon membrane are hybridized to α-^{32}P labelled DNA and autoradiography is used to locate the position of hybrid DNA complementary to the radioactive probe.

Materials

Depurinating solution: 0.25 M HCl. Denaturing solution: 0.5 M NaOH; 0.5 M NaCl. Neutralizing solution: 0.5 M Tris; 2 M NaCl, pH 7.2 (with conc. HCl). 20 x SSC: 175.3 g/l; NaCl: 88.2 g/l Sodium citrate, pH 7.0 (with 10 M NaOH). 2 x SSC stock: 10-fold dilution of 20 x SSC. Nylon membrane: Hybond-N (Amersham, UK). Paper towels. Whatman filter paper: 3 MM Chr. Pyrex dish: 30 x 20 cm. Gladwrap.

Method

The DNA to be transferred was separated by gel electrophoresis, stained, visualized and photographed. The gel was soaked in depurinating solution for 15 min in a Pyrex

dish. The blue tracking dye was used as an indicator of acid penetration as bromocresol purple goes yellow at low pH. The depurinating solution was sucked off by a vacuum. The gel was washed with deionised water and soaked in denaturing solution (NaOH/NaCl) for 15 min and any of the bromocresol purple in the gel returned to it's original blue colour. The gel was washed again with deionised water and soaked in neutralizing solution for 15 min. Four layers of Whatman 3 MM filter paper were cut (to approx. 15 x 22 cm) and laid in the bottom of a Pyrex dish 31 x 20 cm. A piece of Gladwrap was placed over the whole dish so that it could be pressed down on the paper and still overlap the edges of the dish. The Gladwrap was smoothed out and a hole slightly smaller than the gel was made. To avoid preferential transfer of 20 x SSC through the wells in the gel, this hole was cut to such a size that the wells were placed over the top of some of the Gladwrap. The Whatman 3 MM 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 the membrane was floated on the surface of a dish of deionised water. When the gel was ready, it was placed over the hole in the Gladwrap so that all four edges overlapped the hole, as did the wells. 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. No bubbles were allowed between the membrane and the gel and a gloved hand was sometimes required to push out any bubbles that remained. A sheet of Whatman 3 MM paper slightly smaller than the membrane which had been previously wetted with 20 x SSC was placed on the membrane. Two sheets of Whatman 3 MM paper of the same size were placed on this sheet and a stack of paper towels 5-8 cm deep was placed above the Whatman 3 MM paper. Finally a glass plate was placed on the paper towels and weight of about 300 g on the glass plate. The whole apparatus was left overnight after adding enough 20 x SSC to keep the 3 MM paper at the bottom of the dish moist for a few hours. The membrane was washed briefly with 2 x SSC, placed between two sheets of Whatman 3 MM paper and baked for 2 hours at 80°C under vacuum. Blots were labelled and stored at room temperature until required for probing. The blotted gel was restained to confirm the transfer of DNA to the membrane.

2. 15 DNA labelling

Feinberg and Vogelstein (1983; 1984) introduced the use of random sequence hexanucleotide to prime DNA synthesis on denatured DNA template at numerous sites along its length. The primer-template complex is a substrate for the "Klenow" fragment of DNA polymerase 1. The absence of the 5'-3' exonuclease activity

associated with DNA polymerase 1 ensures that labelled nucleotides incorporated by the polymerases are not subsequently removed as monophosphates. Two methods were used for labelling DNA, viz., using the Megaprime^{TM} DNA labelling system (Amersham) and the Ready-To-Go DNA labelling kit (Pharmacia). In both methods very small amounts of input DNA are required, enabling the production of high specific activity DNA probes from relatively small quantities of radioactive nucleotides.

2. 15. 1 Megaprime^{TM} DNA labelling system

Materials

Megaprime DNA labelling system kit (RPN1607): Solution 1 (Primer solution): Random nonamer primers in an aqueous solution; Solution 2 (Megaprime reaction buffer): dATP, dGTP and dTTP in concentrated reaction buffer containing Tris-HCl, pH 7.5; magnesium chloride and 2-mercaptoethanol; Solution 3 (Enzyme solution): 1 unit per µl DNA polymerase 1 "Klenow" fragment (cloned) in 50 mM potassium phosphate pH 6.5, 10 mM 2-mercaptoethanol and 50% glycerol. (α-^{32}P) dCTP: 3000 Ci/mmol in 10 mM Tricine, (1 Ci = 3.7 x 10^{10} Bq). DNA: probe template, 25-50 ng. 2 M HCl. 0.2 M EDTA. Sephadex G-50 slurry: 1 g Sephadex in 20 ml column buffer (equilibrate overnight). Column wash buffer: 1.0 M Tris-HCl, pH 8.0, 1 ml; 0.2 M EDTA, 0.05 ml; β-mercaptoethanol, 0.03 ml; Distilled water to 100 ml. All protective equipment necessary for safe operation with radioactive materials. Polyethyleneimine Cellulose Ion Exchange Resin (PEI) paper. Scintillation vials. Syringes: 1 ml. Scintillation counter.

Method

A restriction digest of double stranded template DNA (25-50 ng in 1-10 µl) and primer solution 1 was made up to 50 µl, denatured by immersion in a boiling water bath for 2-3 min and immediately transferred to an ice bath. Megaprime reaction buffer (solution 2) (10 µl), radiolabelled dNTP (5 µl) and Enzyme (solution 3) (2 µl) were added. The solution was mixed gently, centrifuged briefly at 15,600 x G and incubated in a water bath at 37°C for 30 min. The reaction was stopped by adding 5 µl of 0.2 M EDTA. Incorporation of (α-^{32}P) dCTP was checked with Polyethyleneimine cellulose Ion Exchange resin (PEI) paper. The reaction mix (1 µl) was spotted approximately 1 cm from the base of the paper, the PEI paper was placed upright in a beaker containing a few ml of 2 M HCl. The strip was left for 5-10 min to allow the liquid to rise up the paper by capillary action. The paper was cut in half, the halves placed in separate

scintillation vials and the radioactivity of each portion measured in a liquid scintillation counter (Beckman LS 7000). The vial with the lower portion of the PEI strip contained incorporated label, and the vial with the upper portion contained unincorporated nucleotides. The two readings were compared to determine the percent incorporation of α-^{32}P and approximate specific activity of the probe.

If more than 50% of the label was incorporated into the DNA, the probe was used directly for hybridization. If the incorporation was low, the unincorporated label was separated on a mini-spin column.

2. 15. 1. 1 Mini-spin column procedures

In mini-spin column, a 1 ml syringe plunger was used to push a little glass-wool into the bottom of a 1 ml syringe barrel and a hot wire was used to make a hole in the cap of a 1.5 ml Eppendorf tube, the hole was immediately enlarged with the 1 ml syringe so that a close fit was obtained. Any liquid passing through the syringe barrel would flow into the Eppendorf tube. The syringe/Eppendorf tube assembly was placed in a Falcon tube and packed with Sephadex G-50 and equilibrated in column buffer. The assembly was centrifuged at 1768 x G for 5 min and the liquid discarded. This process was repeated until the syringe barrel contained 0.8-0.9 ml of Sephadex G-50 resin, after which another 1.5 ml Eppendorf tube was attached to the syringe barrel, the labelling mixture was added to the column, allowed a few minutes to become adsorbed by the Sephadex G-50 resin. Column wash buffer (300 µl) was added and the assembly was centrifuged at 1768 x G for 5 min. Finally the labelled probe was diluted in a 1.5 ml Eppendorf tube, and used as a probe or stored at -20°C for further use.

2. 15. 2 Ready-To-Go DNA labelling system

Materials

Ready-To-Go DNA labelling kit (Pharmacia): 27-9251-01: Reaction mix: A room-temperature-stable preparation vitrified in a buffered solution containing dATP, dGTP, dTTP, FPLC*pure*^{r} Klenow Fragment (4-8 units) and random oligodeoxyribo-nucleotides, primarily 9-mers. DNA: probe template, 25-50 ng. Remaining materials are mentioned in section 2. 15. 1.

Method

The template DNA (25-50 ng) was dissolved in water (45 µl) denatured by boiling in a water bath for 2-3 min, immediately transferred to an ice bath, and, after cooling, centrifuged for 10 sec at 15,600 x G. The following components were added to the reaction mix: denatured DNA, and (α-^{32}P) dCTP (5 µl). The mixture was mixed gently by pipetting up and down several times, centrifuged briefly and incubated at 37°C for 30 min. The reaction was stopped by adding 5 µl of 0.2 M EDTA. The method of measuring the percentage incorporation of radiolabelled dCTP in the probe and the removal of unincorporated DNA are described in section 2. 15. 1. 1.

2. 16 Hybridization of Southern blots (Sambrook *et al.*, 1989)

Materials

Hybridization buffer: 1 M HEPES buffer, 25 ml, pH7.0; 20 x SSC, 75 ml; Herring Sperm DNA, 3 mg/ml (purified by phenol/chloroform extraction), 3 ml; 20% SDS, 2.5 ml; Ficoll 70 000, 1 g; Bovine Serum Albumin (BSA), 1 g; Polyvinyl pyrollidone (PVP), 1 g; Made up to 500 ml with deionised water, stored at 4°C, warmed to 37°C before use. 2 x SSC: section 2.14. 0.1 x SSC: 20-fold dilution of 2 x SSC. Hybridization tubes. Plastic box: large enough to fit the membrane. Oven (Bachofer, Germany).

Method

Using clean gloves and tweezers, the blot (section 2. 14) was placed in a clean hybridization tube and prehybridized with about 30 ml of hybridization buffer by rotation at 65°C for 2 hours. The buffer was discarded and 15 ml of hybridization buffer, added. The probe was placed behind perspex shields to thaw out, boiled for 3 min to denature the DNA, centrifuged briefly at 15,600 x G and immediately put on ice. Probe (3-5 x 10^7 cpm) was added to the hybridization tube, and the tube was rotated overnight at 65°C. During this time hybridization took place. After overnight hybridization, the tube was taken from the oven and the hybridization mixture poured off. The membrane was removed, placed in a plastic box on a shaker and washed twice in a box with 2 x SSC for 15 minutes at room temperature. This was followed by a third wash using 0.1 x SSC, in a box on a shaking water bath at 50°C. The washing stringency was vital because it determined the conditions required to obtain a minimum of non-specific binding between the probe DNA and heterologous sequences. The membrane was dried on a sheet of Whatman 3 MM paper, covered with Gladrap and applied to X-ray film (Kodak; Scientific imaging film, X-OMAT,

AR or Fuji medical X-ray film, Nif, Rx) in X-ray cassettes (Cronex, DuPont) with an intensifying screen (DuPont Cronex^{R} Lighting-Plus) at -70°C for 1-5 days for autoradiographic images. The film was developed in a Kodak X-Omat automatic processor.

2. 17 DNA-DNA hybridization

DNA reassociation techniques were used to study relatedness between soil bacteria and recognized *Rhizobium* type strains. Relative levels of hybridization were estimated by using a modification of Biodyne protocol manual, (Grunstein and Hogness, 1975).

Materials

Scintillation fluid contained per liter: 2, 5 diphenyloxazole (PPO), 4.0 g; 2, 2' paraphenylene-bis-5 phenyloxazole (POPOP), 100 mg; tolune, 667 ml, Triton X-100, 333 ml). 10 x SSC: 2-fold dilution of 20 x SSC.

Method

Total DNA was isolated from soil bacteria expressing nodulation genes from *Rhizobium leguminosarum* biovar trifolii and type strains of *Rhizobium leguminosarum* biovar trifolii strains ICMP2163 and ATCC10004, *Rhizobium tropici* IIA strain CFN299, *Rhizobium loti* strain ATCC33669, and *Rhizobium meliloti* strain NZP2011 (section 2. 8). Purity and concentration were measured and the DNA solutions were adjusted to approximately 1 mg/ml (section 2. 9) and digested with *Eco*R I (section 2. 10). The digested DNA samples were denatured by boiling in a water bath (95°C) for 2-3 min and chilled on ice. 1 volume of 20 x SSC (section 2. 14) was added and the DNA spotted (2 µl) onto the nylon membrane prewetted with 10 x SSC, allowed to dry between each aliquot. The wet membrane was transferred to denaturing solution (section 2. 14) for 5 minutes and then to neutralizing solution (section 2. 14) for 1 minute. The membrane was dried and baked in an oven at 80°C for two hours under vacuum to fix the DNA. Membranes were hybridized at 65°C (section 2. 16) with (α-^{32}P) dCTP labelled DNA of high specific activity (10^{8} cpm/ug) from the (section 2. 15) type strains. Membranes were autoradiographed (section 2. 16) overnight at -70°C and cut up so that the radioactivity associated with each spot could be determined separately. Each piece was put into a scintillation vial containing 10 ml scintillation fluid. The scintillation vials with the filters were counted using a Beckman LS7000 scintillation counter. Relative hybridization values were obtained by expressing the

counts obtained with heterologous DNA's as a percentage of the counts per minute obtained with homologous DNA.

2. 18 Ribosomal (rRNA) fingerprinting

The aim of rRNA fingerprinting is to reduce the number of DNA restriction fragments in a genomic digest (Grimont and Grimont, 1991). This is achieved by visualizing only those DNA fragments that hybridize with a given probe. In this project total DNA was isolated (section 2. 8) from soil bacteria expressing nodulation genes and known *Rhizobium* type strains (section 2. 17). Purity and concentration were measured (section 2. 9). The DNA's were digested with *Eco*R I (section 2. 10), separated by electrophoresis in 0.7% agarose (section 2. 11), blotted on nylon membrane (section 2. 14), hybridized at 65°C (section 2. 16) with (α-^{32}P) dCTP labelled (section 2. 15) plasmid pKK3535 (rRNA operon probe), (Brosius *et al.*, 1981) and autoradiographed (section 2. 16). The patterns formed by the DNA fragments that hybridized with the probe were compared to detect relationships between the various strains of bacteria.

2. 19 Design and preparation of primers

Primers were selected on the basis that they would hybridize efficiently to the sequence of interest with negligible hybridization to other sequences present in the sample. Selection was based on complementarity to template, primer length (normally 24 bases), G-C content and avoidance of "primer-dimer" formation. The primers for rRNA amplification were chosen by inspection of the published bacterial sequences available in the EMBL/Genbank data library and are complementary to conserved 16S rRNA gene sequences (Young *et al.*, 1991). The primers were synthesized by the Separation Science Unit, Massey University, Palmerston North, New Zealand or Oligos Etc. Inc. (USA). The forward primer Y1 (5'-TGGCTCAGAACGAACGCTGG CGGC-3') corresponds to positions 20-43 in the *Escherichia coli* 16S rRNA sequence (Brosius *et al.*, 1981) and the reverse primer Y2 (5'-CCCACTGCTGCCTC CCGTAGGAGT-3') to *E. coli* positions 361-368. The primers for the amplification of *nod*A sequence were selected using Primer Designer (Version 1.01; 1990; Scientific and Education Software) on the published sequences of Rossen *et al.*, 1984 (Accession number Y00548) and synthesized by the Separation Science Unit, Massy University. The forward primer *nod*AR (5'-TCATAGTTCCGACCCGTTTCG TTC-3') and the reverse primer *nod*AL (5'-ATGTCTTCTGAAGTGCGATGGAAA-3') were used.

2. 20 Polymerase chain reaction

The polymerase chain reaction (PCR) is an *in vitro* method for the enzymatic amplification of specific DNA sequences, using two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the target DNA. A repetitive series of cycles involving template denaturation, primer annealing, and extension of the annealed primers by DNA polymerase results in the exponential accumulation of a specific fragment whose termini are defined by the 5' ends of the primers. The primer extension products synthesized in one cycle can serve as a template in the next, thus the number of target DNA copies approximately doubles at every cycle. Thus 20 cycles of PCR yields about a million-fold (2^{20}) (Erlich, 1989).

Materials

Ultrapure dNTP set: 2'-Deoxynucleoside 5' Triphosphates (Pharmacia). *Taq* DNA polymerase. *Taq* DNA polymerase 10X buffer: 500 mM KCl; 100 mM Tris-HCl (pH 8.8) 15 mM $MgCl_2$ and 1.0% Triton X-100. Buffer is optimized for use with 0.2 mM for each of dNTPs. DNA template: section 2. 8. 2. Primer DNA: as explained in section 2. 19. Paraffin oil. ART^R Aerosol resistant tips (Biotek). Sterile water and Eppendorf tubes (1.5 ml and 750 µl). Gloves. Techne thermal cycler (type PHC-3).

Method

The components in the reaction were added to a 750 µl Eppendorf tube in an ice bucket in the following order: sterile water to make a final volume of 20 µl; *Taq* polymerase 10 x buffer, 2 µl; dNTPs (4 µl of a mixture containing 2 mM of each of the four bases, dATP, dCTP, dGTP and dTTP; DNA Template, 1-2 µl of a 25-50 µg/ml stock solution (section 2. 8. 2); primer (section 2. 19), 0.5 µl of a 20 pmol/ml stock; *Taq* DNA polymerase 1 µl. All dilutions were made on the same day and stored at 4°C. A negative control contained sterile water, 15 µl of paraffin oil was added and centrifuged briefly at 15,600 x G. A Techne thermal cycler, model PHC-3, was used for these experiments. Three different files on this instrument were used for all experiments as follows: file #13, 93°C for 3 min (Template denaturation), 63°C for 30 sec (Primer annealing), 72°C for 30 sec (Extension of the annealed primers), (1 cycle); file #14, 93°C for 45 sec, 63°C for 30 sec, 72°C for 30 sec, (40 cycles); file #15, 93°C for 45 sec, 63°C for 30 sec, 72°C for 3 min, (1 cycle), file #16, 04°C for 99 hrs (1 cycle). In the case of *nod*A probe primer annealing was at 55°C (section 2. 21. 1). The amplified products were examined by electrophoresis, (section 2. 11) to determine their size and verify the absence of product in the negative control, purified (section 2. 13) and stored at 4°C, -20°C or -70°C for further use.

2. 21 Amplification of genes coding for 16S rRNA

Total genomic DNA isolated (section 2. 8. 2) from soil bacteria expressing noduation genes from *Rhizobium leguminosarum* biovar trifolii was amplified (section 2. 19 and 2. 20) using the forward primer Y1 and reverse primer Y2 (section 2. 19). The amplified product was checked for the correct size (approximately 348 bp), (section 2. 11) and purified (section 2. 13). The amplified product was sequenced directly (section 2. 23).

2. 21. 1 Amplification of *nod*A probe

The *nod*A probe was amplified (section 2. 19 and 2. 20) by the forward primer *nod*AR and reverse primer *nod*AL (section 2. 19) using total genomic DNA (section 2. 8. 2) from type strain *Rhizobium leguminosarum* biovar trifolii, strain ATCC10004. The amplified product was checked for its correct size (590 bp), (section 2. 11), purified (section 2. 13) and used as a probe (section 2. 15) to show the presence of the nodulation genes in the transconjugant soil bacteria isolated from nodules.

2. 22 16S rDNA sequence determination

The chain termination (Sanger *et al.*, 1977, 1978) method was used to directly sequence PCR products from 16S rRNA (section 2. 21) by a modified method known as cycle sequencing using the *fmol*^{TM} DNA sequencing system (Promega). The *fmol*^{TM} DNA sequencing system is an enzymatic sequence analysis system which takes advantage of the thermal stability of DNA polymerase isolated from *Thermus aquaticus*. Promega's Sequencing Grade *Taq* DNA Polymerase is modified to give superior results on double-stranded DNA templates.

2. 22. 1 Preparation of labelled fragments for sequence determination

Materials

fmol^{TM} DNA sequencing system: Sequencing grade *Taq* DNA Polymerase (5 U/µl); *fmol*^{TM} Sequencing 5X buffer: 250 mM tris-HCl, pH 9.0; 10 mM $MgCl_2$; d/ddNTP nucleotide mixes (Deaza); *fmol*^{TM} Sequencing stop solution: 10 mM NaOH; 95% formamide; 0.05% bromophenol blue; 0.05% xylene cyanole; (Promega). DNA template 500 fmol. Primer: 3.0 pmol; [α-^{35}]dATP (> 1,000 Ci/mmol, 10 µCi/µl). *fmol*^{TM} Sequencing 5 x buffer. Sterile water. Thermal cycler, PHC-3, Techne). Sterile

gel loading natural tips (Bioteck) and microcentrifuge tubes. Paraffin oil. Eppendorf tubes.

Method

Four 750 µl microcentrifuge tubes were labelled A, G, C, T and the appropriate d/ddNTP solution (2 µl) added to each tube. Each tube was capped and stored on ice until needed. For each set of four sequencing, reactions the following reagents were mixed in a microcentrifuge tube: Template DNA (1 µl of amplified PCR product), Primer 1 µl (Y1 or Y2), [α-^{35}S] dATP (10 µCi/µl), 0.5 µl, 5 x *fmol*^{TM} Sequencing buffer, 5 µl and made up the final volume to 16 µl. *Taq* DNA polymearse (1 µl) was added to the primer/template mix and mixed briefly by pipetting up and down. 4 µl of the enzyme/primer/template mix were added to the inside wall of each tube containing d/ddNTP mix and mixed. Mineral oil (15 µl) was added to each tube and the tubes were briefly centrifuged. The reaction tubes were placed in a thermal cycler preheated to 95°C and the program set at 95°C for 2 minutes, then 95°C for 30 seconds (denaturation), 67°C for 30 seconds (annealing) and 70°C for 1 minute (extension), for a total of 30 cycles and then held at 4°C. After the thermocycling program was completed, 3 µl of *fmol*^{TM} sequencing stop solution was added to the inside wall of each tube and the tubes were briefly spun in a microcentrifuge to terminate the reactions. Reactions were stored at -20°C for further use.

2. 22. 2 Preparation of denaturing polyacrylamide gels

Materials

40% acrylamide solution (stock solution): acrylamide (DNA-sequencing grade), 380 g; N, N'-methylenebisacrylamide, 20 g; made upto 1 l of deionised water and stored in dark bottles at 4°C. 10 x Tris Borate Buffer (TBB) pH 8.9: Tris, 324 g; Boric acid, 55 g; EDTA, 19 g; 6% acrylamide/urea solution: 288 g of urea was added to 210 ml of deionised water and 90 ml of 40% stock solution of acrylamide. A tea spoon of analytical grade mixed bed resin AG^{R} 501-X8 (20-50 mesh) was added and the mixture was deionised on a magnetic stirrer, filtered and 60 ml of 10 x TBB was added. The final volume was 600 ml and the solution was stored in brown bottles at 4°C. 10% Ammonium persulphate. TEMED (N, N, N', N'-Tetramethylethylene-diamine). Acetone. 2% Dimethyl-dichlorosilane in CCl_4. Glass plates (gel dimension, W x H) : 31.0 x 38.5 cm or 17 x 60 cm. 0.4 mm vinyl spacer. Spacer foam blocks. Vinyl sharks tooth combs. Spring clips. Gel sealing tape (Sleek tape, Smith and Nephew). Handee towels. 250 ml clean Duran bottle (Schott). Gladrap.

Method

The glass plates, spacers and space foam blocks were washed in detergent solution, and rinsed thoroughly in tap water and deionised water. Plates were held by the edges to avoid contamination on the working surfaces and cleaned meticulously with acetone to ensure that air bubbles did not form when the gel was poured. The plates were allowed to dry and the small plate was siliconised with 2% silicone solution to prevent the gel from sticking to it. The larger plate was placed flat on the bench and the two spacers placed on the long sides of the plate. The smaller plate was placed on the spacers. The sides and bottom of the plates were tapped together with gel sealing tape and clamped with spring clips. Eighty millitres of 6% acrylamide/urea solution was added to a 250 ml Duran bottle and then 50 µl of TEMED and 500 µl of 10% ammonium persulphate. The contents of the bottle were mixed by swirling them and poured gently into the space between the plates by holding the plates with the left hand, at an angle of 45°C. To avoid producing air bubbles, the gel solution was poured in a continuous stream until the plates were filled with the solution. The plates were laid on the bench with about 5 cm high support at the open end and the flat side of a shark's tooth comb was inserted approx. 0.5 cm into the gel solution. Only the open end of the plates was then clamped with spring clips and covered with Gladrap and the gel left to polymerize overnight.

2. 22. 3 Loading the reaction mixtures and separation of oligonucleotides

Materials

1 x TBB: 100 ml of 10 x TBB (section 2. 22. 2) was made upto 1 l. Polymerized gel: section 2. 22. 2. Electrophoresis apparatus, Model SA or S2, BRL. Thermocycling apparatus at 80°C. Power supply (ECPS 3000/150, Pharmacia). Sterile gel loading natural tips.

Method

The polymerized gel plates were cleaned with damp paper towels to get rid of dried polyacrylamide/urea. The shark's tooth comb was carefully removed from the top of the gel, and the Sleek tape stripped from the bottom of the gel plate. The plates were tightly attached to the electrophoresis apparatus. The top and bottom reservoir were loaded with 1 x TBB. A Pasteur pipette attached to a rubber bulb was used to squirt TBB across the submerged flat loading surface of the gel to remove fragments of urea

and polyacrylamide. The shark's tooth comb was reinserted with its teeth just sticking into the loading surface and the slots washed out with TBB to remove urea and polyacrylamide. The apparatus was connected to a power pack and run for 30 minutes at constant Watts (65 W) to warm the gel. Then the power was disconnected and TBB was used to wash out the slots. The sequencing reactions were heated to 80°C for 2 min and immediately loaded into the freshly rinsed slots (3 µl per reaction per slot) in the order A, G, C and T. After the samples were loaded the electrodes were connected to the power pack and the power set at 65 W. The time required to complete a separation was (5-6 hours) estimated by monitoring the migration of the marker dyes in the loading buffer (*fmol*™ sequencing stop solution: section 2. 22. 1). After electrophoresis the gel is fixed, dried and autoradiographed (section 2. 22. 4).

2. 22. 4 Autoradiography and reading of sequencing of gels

Materials

Fixing solution: 10% methanol and 10% acetic acid in water. Gel: section 2. 22. 3. Plastic trays. Gladrap. Whatman 3 MM paper. X-ray cassette and film: section 2. 16. Slab gel drier (BioRad, Model 483) or Gel drier (BioRad, Model 583).

Method

The power was turned off and electrophoresis buffer discarded. The plates were removed from the sequencing apparatus and placed on the working area with the smaller notched plate uppermost. The Sleek tape was removed and a metal spatula was used to separate the plates at one end. The glass plate with the gel attached was carefully submerged in a tray of fixing solution for 20 min. The plate was removed from the fixing solution after tilting it carefully to allow excess fixation fluid to drain away, taking care not to allow the gel to slide off the plate. A piece of Whatman 3 MM paper slightly larger (2-3 cm) than the gel in both the length and the width was placed on top of the gel and gently pressed so that the gel becomes firmly attached to the rough surface of the paper. The 3 MM paper was held with one hand and supporting plate picked up and quickly flipped over and laid on a dry piece of paper. The gel would stick to the 3 MM as it is peeled from the glass plate. The 3 MM paper (gel uppermost) was covered with Gladrap and dried for one hour under vacuum on a commercial gel drier set at 80°C. The dried gel was removed from the drier and the Gladrap removed from the gel. The gel is autoradiographed by exposing it to X-ray film (Kodak; Scientific imaging film, X-OMAT) for 1-5 days and developed as described in section 2. 16. The bands on autoradiographs reflect the relative migration

distances of DNA fragments according to their size. These distances indicate the positions of the respective 3' terminal bases in the sequences relative to the priming site. The sequence is read in the 5' to 3' direction from the bottom of the autoradiograph (gel) upward. The X-ray film was placed on a light box and the sequence was read in the following order A, G, C, T and the sequence analyzed (section 2. 23).

2. 23 Analysis of sequence data

The double stranded 16S rRNA sequences were analyzed using the following programs: The GCG Fragment Assembly System (FAS) (section 2. 23. 1), The BLAST search (section 2. 23. 2) and PILEUP (section 2. 23. 3). FAS and PILEUP were run on The Genetics Computer Group Sequence Analysis Package, Version 7 on a VAX computer. The aligned sequences were latter used to construct unrooted phylogenetic trees (section 2. 24).

2. 23. 1 The GCG Fragment Assembly System (FAS)

The GCG Fragment Assembly System (FAS) is a series of programs used to assemble overlapping fragment sequences. FAS was used here to 1) store fragment sequences; 2) recognize overlapping sequences and align sequences (Staden, 1980). Each of the double stranded 16S rRNA sequences was assembled using FAS and the nucleotide sequences were double checked against original autoradiographs for extra bases or mismatched base pairs.

2. 23. 2 The BLAST Search

The method of Altschul *et al.*, (1990), was used in this program. The program is optimized to find nearly identical sequences rapidly. An E-mail message was sent to "blast @ncbi.nlm.nih.gov" with the following text information on each of the 16S rRNA sequences assembled in FAS (section 2. 23. 1).

PROGRAM blastn
DATALIB nr
BEGIN
> Soil isolate KJ13
AGGCTTAACACATGCAAGTCGAGCGGGCGTAGCAATACGTCAGCGGCAGA
CGGGTGAGTAACGCGTGGGAACATACCTTTTGGTTCGGAACAACACAGGG
AAACTTGTGCTAATACCGGATAAGCCCTTACGGGGAAAGATTTATCGCCG

AAAGATTGGCCCGCGTCTGATTAGCTAGTTGGTAGGGTAATGGCCTACCA
AGGCGACGATCAGTAGCTGGTCTGAGAGGATGATCAGCCACATTGGGACT
GAGACACGGCCCAA

The search and the computation was performed at the NCBI (National Center for Biotechnology Information at the National Library of Medicine, USA).

2. 23. 3 PILEUP

Pileup aligns a group of related sequences using the progressive pair-wise alignment methods of Feng and Doolittle, 1987. The procedure begins with the pair-wise alignment of the two most similar sequences. The final alignment is achieved by a series of progressive, pair-wise alignments that include increasingly dissimilar sequences and clusters until all sequences have been included in the final pair-wise alignment.

2. 24 Construction of unrooted phylogenetic trees

Felsenstein's PHYLIP (Phylogeny Inference Package) version 3.5c program was used in the construction of the phylogenetic trees. The program was obtained by connecting to evolution.genetics.washington.edu or 128.95.12.41 using FTP (File Transfer Program). For the 386 PCDOS three archived executables were obtained, viz., phylip.exe, phylip3x.exe and phylip3y.exe. The three programs were put in one directory and self extracted for further use. The purpose of the phylogenetic tree is to relate the 16S rRNA sequences of soil bacteria expressing nodulation genes from *Rhizobium leguminosarum* biovar trifolii to those of named strains. This involved four different steps within the PHYLIP 3.5c program namely, 1. Seqboot (Bootstrap), 2. Dnadist, 3. Neighbor and 4. Consense, described below.

2. 24. 1 Seqboot (Bootstrap)

Seqboot is a general bootstrapping tool and was used to generate multiple data sets (1000) from aligned sequences of 16S rRNA (section 2. 23. 1, 2. 23. 2 and 2. 23. 3). Bootstrapping was invented by Bradely Efron in 1979 and was introduced in phylogeny estimation (Felsenstein, 1985).

2. 24. 2 Dnadist

This program was used to compute a distance matrix from the multiple data set generated from Seqboot. The model used was that of Jukes and Cantor, (1969). This model assumes that there is independent change at all sites, with equal probability, and when a change occurs there is an equal probability of ending up with each of the other three bases.

2. 24. 3 Neighbor

The distance matrix generated from by Dnadist was used to construct a tree by the successive clustering of the lineages, setting branch lengths as the lineages join using the Neighbor-Joining (Saitou and Nei, 1987) algorithm.

2. 24. 4 Consense

Consense read a file of computer-readable trees created by the Neighbor-Joining method to find a consensus tree. It carries out a family of consensus tree methods (Margush and McMorris, 1981) which includes strict consensus and majority rule consensus.

2. 25 Identification of soil bacteria based on total fatty acid analysis

The fatty acid composition of bacteria was used to identify them. This work was carried out by S W Tighe of Analytical Services Inc., P O Box 626, Essex Junction, VT 05453, USA.

Cells were subcultured three times on TYEA agar (Section 2. 3. 8) for 48 hr at 28°C to stabilize their fatty acid composition. The final subculture was made on medium which was not more than 48 hr old in order to standardize the osmotic pressure of the medium. Standard methods described by Jarvis and Tighe, (1994) were used to saponify, methylate and extract the fatty acids. The fatty acids separated by gas chromatography and identified by calculation of their equivalent carbon chain lengths and comparison with the MIS (Microbial ID Inc. Newark, DE) software peak naming table. Quantities of fatty acids were expressed as percentages of the total named fatty acids. The fatty acid profile of each strain is compared with the composite profile of all named species and a Similarity Index (SI) is computed. An $SI \geq 0.400$ indicates a good match.

3. RESULTS

3. 1 Origins of soil samples

Gram negative soil bacteria were isolated from 16 soil cores. Four cores each were taken as samples from Ramiha silt loam, Tokomaru silt loam, Kairanga silt loam and Manawatu sandy loam. A summary of the soil samples and some of their agricultural properties are shown in Table 3 (Cowie *et al.*, 1972). The Ramiha silt loam, Tokomaru silt loam and Kairanga silt loam were from ryegrass-white clover permanent pastures. Manawatu sandy loam was from a fallow land with shrubs of *Lupinus* sp. These pastures had no recent history of *Rhizobium* inoculation but were probably inoculated when they were sown.

3. 2 Isolation and screening of soil bacteria

Soils were plated on YMG agar or SE agar and incubated for 5 to 7 days at 28°C. Colonies were purified by streaking on TY medium. The 100 strains of Gram negative rod shaped soil bacteria with various colony morphologies were tested for their ability to nodulate and fix atmospheric nitrogen on white clover (*Trifolium repens* cultivar Grasslands Huia). Four (2%) of the soil isolates formed nodules on clover plants after 4 to 6 weeks of incubation. The remaining soil bacteria (98%) were used as recipients in conjugation experiments in which the co-integrate plasmid pPN1 was donated by *Escherichia coli* strain PN200.

3. 3 Examination of pPN1 and its transfer to non-nodulating soil bacteria

3. 3. 1 Examination of pPN1 in donor cells

The plasmid pPN1 is a stable co-integrate plasmid comprising the broad host range plasmid, R68.45, and the symbiotic plasmid pRtr514a. The Eckhardt (1978) method was used to verify that donor cells contained a co-integrate and that it formed a single band (219 Mdal). Reference plasmids from *Agrobacterium tumefaciens* strain [pTi-C58 (130 Mdal) (Holsters *et al.*, 1978) and pAt-C58 (275 Mdal) (Denarie *et al.*, 1981)] were used to determine its size. The observed results suggested the presence of an intact pPN1.

Table 3. Summary of soil samples and some of their agricultural properties

Soil name	Parent material or rock	Distinguishing features of soil and environment	Common name and natural nutrient status
Ramiha silt loam	Loess[a], solifluction[b] material, and slope deposits	On rolling uplands between 1,000 and 2,000 ft (305-610m), under moderate (50- 70in.; 1270-1780 mm) rainfall with no summer dry seasons. Has dark brown top soil with strong nut structures overlying yellowish brown very friable silt loam	Strongly leached yellow-brown earth, and gleyed[c] yellow-brown earth, low to medium P, low Ca, and K. pH 5.0-5.5
Toko-maru silt loam	Loess	On higher terraces from silty to fine sandy loess under low to moderate rainfall (35-45in.; 890-1140 mm) with summer dry seasons. Poorly drained pale olive compact clay loam with many to abundant brown mottles and moderately thick clay coatings in lower part	Weakly leached moderately to strongly gleyed yellow-grey earths, moderate to low P and Ca, medium K. pH 5.7-6.0
Kai-ranga silt loam	Alluvium[d]	In occasionally flooded river basins and on flats. Poorly drained with greyish brown silty loam over grey clay loam with brown mottles	Weakly leached slowly accumulating gley recent soil, high P and Ca, medium K. pH 5.6-5.8
Mana-watu sandy loam	Alluvium	Well to somewhat excessively drained with sandy profiles on sand and gravel	Weakly leached slowly accumulating recent soils, high P and Ca, medium K. pH 5.8-6.1

[a]: a homogenous, porous, fine grained, aeolean deposit consisting predominantly of silt sized particles, with subordinate clay and fine sand; [b]: the slow downhill movement of soil as result of the alternate freezing and thawing of the contained water; [c]: mottled pale grey and rusty brown colours where reduction and oxidation processes alternate as under conditions of a fluctuating water table; [d]: Detrital material which is transported by river and deposited usually temporarily at points along the flood plain of a river.

3. 3. 2 Fate of pPN1 in soil bacteria

On conjugation with soil bacteria, the fate of the co-integrate plasmid pPN1 in recipient soil bacteria was verified by Eckhardt gels. Figure 10 shows plasmid R68.45 from *Escherichia coli* strain PN298, (lane 1) an intact co-integrate plasmid pPN1 from the donor *Escherichia coli* strain PN200 (lane 2) and plasmid bands from the recipient non-nodulating soil bacterium KJ30 (lane 3) and the tranconjugant of soil bacterium KJ30 which contained pPN1 and R68.45 (lane 4). Routine screening was done to assess the fate of the pPN1 in soil bacteria. Transconjugants of strains KJ1 and KJ3 contained R68.45. Transconjugants of strains KJ13, KJ19, KJ23, KJ26, KJ30 and KJ44 contained pPN1 and R68.45. Transconjugants of strains KJ5, KJ17, KJ27, KJ57, KJ203 and PN165 contained only pPN1.

3. 4 Frequency of transfer and expression of pPN1

3. 4. 1 Frequency of transfer and expression

Strain PN200, a derivative of *Escherichia coli* strain HB101 which contained the co-integrate plasmid pPN1 was used to introduce by conjugation on TY plates a symbiotic plasmid (pRtr514a; originally from *Rhizobium leguminosarum* biovar trifolii strain NZP514) into Rifampicin resistant derivatives of soil bacteria. The mixture of transconjugants, donor, and recipient was either used to inoculate clover seedlings directly or plated onto selective medium. This contained Rifampicin and Kanamycin and was used to select for transconjugants which were subsequently used to inoculate clover seedlings. A total of 11 soil isolates out of 100 crosses (11%) formed nodules (Figure 11) on clover plants. Table 4 shows that the mean transconjugation frequency was 2.91×10^{-5}. Nodules were observed 4 to 6 weeks after inoculation. A range of 40% to 70% plants nodulated with each strain and 3 to 6 nodules were formed by each plant. Comparison of the mean values with those of the positive control (strain PN165) indicated that the soil bacteria which could accept pPN1 did so with comparable frequency and were usually able to express pSym.

3. 4. 2 Plant Test

All transconjugants were purified and tested for their ability to form nodules on white clover. Representative results are shown in Figure 11.

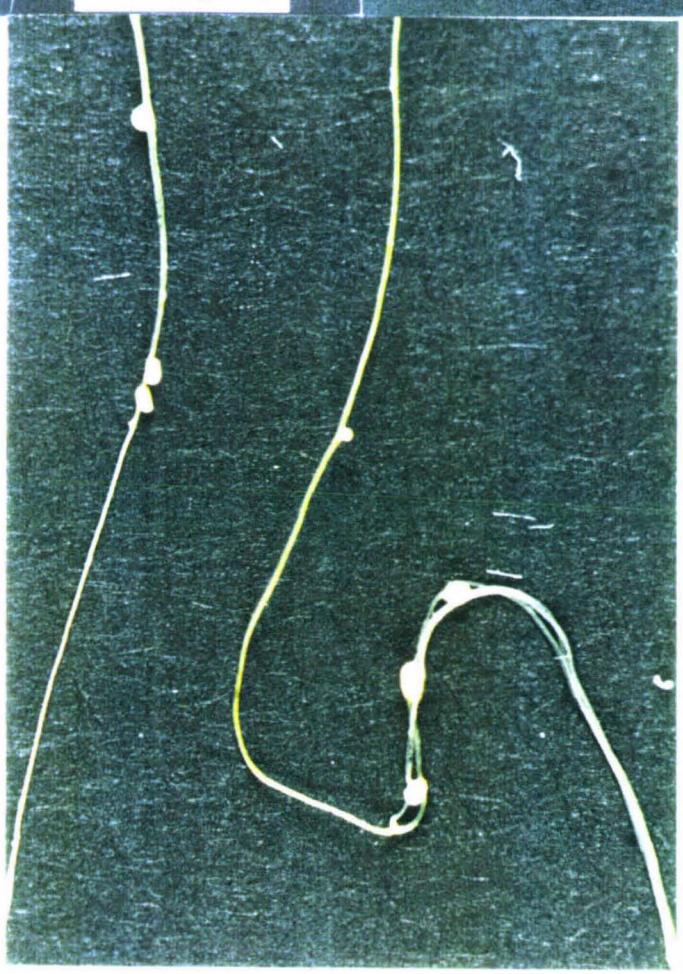
Figure 10. Eckhardt gel of the Gram negative soil bacterium KJ30 and a transconjugant of KJ30 which received pPN1 from *Escherichia coli*, strain PN200. The Eckhardt gel contained 0.8% (w/v) of agarose in 1 x TBE pH 8.0. Lane: 1, R68.45 (broad host range plasmid); Lane: 2, PN200 (pPN1); Lane: 3, Gram negative soil isolate KJ30; Lane: 4, Transconjugant of soil isolate KJ30.

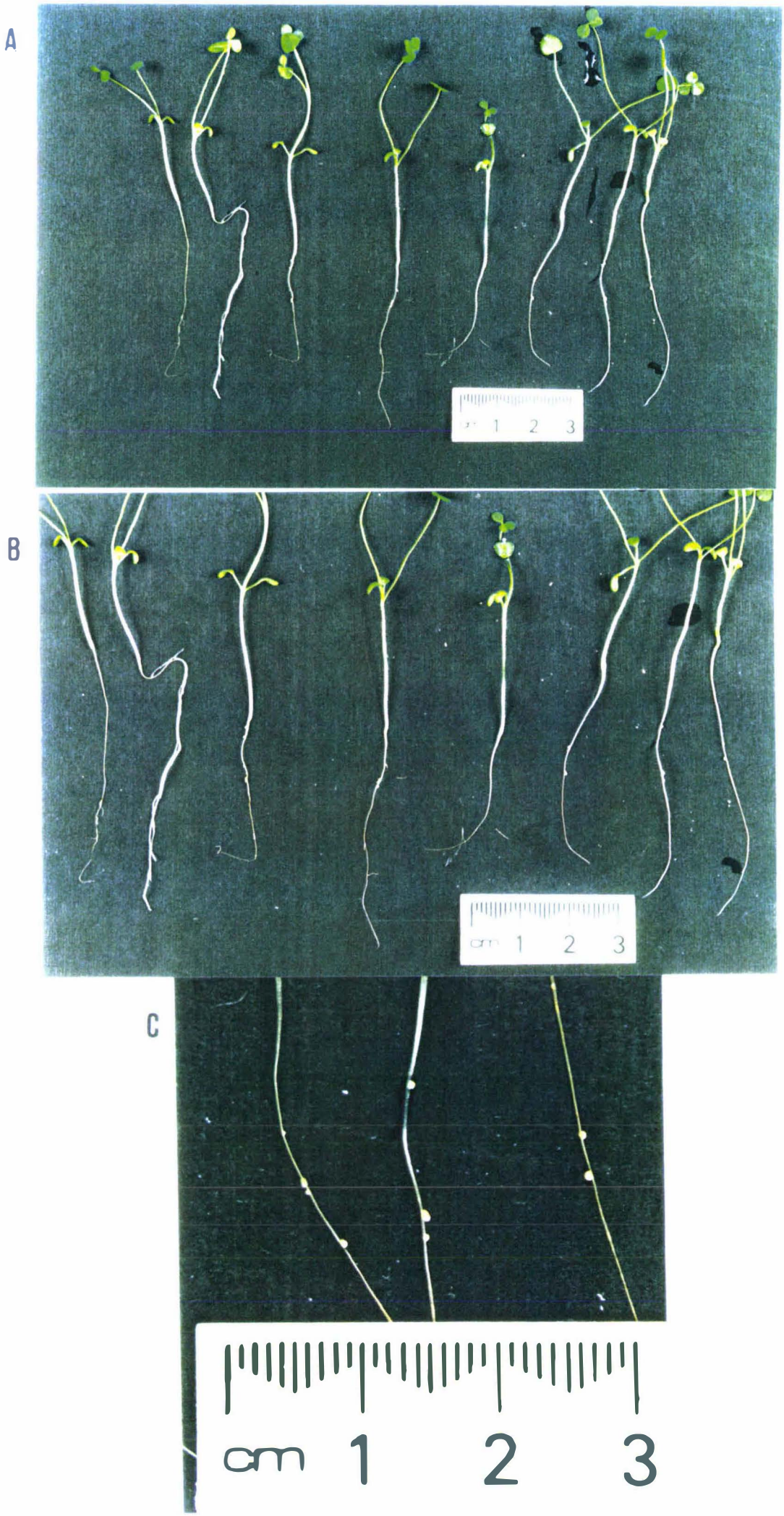
Figure 11. Nodule formation on white clover (*Trifolium repens*, cultivar Grasslands Huia) 4 to 6 weeks old. (A) Seedlings after inoculation with (left to right), sterile water, *Escherichia coli* strain PN200, *Rhizobium leguminosarum* biovar trifolii strain ICMP2163, *Rhizobium leguminosarum* biovar trifolii strain ICMP2163, soil isolate KJ13, transconjugants of KJ13, KJ30 and KJ203. (B) Seedling roots from (A) in the same order as above. (C) Seedling roots from (A) with (left to right) transconjugants of KJ13, KJ30, and KJ203. (D) Seedlings after inoculation with (left to right), *Escherichia coli* strain PN200 and transconjugants of soil bacteria KJ26, KJ27 and KJ57. (E) Seedling roots from (D) in the same order as above. (F) Seedling roots from (D) after inoculation with (left to right) transconjugants of soil bacteria KJ26 and KJ27.

D

E

F




A
B
C
cm 1 2 3
cm 1 2 3
cm 1 2 3

1 2 3 4

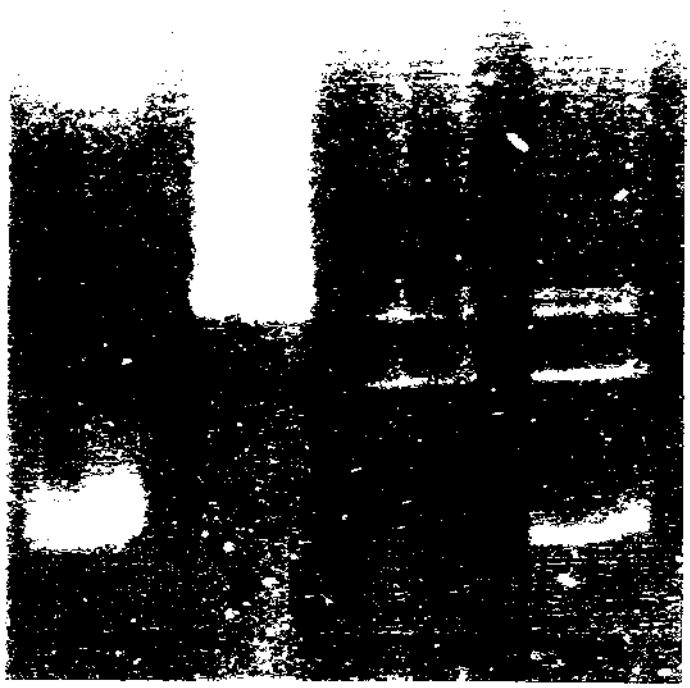


Table 4. Frequency of transfer and expression of pPN1 in soil bacteria

Bacteria	Frequency of transfer[a]	Nodulation test[c]		
		Weeks after inoculation[b]	Number of plants nodulated[d]	Nodule number/ plant
Soil bacteria				
KJ1	1.30×10^{-5}	Did not nodulate		
KJ3	1.40×10^{-6}	6	5	3
KJ5	[e]	5	4	3
KJ13	1.30×10^{-6}	6	5	3
KJ17	[e]	5	6	3
KJ19	1.25×10^{-5}	4	4	5
KJ23	2.00×10^{-4}	4	5	4
KJ26	1.40×10^{-6}	6	4	4
KJ27	[e]	6	5	4
KJ30	1.50×10^{-6}	4	7	6
KJ44	1.59×10^{-6}	5	5	5
KJ57	[e]	6	5	5
KJ203	[e]	6	4	3
Mean	2.91×10^{-5}	5	5	4
Control				
PN200[f]	Did not nodulate			
E. coli[g]	Did not nodulate			
PN165[h]	1.60×10^{-5}	4	7	6
R. legum[i]		4	8	7

[a], Frequency of transfer = number of transconjugants expressing pPN1 per total recipients, recipient isolates showing the transfer frequency were made resistant to Rifampicin, conjugated with PN 200 (Kanamycin resistant) and selected on antibiotic medium (Km + Rif) for transconjugants; [b], weeks after inoculation of plants; [c], Nodulation test to confirm the expression of the Sym::R68.45 was performed using *Trifolium repens* (white clover) cultivar Grasslands Huia; [d], out of ten clover seedlings inoculated for each tranconjugant or transconjugant mixture; [e], Transfer frequency is not indicated because these recipients were resistant to both antibiotics (Km + Rif), and the transconjugant mixture was inoculated on clover plants to confirm the expression of *Sym*::R68.45; [f], Donor, *Escherichia coli* strain PN200 containing plasmid pPN1; [g], negative control for nodulation test; [h], positive control, strain PN165 to confirm the transfer of pPN1; [i], *Rhizobium leguminosarum* biovar trifolii strain ICMP2163 was used as a positive control for plant nodulation test.

Three negative controls were used in the plant test, namely, sterile water, the donor *Escherichia coli* strains PN200 or ATCC9637 and the recipient soil bacteria. The positive control *Rhizobium leguminosarum* biovar trifolii strain ICMP2163 nodulated clover and so did the transconjugant soil bacteria harbouring pSym. It was concluded that the transconjugant soil bacteria had accepted and expressed pSym on white clover.

The nodules that formed on white clover were subsequently studied by light and electron microscopy.

3. 5 Presence of bacteroids in the nodules formed by transconjugant soil bacteria

Representative results of light and electron microscopy for strain KJ30 are shown in Figure 12.

Figure 12A shows the xylem and pholem tissue with the whole cross section of the nodule protruding away from the root. In Figure 12B the colonization of bacteroids within the cytoplasm of the plant cell within the nodule can be observed and some nodule cells are vaculated and do not contain many bacteroids. In contrast the nodule cells of bacteria inoculated with the inoculant bacteria *Rhizobium leguinosarum* biovar trifolii strain ICMP2163 were filled with bacteroids. Electron microscopy (Figure 12C) clearly indicated bacteroids within the plant cell and Figure 12D shows bacteroids within the peribacteroid membrane.

A molecular approach was next adopted to probe for the presence of pSym in the whole genome of transconjugant soil bacteria. Such a presence would be consistent with the nodule formation having resulted from gene expression in the transconjugant soil bacteria.

3. 6 Use of *nod*A probe to probe for pSym in transconjugant soil bacteria isolated from nodules

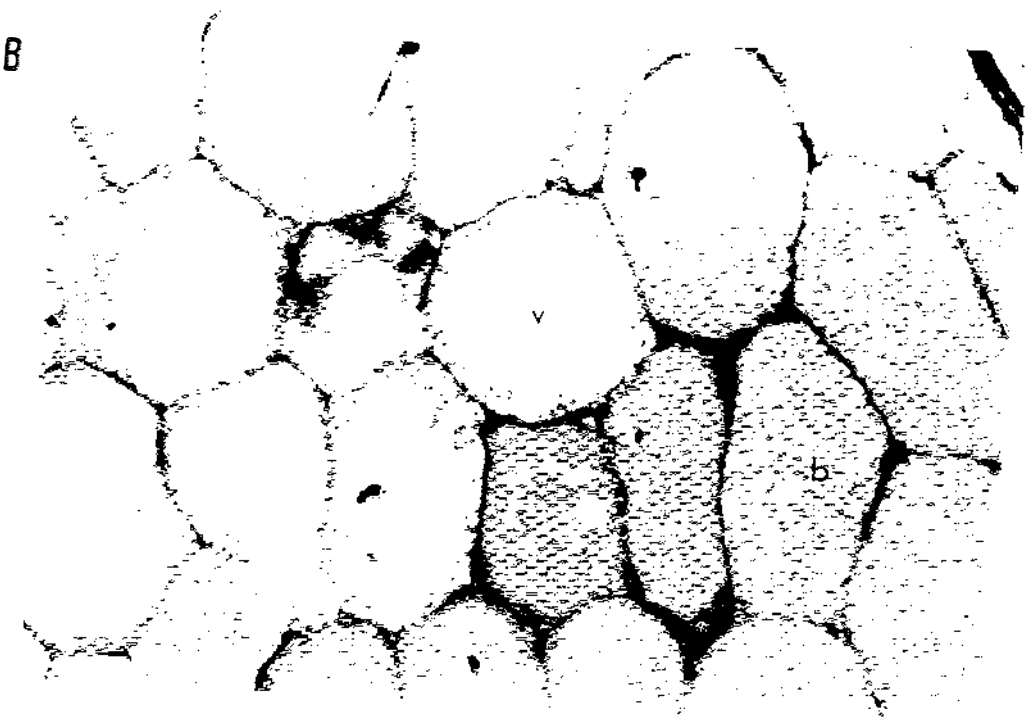
3. 6. 1 Amplification of *nod*A sequence

Since the sequence of *nod*A was known (Rossen *et al.*, 1984) primers were designed which permitted amplification of a 590 bp region in the *nod*A gene by the polymerase chain reaction. The amplified *nod*A sequence is shown in Figure 13. Lane 2 shows the negative control used to test for contamination.

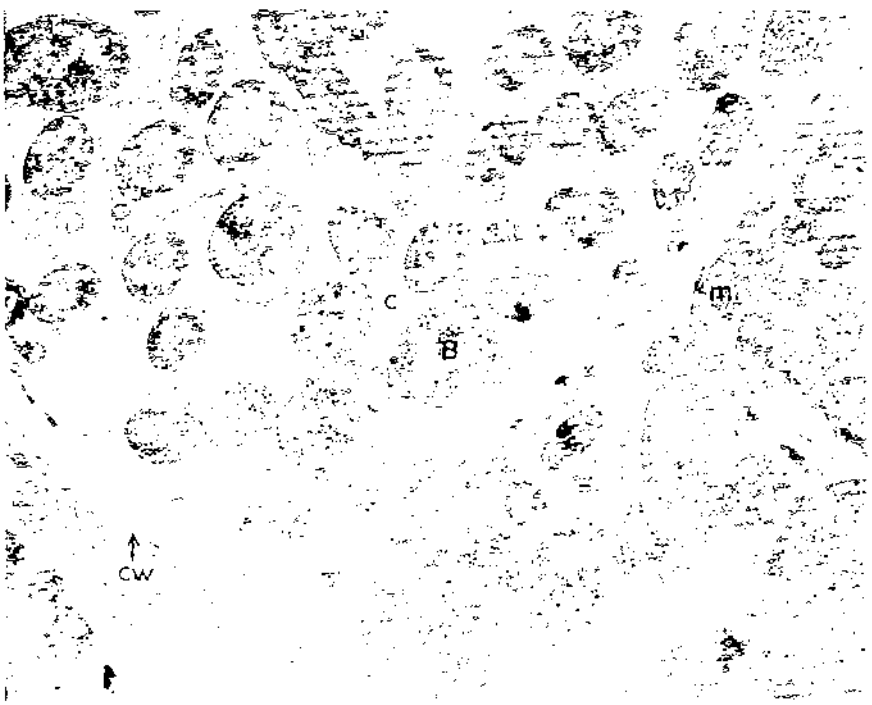
Figure 12. Sections across the nodules formed by the transconjugant soil bacterium KJ30 on white clover (*Trifolium repens*) cultivar Huia. (A) Light microscopy section (160X). (B) Light microscopy section (1000X). (C) Electron microscopy section (13,400X). (D) Electron microscopy section (38,200X). x: Xylem; p: Pholem; v: Vacuole; b: Bacteroids; B: Bacteroid; cw: Cell wall; m: Mitochondrion; pm: Bacteroid cytoplasmic membrane.

A

B



C



D

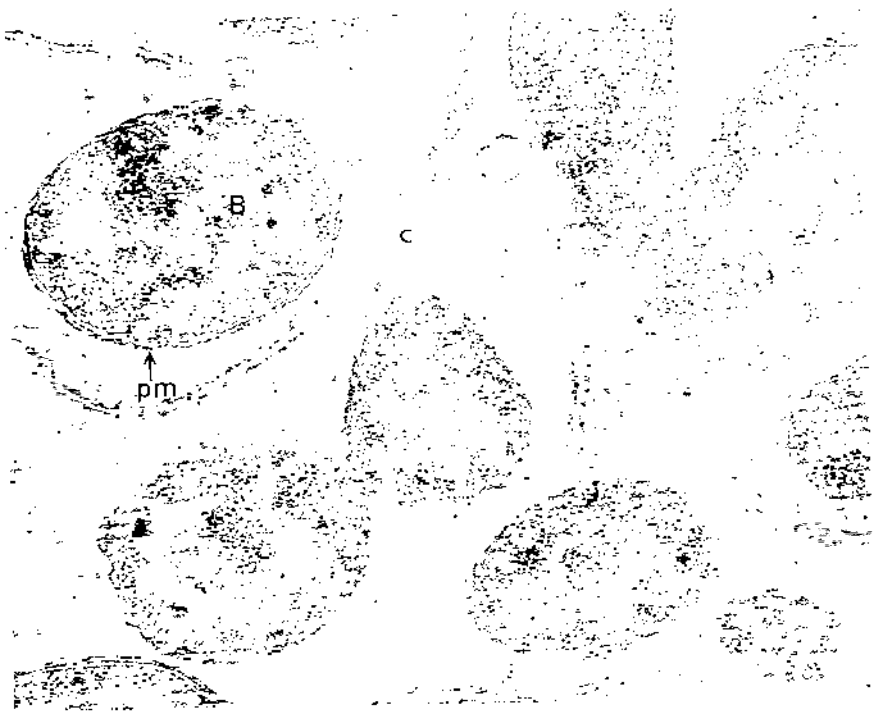
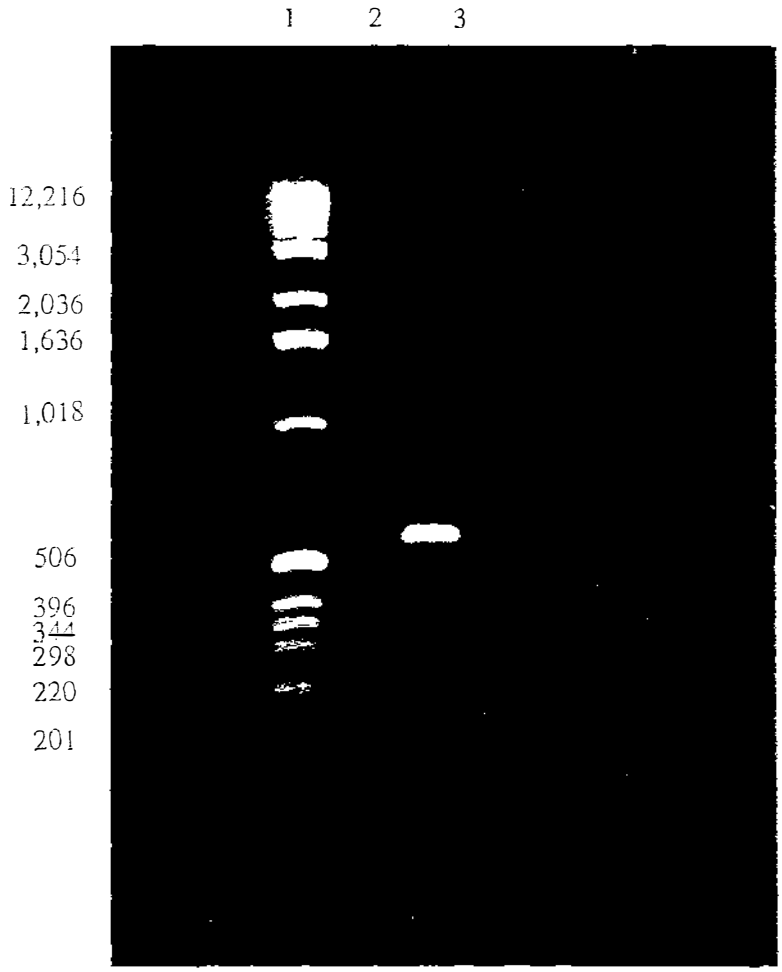


Figure 13. Amplification of a *nod*A sequence in *Rhizobium leguminosarum* biovar trifolii ATCC10004. Lane: 1, 1 Kb (GIBCO, BRL) ladder; Lane: 2, Negative control; Lane: 3, Amplified *nod*A sequence.


1
2
3
12,216
3,054
2,036
1,636
1,018
506
396
344
298
220
201

Lane 3 shows the amplified *nod*A sequence from the type strain of *Rhizobium leguminosarum* biovar trifolii strain ATCC10004. This amplified product was used as a probe to test for the presence of pSym in transconjugant soil bacteria obtained from nodules on white clover (*Trifolium repens* cultivar Grasslands Huia).

3. 6. 2 Probing for the presence of pSym

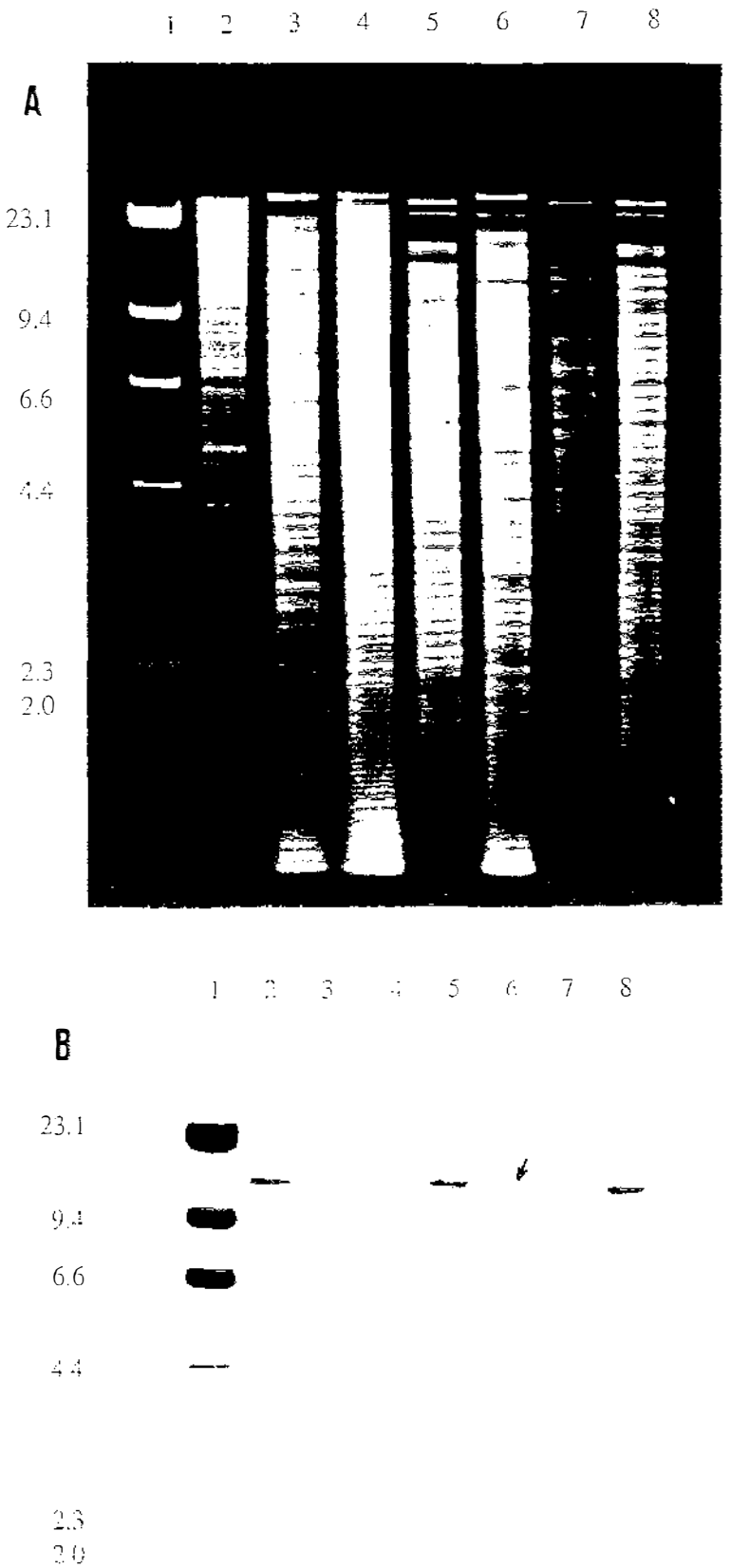
DNAs from the donor (strain PN200), recipient soil bacteria and transconjugant soil bacteria (isolated from nodules) were digested with *Eco*R I and transferred to nylon membrane. Southern blots were probed with the amplified *nod*A probe. Examples of the results obtained are shown in Figure 14A-D. Here are shown representative genomic DNA digests of the donor strain PN200, and transconjugant soil bacteria from nodules. Fig 14B shows a Southern blot of Figure 14A probed with the 590 bp *nod*A probe. Lane 2 is strain PN200 (pPN1) where hybridization occurred at 11.7 Kb with the *nod*A probe, lane 5, 6, 7 and 8 showed similar results indicating the transfer of pSym by conjugation to soil bacteria KJ44, KJ57, KJ19 and KJ23. Lane 3 and 4 have not hybridized to the *nod*A probe indicating that pPN1 was not stably maintained in strains KJ1 and KJ3. A faint band was observed in lane 6 (arrow).

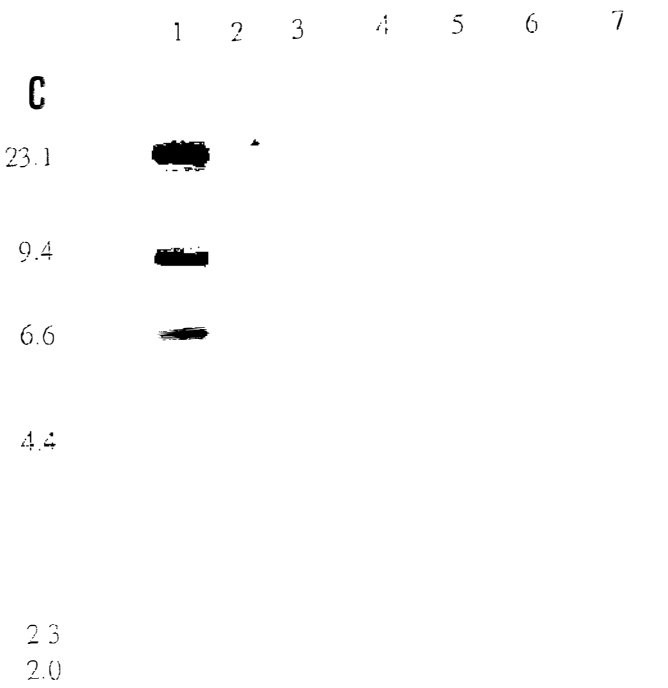
In figure 14C, lane 2 shows DNA from strain PN200 which has hybridized to the *nod*A probe. Lane 3 is blank. Lanes 4 and 6 shows DNAs from recipient soil bacteria KJ5 and KJ13 which have not hybridized to the *nod*A probe. Lanes 5 and 7 shows DNAs from transconjugants of strains KJ5 and KJ13 which have hybridized to the *nod*A probe at 11.7 Kb. Figure 14D shows the presence of pSym in transconjugants of strains KJ26 and KJ27. Eleven strains of transconjugant soil bacteria hybridized with the *nod*A probe. Hybridization occurred at 11.7 Kb in tracks containing DNA from the donor and transconjugant soil bacteria. DNA from the recipient soil bacteria did not hybridize to the *nod*A probe. This indicated that the donor had transferred pPN1 to the recipient soil bacteria and that nodulation was due to the presence of such transferred DNA.

3. 6. 3 Soil isolates from different soil types which contained pSym

Table 5 shows strain numbers for soil isolates from different soil types which contained pSym.

Figure 14. Detection of pSym in transconjugant soil bacteria. (A) Genomic DNA digested with the restriction enzyme *Eco*R I and separated by electrophoresis in a 0.7% (w/v) of agarose gel. Lanes 1 to 8, (left to right) Lambda DNA cut with *Hind* III, DNA from *Escherichia coli* strain PN200 (pPN1) and DNAs from transconjugants of soil bacteria KJ1, KJ3, KJ44, KJ57, KJ19 and KJ23. (B) Autoradiogram of a blot of the above gel probed with a 590 bp *nod*A sequence. (C) Autoradiogram of a blot probed with a 590 bp *nod*A sequence. Lanes 1 to 6, (left to right) Lambda DNA cut with *Hind* III, DNA from *Escherichia coli* strain PN200, blank, DNA from recipient non-nodulating soil bacterium KJ5, DNA from transconjugants of soil bacterium KJ5, DNA from recipient non-nodulating soil bacterium KJ13 and DNA from transconjugants of soil bacterium KJ13. (D) Autoradiogram of a blot probed with a 590 bp *nod*A sequence. Lanes 1 to 7, (left to right) Lambda DNA cut with *Hind* III, DNA from *Escherichia coli* strain PN200, blank, DNA from recipient non-nodulating soil bacterium KJ26, DNA from transconjugants of soil bacterium KJ26, DNA from recipient non-nodulating soil bacterium KJ27 and DNA from transconjugants of soil bacterium KJ27. Arrowhead is explained in text.


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


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

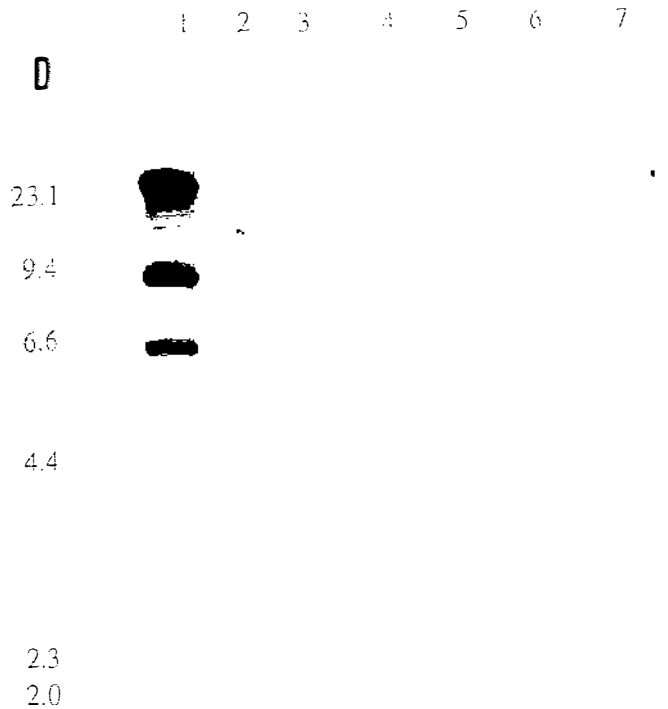

1 2 3 4 5 6 7
D
23.1
9.4
6.6
4.4
2.3
2.0

Table 5. Soil isolates expressing pSym

Soil type[a]	Transconjugant soil bacteria[b]	
	Nodulate white clover	Contain *nod*A gene
Manawatu sandy loam	19	19
Ramiha silt loam	3, 17, 30	17, 30
Tokomaru silt loam	5, 23, 26 44, 57, 203	5, 23, 26 44, 57, 203
Kairanga silt loam	13, 27	13, 27
Totals	12	11

[a]: Strain numbers listed should be pre-fixed by the letters KJ; [b]: Soil types are described in Table 1. NB. I left out KJ1 because it did not nodulate or contain *nod*A so it was not one of the strains of interest.

Note that KJ3 nodulated but *nod*A was not detected and further nodulation tests were not carried out on this particular strain. It was assumed that pPN1 was unstable in this background. However R68.45 was detected (section 3. 3. 2). These results show that bacteria which can accept and express pPN1 are present in several soil types, including leached, low P and low pH soil such as Ramiha silt loam.

The soil isolates which expressed the pSym were identified based on rRNA fingerprinting, 16S rRNA sequences, DNA-DNA hybridization and total fatty acid content.

3. 7 Ribosomal hybridization RFLPs

Total DNA *Eco*R I digests were transferred to membranes and these were hybridized with plasmid pKK3535 (Figure 15). This plasmid carries a 7.5 Kb *Bam*H I fragment containing the *Escherichia coli rrn*B operon comprising 5S, 16S and 23S rRNA genes cloned in plasmid pBR322. The representative hybridization patterns are shown in Figure 16.

Soil isolates KJ19, KJ23, KJ30 and KJ44 in Figure 16A and KJ27 in Figure 16B have three bands with identical mobility. Strains KJ19, KJ23, KJ30 and KJ44, had a fourth band with identical mobility which was absent in KJ27. However the fourth band was very faint (arrow) in strains KJ19 and KJ23. Strains KJ19, KJ23, KJ27, KJ30 and KJ44 showed a similar pattern to *Rhizobium leguminosarum* biovar trifolii strains ICMP2163 and ATCC10004. These are shown in Figure 16C. This similarity of pattern suggests that the unknowns mentioned are also *Rhizobium leguminosarum* biovar trifolii, a result consistent with the sequence data to be presented. Soil isolate KJ57 in Fig 16B had a similar pattern to *Rhizobium tropici* IIA strain CFN299 which is shown in Figure 16C and this grouping also agreed with the sequence data to be presented.

Interestingly, the results show that within the one reference species of *Rhizobium leguminosarum* biovar trifolii there are differences between strains. If such variation is diagnostic of such strains then ribosomal RNA fingerprinting might generally be applicable for distinguishing strains within species. The marked differences between some *Rhizobium* species also suggests its usefulness for identification purposes at this level. However more study is required to investigate the variability of these patterns before the fingerprint method could be sufficiently useful for the overall aims in the present study. Nevertheless the observations show that some of the unknowns are very

Figure 15. Plasmid pKK3535 used in ribosomal hybridization.

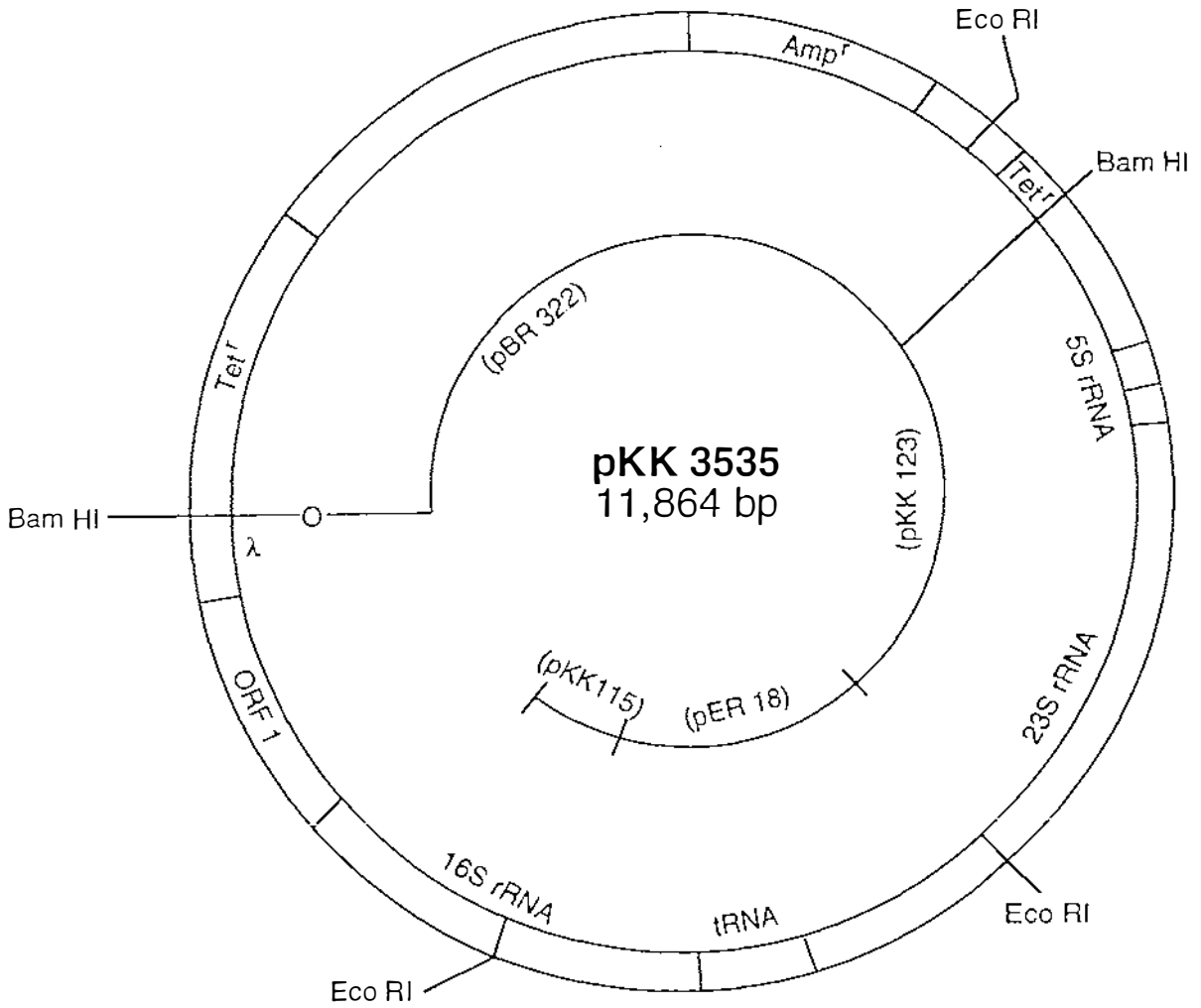
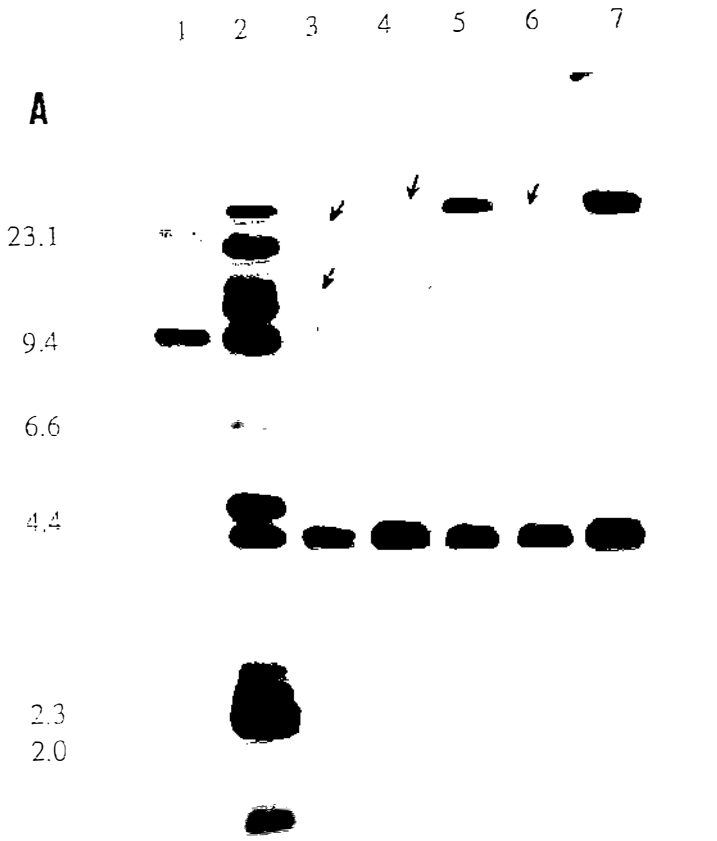
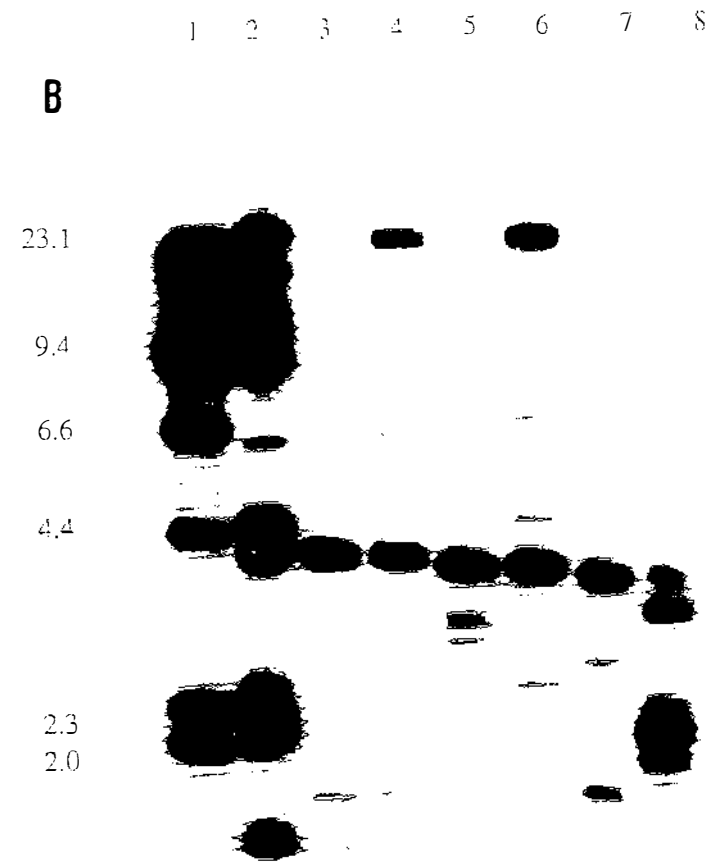
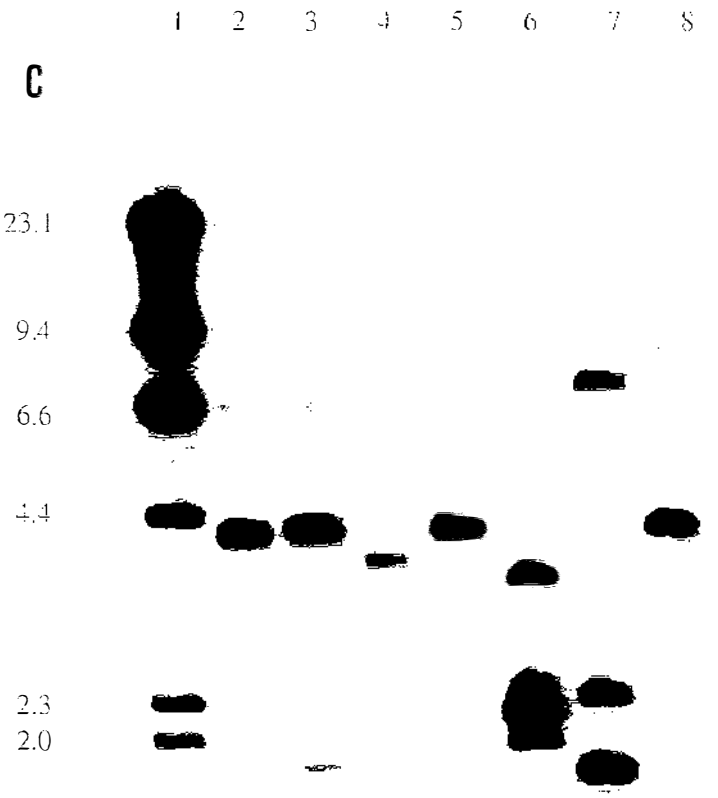

pKK 3535
11,864 bp
Eco RI
Amp^r
Bam HI
Tet^r
5S rRNA
23S rRNA
Eco RI
tRNA
16S rRNA
Eco RI
ORF 1
Tet^r
Bam HI
λ
O
(pBR 322)
(pKK 123)
(pKK115)
(pER 18)

Figure 16. Autoradiograph of total DNA *Eco*R I digest blot which was hybridized with plasmid pKK3535 containing the rRNA operon from *Escherichia coli*. (A) Autoradiogram of a blot probed with pKK3535. Lanes 1 to 8, (left to right) Lambda DNA cut with *Hind* III, DNAs from *Escherichia coli* strain PN200, *Rhizobium leguminosarum* biovar trifolii strain ICMP2163, soil isolates KJ19, KJ44, KJ23 and KJ30. (B) Autoradiogram of a blot probed with pKK3535. Lanes 1 to 8, (left to right) Lambda DNA cut with *Hind* III, DNAs from *Escherichia coli* strain PN200, *Rhizobium leguminosarum* biovar trifolii strain ICMP2163, soil isolates KJ27, KJ203, NR42, KJ5 and KJ57. (C) Autoradiogram of a blot probed with pKK3535. Lanes 1 to 8, (left to right) Lambda DNA cut with *Hind* III, DNAs from *Rhizobium leguminosarum* biovar trifolii strain ICMP2163, *Rhizobium leguminosarum* biovar trifolii strain ATCC10004, *Rhizobium loti* strain ATCC33669, *Rhizobium meliloti* strain NZP2011, *Rhizobium tropici* IIA strain CFN299, *Rhizobium fredii* strain USDA205 and *Rhizobium leguminosarum* biovar trifolii strain ATCC14480. Arrowhead is explained in text.


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


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


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

similar to *Rhizobium leguminosarum* biovar trifolii, whilst others are very different. This suggests that a considerable heterogeneity exists in the soil bacteria which can express pSym. To help resolve relationships which remained ambiguous after the fingerprint analysis 16S rDNA sequences were determined from the soil isolates. This also provided a means of testing the relationships suggested from the fingerprint study. Recently many bacterial type strains have been sequenced for their 16S rDNA. This sequence data is now available from electronic data bases. Such information was used to further characterize the soil isolates.

3. 8 16S rRNA sequence data of soil bacteria expressing pSym

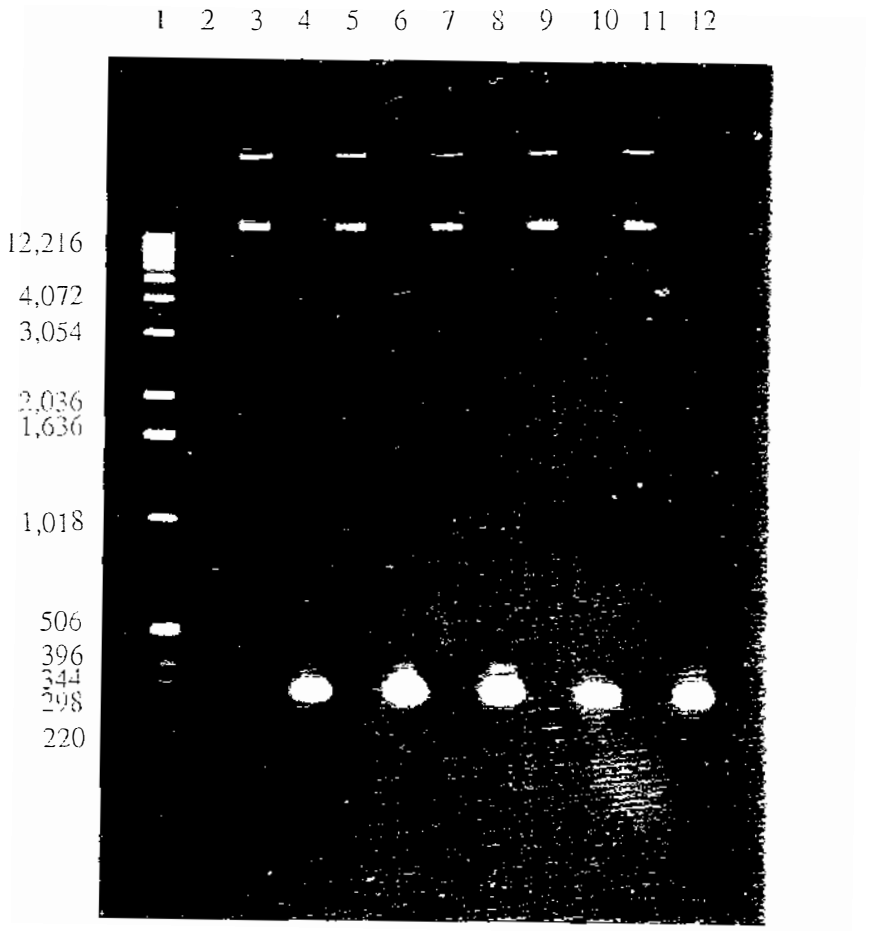
3. 8. 1 Amplification of 16S rRNA

A 260 bp variable region from the 16S rRNA gene was amplified and sequenced from each of the soil bacteria capable of expressing pSym. Figure 17 shows a representative electrophoretic gel of the total genomic DNA and PCR products from several of the soil bacteria. The forward primer Y1 corresponds to positions 20 to 43 in the *Escherichia coli* 16S rRNA sequence and the reverse primer corresponds to *E. coli* positions 361 to 338. The primers themselves, are complementary to conserved sequences and have previously been shown to readily amplify the correct fragment from all alpha *Proteobacteria* tested (Young *et al.*, 1991). An amplified product of approximately 340 bp was observed on a 2% agarose gel (Figure 17). The negative control (lane 1) confirmed no contamination of the amplified products.

3. 8. 2 Known sequences used in the alignment of unknown 16S rDNA sequences

The known sequences used in the alignment of the 16S rDNA sequences and their accession numbers were from: *Bradyrhizobium japonicum* strain USDA31; (GenBank M55487), *Azorhizobium caulinodans* strain ORS571; (GenBank M55491), *Rhodomicrobium vannielii*; (GenBank M34127), *Rhizobium loti* strain NZP2213; (EMBL X67229), *Rhizobium galegae* strain HAMBI540; (EMBL X67226), *Agrobacterium vitis* strain NCPPB3554; (EMBL X67225), *Agrobacterium tumefaciens* strain DMS30150; (GenBank M11223), *Rhizobium etli* strain Or191; (GenBank M55236), *Rhizobium meliloti* strain NZP4017; (GenBank M55495), *Rhizobium fredii* strain USDA205; (GenBank M74163), *Agrobacterium rhizogenes* strain ATCC15834, (EMBL X67224), *Rhizobium tropici* IIA strain CFN299; (EMBL X67233), *Rhizobium*

Figure 17. Total genomic DNAs and amplified fragments of the 16S rRNA of soil bacteria expressing pSym obtained by the polymerase chain reaction. Lane 1 is the 1 Kb ladder (GIBCO, BRL), lane 2 is the negative control. Lanes 3, 5, 7, 9, and 11 indicate total genomic DNA of soil isolates KJ19, KJ23, KJ26, KJ27 and KJ30 respectively. Lanes 4, 6, 8, 10 and 12 indicate the amplified sequences of soil isolates KJ19, KJ23, KJ26, KJ27 and KJ30 respectively.


1 2 3 4 5 6 7 8 9 10 11 12
12,216
4,072
3,054
2,036
1,636
1,018
506
396
344
298
220

leguminosarum biovar trifolii strain ATCC14480; (EMBL X67227). These sequences were obtained from the Genetic Sequence Data Bank (GenBank) and European Molecular Biology Laboratory (EMBL) data libraries.

3. 8. 3 Alignment with unknown sequences of 16S rRNA

The 16S rDNA sequences derived by direct sequencing of the amplified fragments from the 15 strains of Gram negative soil bacteria shown to express the pSym were aligned with the corresponding sequences from the data banks. Such an alignment was unambiguous as there were no deletions or insertions between any of the compared sequences. These 260 bp fragments corresponded to positions 44 to 337 in the *Escherichia coli* 16S rDNA sequences. This permits quantification of the differences based on Figure 18.

The alignment in Figure 18 shows that the sequences of soil isolates KJ19, KJ23, KJ27, KJ30, NR41 and KJ44 are similar to *Rhizobium leguminosarum* biovar trifolii strain ATCC14480. Gram negative soil isolate KJ57 showed sequences similar to *Rhizobium tropici* IIA strain CFN299, and NR42 showed sequences similar to *Rhizobium meliloti* strain NZP4017 and *Rhizobium fredii* strain USDA205. Soil isolate KJ203 showed sequences similar to *Rhizobium etli* strain Or191 and the sequence of *Rhizobium loti* strain NZP2213 showed sequences similar to that of soil isolates KJ13 and KJ5.

The sequences of soil isolates KJ17 and KJ26 were similar to each other and are different to those of strain NR64, but were most closely related to the sequence of *Rhizobium etli* strain Or191 and showed similarities of 98% and 95% respectively. The sequences of strain OR168 was most closely related to *Rhizobium leguminosarum* biovar trifolii strain ATCC14480 with similarities of 98%.

3. 9 Phylogenetic relationships

An analysis of phylogenetic relationships between Gram negative soil isolates expressing pSym and bacterial strains belonging to rhizobia, agrobacteria, and other members of the alpha-subgroup of the *Proteobacteria* is shown in Figure 19 A-B. The distance matrix shown in Table 6 was derived from the sequences given in Appendix 1 and previously published sequences available from the Genbank and EMBL data

Figure 18. Aligned sequences of part of the 16S rRNA gene from fifteen Gram negative soil bacteria which could express pSym from *Rhizobium leguminosarum* biovar trifolii and some related bacteria. Sequences are aligned to known sequence from the Genbank and EMBL Data Library. *R. legumin.*, *Rhizobium leguminosarum* biovar trifolii strain ATCC14480 (EMBL accession number X67227); *R. tropici*, *Rhizobium tropici* IIA strain CFN299 (EMBL accession number X67233); *A. rhizoge.*, *Agrobacterium rhizogenes* strain ATCC15834 (EMBL accession number X67224); *R. melilot.*, *Rhizobium meliloti* strain NZP4017 (GenBank accession number M55495); *R. fredii*, *Rhizobium fredii* strain USDA205 (GenBank accession number M74163); *R. etli*, *Rhizobium etli* strain Or191 (GenBank accession number M55236); *Agro. tum.*, *Agrobacterium tumefaciens* strain DMS30150 (GenBank accession number M11223); *A. vitis*, *Agrobacterium vitis* strain NCPPB3554 (EMBL accession number X67225); *R. galega*, *Rhizobium galegae* strain HAMBI540 (EMBL accession number X67226); *R. loti*, *Rhizobium loti* strain NZP2213 (EMBL accession number X67229); *Rho. vanni.*, *Rhodomicrobium vannielii* (GenBank accession number M34127); *Azo. cauli.*, *Azorhizobium caulinodans* strain ORS571 (GenBank accession number M55491); *Bradyrhiz.*, *Bradyrhizobium japonicum* strain USDA31 (GenBank accession number M55487).

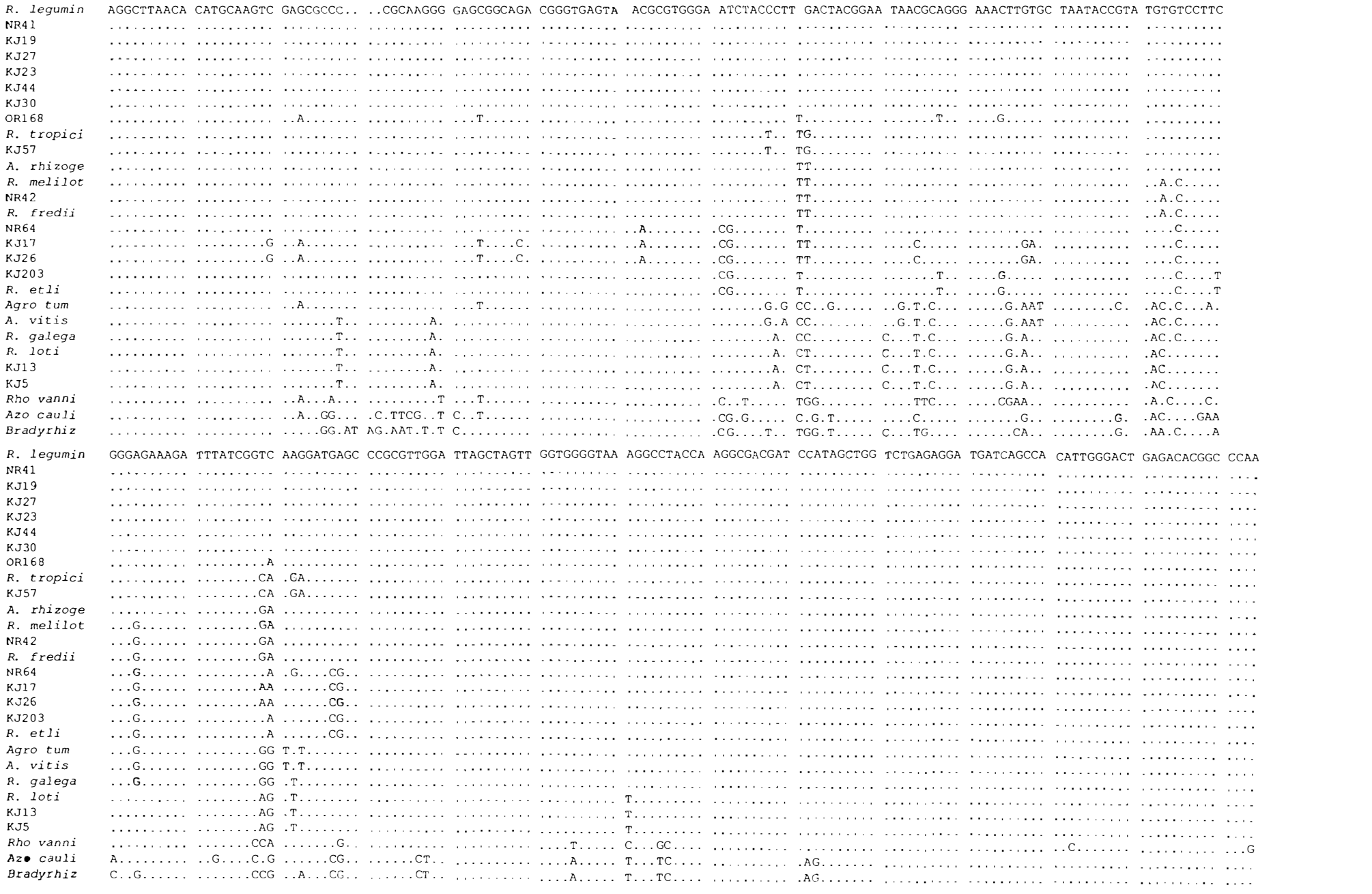
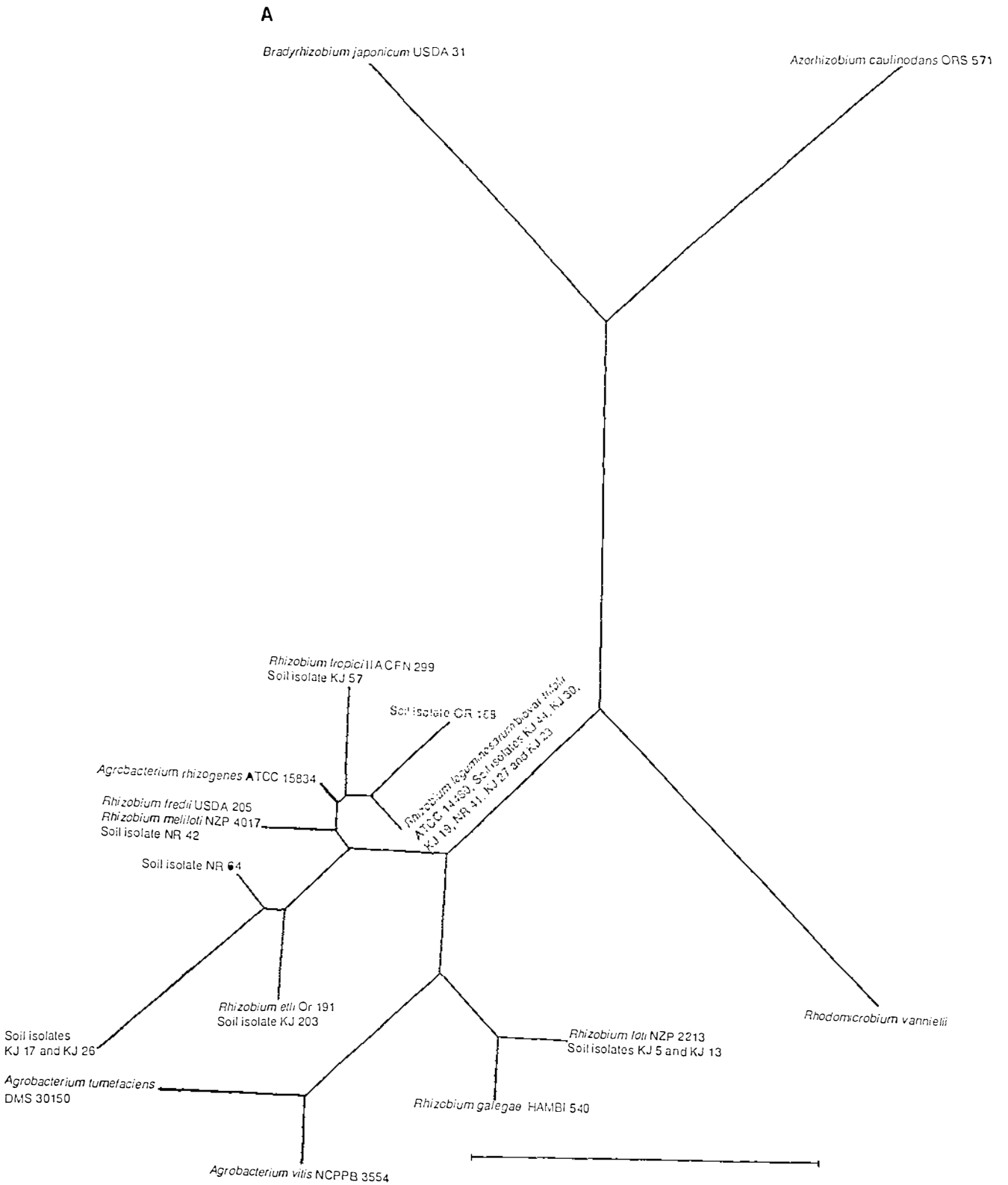

R. legumin AGGCTTAACA CATGCAAGTC GAGCGCCC.. ..CGCAAGGG GAGCGGCAGA CGGGTGAGTA ACGCGTGGGA ATCTACCCTT GACTACGGAA TAACGCAGGG AAACTTGTGC TAATACCGTA TGTGTCCTTC
NR41
KJ19
KJ27
KJ23
KJ44
KJ30
OR168
R. tropici
KJ57
A. rhizoge
R. melilot
NR42
R. fredii
NR64
KJ17
KJ26
KJ203
R. etli
Agro tum
A. vitis
R. galega
R. loti
KJ13
KJ5
Rho vanni
Azo cauli
Bradyrhiz
R. legumin GGGAGAAAGA TTTATCGGTC AAGGATGAGC CCGCGTTGGA TTAGCTAGTT GGTGGGGTAA AGGCCTACCA AGGCGACGAT CCATAGCTGG TCTGAGAGGA TGATCAGCCA CATTGGGACT GAGACACGGC CCAA
NR41
KJ19
KJ27
KJ23
KJ44
KJ30
OR168
R. tropici
KJ57
A. rhizoge
R. melilot
NR42
R. fredii
NR64
KJ17
KJ26
KJ203
R. etli
Agro tum
A. vitis
R. galega
R. loti
KJ13
KJ5
Rho vanni
Azo cauli
Bradyrhiz

Figure 19. (A) Unrooted phylogenetic tree, obtained by the Neighbour-Joining method from a distance matrix of 16S rRNA sequences (with Jukes-Cantor corrections), showing the relationship of soil bacteria expressing pSym, *Rhizobium*, *Agrobacterium* species and related taxa from the alpha-2 subgroup of the *Proteobacteria*. Bar = 0.05 evolutionary distance units as calculated by the Jukes-Cantor method. (B) Unrooted (and unweighted) majority rule consensus tree. The tree was constructed from 16S rDNA sequences using Jukes-Cantor pairwise distance estimates and then Neighbour-Joining. The consensus tree was that found from 1000 bootstrap replicates. The number of times a particular grouping was obtained from the bootstrap samples is indicated by the values on the internal edges of the tree.


A
Bradyrhizobium japonicum USDA 31
Azorhizobium caulinodans ORS 571
Rhizobium tropici IIA CFN 299
Soil isolate KJ 57
Soil isolate OR 155
Rhizobium leguminosarum biovar trifolii
ATCC 14480, Soil isolates KJ 44, KJ 30,
KJ 19, NR 31, KJ 27 and KJ 23
Agrobacterium rhizogenes ATCC 15834
Rhizobium fredii USDA 205
Rhizobium meliloti NZP 4017
Soil isolate NR 42
Soil isolate NR 64
Rhizobium etli Or 191
Soil isolate KJ 203
Soil isolates
KJ 17 and KJ 26
Agrobacterium tumefaciens
DMS 30150
Agrobacterium vitis NCPPB 3554
Rhizobium galegae HAMBI 540
Rhizobium loti NZP 2213
Soil isolates KJ 5 and KJ 13
Rhodomicrobium vannielii

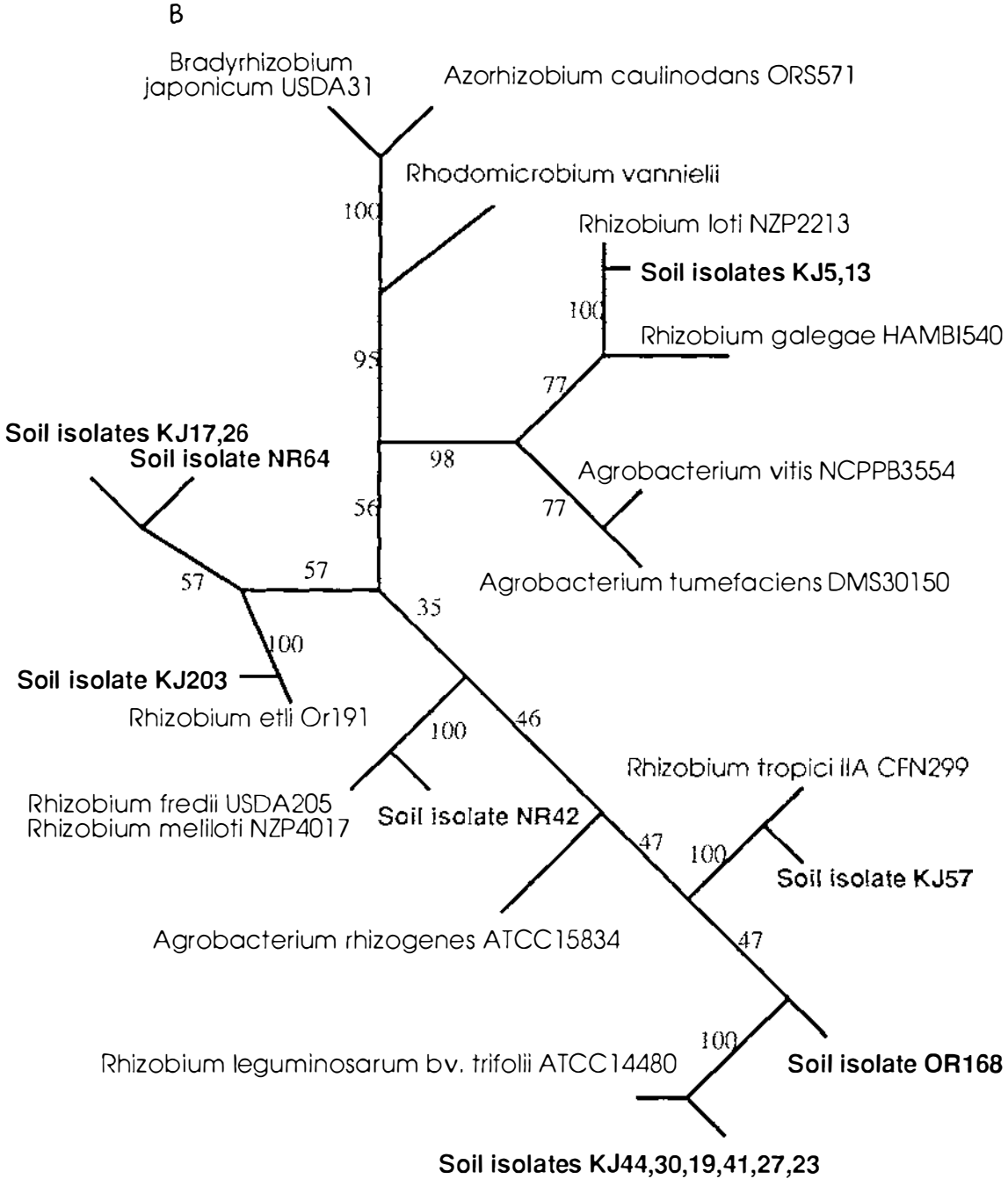

B
Bradyrhizobium japonicum USDA31
Azorhizobium caulinodans ORS571
Rhodomicrobium vannielii
100
Rhizobium loti NZP2213
Soil isolates KJ5,13
100
Rhizobium galegae HAMBI540
95
77
Soil isolates KJ17,26
Soil isolate NR64
98
Agrobacterium vitis NCPPB3554
56
77
57
57
Agrobacterium tumefaciens DMS30150
35
100
Soil isolate KJ203
Rhizobium etli Or191
100
46
Rhizobium tropici IIA CFN299
Rhizobium fredii USDA205
Rhizobium meliloti NZP4017
Soil isolate NR42
47
100
Soil isolate KJ57
Agrobacterium rhizogenes ATCC15834
47
100
Rhizobium leguminosarum bv. trifolii ATCC14480
Soil isolate OR168
Soil isolates KJ44,30,19,41,27,23

Table 6. Distance matrix (with Jukes-Cantor corrections) for the 16S rDNA sequences of rhizobia, Gram negative soil bacteria expressing pSym from *Rhizobium leguminosarum* biovar trifolii and other members of the alpha-2 subgroup of the *Proteobacteria* based on alignment of 264 nucleotides.

Strain	evolutionary distances																										
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	218	22	23	24	25	26	27	28
Bradyrhiz	0.0000	0.1056	0.1645	0.1740	0.1740	0.1740	0.1693	0.1885	0.1934	0.1505	0.1505	0.1693	0.1693	0.1505	0.1551	0.1551	0.1551	0.1693	0.1551	0.1551	0.1885	0.1740	0.1740	0.1740	0.1740	0.1740	0.1740	0.174
Azo cauli		0.0000	0.1693	0.1836	0.1836	0.1836	0.1983	0.2183	0.1934	0.1645	0.1645	0.1645	0.1645	0.1645	0.1836	0.1836	0.1836	0.1740	0.1885	0.1885	0.1645	0.1645	0.1645	0.1645	0.1645	0.1645	0.1645	0.164
Rho vanne			0.0000	0.1232	0.1232	0.1232	0.1322	0.1505	0.1413	0.1277	0.1277	0.1277	0.1277	0.1277	0.1232	0.1232	0.1232	0.1100	0.1144	0.1144	0.1056	0.1188	0.1188	0.1188	0.1188	0.1188	0.1188	0.118
KJ5				0.0000	0.0000	0.0000	0.0192	0.0591	0.0883	0.1012	0.1012	0.1056	0.1056	0.0926	0.0673	0.0673	0.0673	0.0591	0.0715	0.0715	0.0799	0.0632	0.0632	0.0632	0.0632	0.0632	0.0632	0.063
KJ13					0.0000	0.0000	0.0192	0.0591	0.0883	0.1012	0.1012	0.1056	0.1056	0.0926	0.0673	0.0673	0.0673	0.0591	0.0715	0.0715	0.0799	0.0632	0.0632	0.0632	0.0632	0.0632	0.0632	0.063
R. loti						0.0000	0.0192	0.0591	0.0883	0.1012	0.1012	0.1056	0.1056	0.0926	0.0673	0.0673	0.0673	0.0591	0.0715	0.0715	0.0799	0.0632	0.0632	0.0632	0.0632	0.0632	0.0632	0.063
R. galega							0.0000	0.0389	0.0673	0.0883	0.0883	0.1012	0.1012	0.0799	0.0550	0.0550	0.0550	0.0632	0.0757	0.0757	0.0841	0.0673	0.0673	0.0673	0.0673	0.0673	0.0673	0.067
A. vitis								0.0000	0.0309	0.1056	0.1056	0.1100	0.1100	0.1012	0.0715	0.0715	0.0715	0.0799	0.0883	0.0883	0.1012	0.0841	0.0841	0.0841	0.0841	0.0841	0.0841	0.084
Agro tum									0.0000	0.1188	0.1188	0.1056	0.1056	0.1144	0.0841	0.0841	0.0841	0.0926	0.1012	0.1012	0.0969	0.0969	0.0969	0.0969	0.0969	0.0969	0.0969	0.096
R. etli										0.0000	0.0000	0.0509	0.0509	0.0192	0.0389	0.0389	0.0389	0.0429	0.0550	0.0550	0.0349	0.0429	0.0429	0.0429	0.0429	0.0429	0.0429	0.042
KJ203											0.0000	0.0509	0.0509	0.0192	0.0389	0.0389	0.0389	0.0429	0.0550	0.0550	0.0349	0.0429	0.0429	0.0429	0.0429	0.0429	0.0429	0.042
KJ17												0.0000	0.0000	0.0389	0.0550	0.0550	0.0550	0.0591	0.0757	0.0757	0.0632	0.0715	0.0715	0.0715	0.0715	0.0715	0.0715	0.071
KJ26													0.0000	0.0389	0.0550	0.0550	0.0550	0.0591	0.0757	0.0757	0.0632	0.0715	0.0715	0.0715	0.0715	0.0715	0.0715	0.071
NR64														0.0000	0.0349	0.0349	0.0349	0.0389	0.0429	0.0429	0.0469	0.0389	0.0389	0.0389	0.0389	0.0389	0.0389	0.038
R. melilot															0.0000	0.0000	0.0000	0.0115	0.0309	0.0309	0.0349	0.0270	0.0270	0.0270	0.0270	0.0270	0.0270	0.027
NR42																0.0000	0.0000	0.0115	0.0309	0.0309	0.0349	0.0270	0.0270	0.0270	0.0270	0.0270	0.0270	0.027
R. fredii																	0.0000	0.0115	0.0309	0.0309	0.0349	0.0270	0.0270	0.0270	0.0270	0.0270	0.0270	0.027
A. rhizoge																		0.0000	0.0192	0.0192	0.0231	0.0153	0.0153	0.0153	0.0153	0.0153	0.0153	0.015
R. tropici																			0.0000	0.0000	0.0349	0.0270	0.0270	0.0270	0.0270	0.0270	0.0270	0.027
KJ57																				0.0000	0.0349	0.0270	0.0270	0.0270	0.0270	0.0270	0.0270	0.027
OR168																					0.0000	0.0231	0.0231	0.0231	0.0231	0.0231	0.0231	0.023
R. legumin																						0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.000
NR41																							0.0000	0.0000	0.0000	0.0000	0.0000	0.000
KJ19																								0.0000	0.0000	0.0000	0.0000	0.000
KJ27																									0.0000	0.0000	0.0000	0.000
KJ23																										0.0000	0.0000	0.000
KJ44																											0.0000	0.000
KJ30																												0.000

Bradyrhiz, Bradyrhizobium japonicum strain USDA31; Azo cauli, *Azorhizobium caulinodans* strain ORS571; *Rho vanni, Rhodomicrobium vannielii*; *R. loti, Rhizobium loti* strain NZP2213; *R. galega, Rhizobium galegae* strain HAMBI540; *A. vitis, Agrobacterium vitis* strain NCPPB3554; *Agro tum, Agrobacterium tumefaciens* strain DMS30150; *R. etli, Rhizobium etli* strain Or191; *R. melilot, Rhizobium meliloti* strain NZP4017; *R. fredii, Rhizobium fredii* strain USDA205; *A. rhizoge, Agrobacterium rhizogenes* strain ATCC15834, *R. tropici, Rhizobium tropici* IIA strain CFN299; *R. legumin, Rhizobium leguminosarum* biovar trifolii strain ATCC14480.

libraries. Figure 19A was constructed using the Jukes-Cantor pairwise distances shown in Table 6 and the Neighbour-Joining (NJ) method (Saitou and Nei, 1987).

To estimate the relative occurance of patterns in the data which supported the relationships shown in the reconstructed tree a bootstrap sampling analysis of 1000 trees was carried out and is shown in Figure 19B.

The 95% bootstrap value seen in the unrooted phylogenetic tree (Figure 19A, B) indicates strong support for grouping isolates with agrobacteria and rhizobia.

Within the agrobacteria and rhizobia group soil isolates KJ5 and KJ13 form a distinct subgroup with *Agrobacterium tumefaciens* strain DSM30150, *Agrobacterium vitis* strain NCPPB3554, *Rhizobium galegae* strain HAMBI540 and *Rhizobium loti* strain NZP2213 (98% bootstrap support; Fig 19B). The alignment (see section 3. 8. 3), the NJ tree (Fig 19A) and bootstrap (19B) also indicate they are identical to *R. loti.*

Bootstrap values shown in Fig 19B indicate that the exact relationship between some rhizobia and agrobacteria species is not resolved with a great deal of certainty. However most soil isolates can be clearly identified as specific rhizobial species (see Fig 19A, B, section 3. 8. 3).

3. 10 Genomic relatedness between type strains and soil bacteria expressing pSym

The degree of genomic hybridization at 65°C was determined against type strains for each of the soil isolates. The type strains used were *Rhizobium leguminosarum* biovar trifolii strains ICMP2163 and ATCC10004, *Rhizobium tropici* IIA strain CFN299, *Rhizobium loti* strain ATCC33669, *Rhizobium meliloti* strain NZP2011. The results are shown in Table 7. Of the fifteen soil isolates examined five identifications could be made based on their DNA relatedness to known type strains.

Soil isolates KJ5 and KJ13 were 91% and 90% similar to *Rhizobium loti* strain ATCC33669 respectively which is in agreement with the sequence data. Soil isolates KJ19, KJ23, KJ27, KJ30, NR41 and KJ44 were 91% and 90% similar to *Rhizobium leguminosarum* biovar trifolii strains ICMP2163 and ATCC10004 respectively which is also in agreement with the sequence data. Soil isolates KJ17, KJ26, NR64, OR168 and KJ203 showed 77% and 75% similarity with *Rhizobium leguminosarum* biovar trifolii strains ICMP2163 and ATCC10004 respectively. This was in partial agreement

Table 7. DNA-DNA relatedness at 65°C between DNAs from soil bacteria which express pRtr514a and reference DNAs from *Rhizobium leguminosarum* biovar trifolii, *Rhizobium tropici*, *Rhizobium loti* and *Rhizobium meliloti*.

Unlabelled DNA's	Reference labelled DNA's				
	Rhizobium leguminosarum biovar trifolii		*Rhizobium tropici* IIA	*Rhizobium loti*	*Rhizobium meliloti*
	ICMP2163	ATCC10004	CFN299	ATCC33669	NZP2011
Reference strains					
ICMP2163	**100**	**90**	22	14	8
ATCC10004	**93**	**100**	26	15	9
CFN299	22	24	**100**	18	31
ATCC33669	14	15	19	**100**	25
NZP2011	8	9	31	25	**100**
ATCC9737[a]	0	0	0	0	0
Soil isolates					
KJ5	16	14	18	**91**	25
KJ13	15	16	17	**90**	24
KJ17	76	75	25	18	10
KJ19	**89**	**90**	24	15	9
KJ23	**91**	**89**	22	15	7
KJ26	77	72	24	19	9
KJ27	**93**	**92**	23	14	8
KJ30	**92**	**90**	22	14	8
KJ44	**90**	**91**	23	14	9
KJ57	22	24	**92**	18	30
KJ203	75	72	25	16	10
NR41	**90**	**89**	24	14	10
NR42	8	9	32	26	**91**
NR64	78	77	26	15	11
OR168	79	80	23	15	9

[a]: *Escherichia coli*.

with the sequence data and reflects a relatively close relationship between *R. etli* and *R. leguminosarum* and the unavailability of a *R. etli* reference strain. Soil isolate KJ57 was found to be 92% similar to *Rhizobium tropici* IIA strain CFN299 in agreement with the sequence data. Soil isolate NR42 was 91% similar with *Rhizobium meliloti* strain NZP2011 in agreement with the sequence data.

3. 11 Identification of soil bacteria based on total fatty acids

3. 11. 1 Fatty acids used in the identification

Total fatty acid composition of known bacterial species is shown in Table 8. The fatty acids and related compounds were identified by determining their equivalent carbon chain lengths (ECL) and comparing these with known standards using microbial identification system (MIS) software. The quantity of each fatty acid present was expressed as a percentage of the total named fatty acids present in concentrations of more than 0.2%. Mean fatty acid profiles were computed for each known species and a computerized library database was constructed using the major fatty acid components from each taxon as shown in Table 8. Table 8 also indicates minor fatty acids which were omitted from the library database. The major fatty acids used in the identification were 13:0 ISO3 OH, 13:1 AT 12-13, 15:0 2OH, 15:0 ISO 3OH, 16:0, 16:3OH, 16:1 $_{w}$7C, 17:0, 17:0 ISO, 17:0 CYCLO, 18:0, 18:1 2OH, 19:0 CYCLO $_{w}$8C, 19:0 10 METHYL, 20:2 $_{w}$6, 9C, 20:3 $_{w}$6, 9, 12C, summed feature 3 and summed feature 7. In the table summed feature 3 corresponds to 12:0 aldehyde (?), 16:1 ISO I/14:0 3OH, 14:0 3OH/16:1 ISO I fatty acids and an unknown compound at an ECL value of 10.928. Summed feature 7 is composed of 18:1 $_{w}$7Cis/$_{w}$9Trans/$_{w}$12Trans, 18:1 $_{w}$9Cis/$_{w}$12Trans/$_{w}$7Cis and 18:1 $_{w}$12Trans/$_{w}$9Trans/$_{w}$7Cis fatty acids.

3. 11. 2 Identification of soil isolates

The total fatty acid composition of each unknown soil isolate was determined and the fatty acid profile compared to the existing library data base for its identification. Table 9 indicates the total fatty acid profile and the identification of each unknown soil isolate. Identification is based on the quantity of each fatty acid present as well as its presence or absence.

Table 8. Total fatty acid composition of known bacterial strains

Feature	Known bacterial strains							
	Rhizobium tropici		*Rhizobium leguminosarum*		*Rhizobium loti*		*Rhizobium meliloti*	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
10:0	-	-	-	-	-	-	-	-
10:0 3OH	-	-	-	-	-	-	-	-
11:0 2OH	-	-	-	-	-	-	-	-
unknown 11.541	0.02	0.07*	-	-	-	-	-	-
unknown 14.966	-	-	-	-	-	-	-	-
12:0 3OH	-	-	-	-	0.40	0.40*	-	-
13:0 ISO 3OH	-	-	-	-	1.39	0.41	-	-
13:1 AT 12-13	0.07	0.15*	-	-	-	-	-	-
14:0	-	-	-	-	-	-	-	-
15:0	-	-	-	-	-	-	-	-
15:0 ANTEISO	-	-	0.03	0.19*	0.14	0.58*	-	-
15:0 ISO	0.02	0.09*	0.03	0.15*	0.16	0.29*	-	-
15:0 2OH	-	-	0.91	0.52	-	-	-	-
15:0 3OH	-	-	0.06	0.23*	-	-	-	-
15:0 ISO 3OH	3.42	0.45	-	-	-	-	-	-
15:1 $_{w}$8C	-	-	-	-	-	-	-	-
16:0	7.73	1.92	4.29	1.36	16.38	2.21	4.94	1.14
16:0 3OH	3.90	0.24	1.42	0.36	-	-	0.68	0.12
16:0 ISO	-	-	-	-	-	-	-	-
16:1 $_{w}$7C	0.16	0.22*	0.52	0.36	0.45	0.57*	0.34	0.21
17:0	0.02	0.12*	0.43	0.67*	0.59	0.65	0.54	0.24
17:0 ISO	0.93	0.26	-	-	9.11	2.27	-	-
17:0 ISO 3OH	0.31	0.22*	-	-	-	-	-	-
17:0 CYCLO	0.68	0.43	0.13	0.27*	0.82	0.54	0.75	0.31
17:0 3OH	-	-	0.01	0.07*	-	-	0.05	0.10*
17:1 $_{w}$6C	-	-	0.01	0.08*	-	-	-	-
17:1 $_{w}$7C	-	-	-	-	-	-	-	-
17:1 $_{w}$8C	-	-	0.05	0.16*	0.14	0.35*	0.09	0.14*
18:0	2.89	1.22	8.04	2.14	5.01	1.40	2.70	0.72
18:1 2OH	0.83	0.69	0.04	0.22*	0.50	0.71*	-	-
18:1 $_{w}$9C	-	-	0.13	0.29*	0.38	0.49*	0.09	0.07*
19:0	-	-	0.01	0.05*	-	-	-	-
19:0 CYCLO $_{w}$8C	49.54	10.05	14.80	5.75	25.19	9.19	22.56	8.25
19:0 10 METHYL	-	-	0.07	0.21*	1.37	0.51	0.85	0.26
20:0	-	-	-	-	0.08	0.22*	-	-
20:1 $_{w}$9T	-	-	0.01	0.05*	0.19	0.31*	-	-
20:2 $_{w}$6, 9C	0.40	0.21	-	-	0.06	0.16*	0.02	0.07*
20:3 $_{w}$6, 9, 12C	1.86	0.67	2.43	0.61	-	-	2.94	0.33
SUMMED FEATURE 1[a]	-	-	-	-	-	-	-	-
SUMMED FEATURE 2[b]	-	-	0.01	0.05*	-	-	-	-
SUMMED FEATURE 3[c]	2.35	0.68	6.79	1.05	-	-	6.45	0.70
SUMMED FEATURE 4[d]	-	-	-	-	0.09	0.37*	-	-
SUMMED FEATURE 7[e]	24.85	11.13	59.78	6.48	37.56	7.08	57.01	8.27
SUMMED FEATURE 9[f]	-	-	-	-	-	-	-	-

Feature	Known bacterial strains							
	Rhizobium etli		*Rhizobium huakuii*		*Rhizobium galagae*		*Rhizobium fredii*	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
10:0	-	-	-	-	-	-	-	-
10:0 3OH	-	-	-	-	-	-	-	-
11:0 2OH	0.02	0.10*	-	-	-	-	-	-
unknown 11.541	-	-	-	-	-	-	-	-
unknown 14.966	0.01	0.07*	-	-	-	-	-	-
12:0 3OH	-	-	0.28	0.34*	-	-	-	-
13:0 ISO 3OH	-	-	0.97	0.40	-	-	-	-
13:1 AT 12-13	-	-	-	-	-	-	-	-
14:0	-	-	-	-	-	-	-	-
15:0	-	-	-	-	-	-	-	-
15:0 ANTEISO	-	-	-	-	-	-	0.08	0.05*
15:0 ISO	-	-	-	-	-	-	0.01	0.05*
15:0 2OH	0.72	0.59	-	-	-	-	-	-
15:0 3OH	0.01	0.08*	-	-	-	-	-	-
15:0 ISO 3OH	-	-	-	-	-	-	-	-
15:1 $_{w}$8C	-	-	-	-	-	-	-	-
16:0	2.68	0.82	16.42	1.24	9.49	1.78	5.52	1.88
16:0 3OH	1.18	0.30	-	-	3.18	0.63	0.50	0.38
16:0 ISO	-	-	-	-	-	-	0.02	0.12*
16:1 $_{w}$7C	0.21	0.25*	0.59	0.55	0.54	0.34	0.79	0.77
17:0	0.28	0.46*	0.83	1.51	0.44	0.40	0.66	0.47
17:0 ISO	-	-	6.01	1.34	-	-	-	-
17:0 ISO 3OH	-	-	-	-	-	-	-	-
17:0 CYCLO	-	-	0.65	0.55	0.15	0.24*	0.27	0.48*
17:0 3OH	-	-	-	-	0.09	0.19*	-	-
17:1 $_{w}$6C	-	-	0.05	0.21*	-	-	-	-
17:1 $_{w}$7C	-	-	-	-	-	-	-	-
17:1 $_{w}$8C	0.02	0.10*	0.47	0.88*	0.06	0.15*	0.10	0.16*
18:0	9.02	1.42	4.29	0.50	1.29	0.52	4.72	1.23
18:1 2OH	-	-	0.13	0.33*	-	-	-	-
18:1 $_{w}$9C	0.11	0.30*	-	-	0.00	0.02*	0.11	0.17*
19:0	-	-	-	-	-	-	-	-
19:0 CYCLO $_{w}$8C	10.70	6.33	22.66	6.64	31.27	9.12	4.68	3.86
19:0 10 METHYL	0.01	0.06*	1.18	0.51	2.24	0.49	0.51	0.51
20:0	-	-	0.03	0.15*	-	-	-	-
20:1 $_{w}$9T	0.09	0.16*	-	-	-	-	0.10	0.24*
20:2 $_{w}$6, 9C	-	-	0.03	0.14*	0.12	0.17*	-	-
20:3 $_{w}$6, 9, 12C	1.97	0.40	-	-	1.34	0.43	3.34	1.01
SUMMED FEATURE 1[a]	-	-	-	-	-	-	-	-
SUMMED FEATURE 2[b]	-	-	-	-	-	-	-	-
SUMMED FEATURE 3[c]	6.09	0.64	-	-	4.88	0.46	7.96	2.63
SUMMED FEATURE 4[d]	-	-	-	-	-	-	-	-
SUMMED FEATURE 7[e]	66.87	8.07	45.41	7.79	44.91	10.45	70.61	5.53
SUMMED FEATURE 9[f]	-	-	-	-	-	-	-	-

Feature	Known bacterial strains					
	Agrobacterium -biovar 1		*Agrobacterium* -biovar 2		*Agrobacterium* -biovar 3	
	Mean	SD	Mean	SD	Mean	SD
10:0	-	-	0.01	0.04*	-	-
10:0 3OH	-	-	0.23	0.22*	-	-
11:0 2OH	-	-	-	-	-	-
unknown 11.541	-	-	0.08	0.11*	-	-
unknown 14.966	-	-	-	-	-	-
12:0 3OH	-	-	-	-	-	-
13:0 ISO 3OH	-	-	-	-	-	-
13:1 AT 12-13	0.50	0.21	0.34	0.17	-	-
14:0	0.11	0.12*	0.09	0.21*	-	-
15:0	0.29	0.19*	0.01	0.07*	-	-
15:0 ANTEISO	-	-	-	-	-	-
15:0 ISO	-	-	-	-	-	-
15:0 2OH	-	-	-	-	-	-
15:0 3OH	0.03	0.08*	-	-	-	-
15:0 ISO 3OH	-	-	3.08	1.83	-	-
15:1 $_{w}$8C	0.05	0.11*	0.02	0.09*	0.06	0.19*
16:0	9.07	1.29	7.39	2.04	6.02	0.90
16:0 3OH	4.79	1.22	4.81	1.15	2.30	0.77
16:0 ISO	-	-	-	-	-	-
16:1 $_{w}$7C	1.63	0.40	2.10	3.29	5.19	1.16
17:0	0.13	0.14*	0.05	0.10*	-	-
17:0 ISO	-	-	0.46	0.30	-	-
17:0 ISO 3OH	-	-	0.16	0.18*	-	-
17:0 CYCLO	1.60	0.55	0.74	0.39	-	-
17:0 3OH	0.18	0.18*	-	-	-	-
17:1 $_{w}$6C	-	-	-	-	-	-
17:1 $_{w}$7C	-	-	-	-	-	-
17:1 $_{w}$8C	0.22	0.15*	0.02	0.08*	-	-
18:0	0.17	0.17*	1.73	0.92	1.22	0.48
18:1 2OH	-	-	1.84	1.20	1.29	0.43
18:1 $_{w}$9C	-	-	-	-	-	-
19:0	-	-	-	-	-	-
19:0 CYCLO $_{w}$8C	18.88	5.17	27.78	15.25	4.14	2.09
19:0 10 METHYL	1.09	0.30	0.44	0.77*	2.04	0.67
20:0	-	-	-	-	-	-
20:1 $_{w}$9T	-	-	0.03	0.08*	-	-
20:2 $_{w}$6, 9C	0.01	0.04*	0.10	0.15*	-	-
20:3 $_{w}$6, 9, 12C	0.33	0.16	2.01	1.00	1.68	0.38
SUMMED FEATURE 1[a]	-	-	-	-	-	-
SUMMED FEATURE 2[b]	0.03	0.08*	-	-	-	-
SUMMED FEATURE 3[c]	8.19	1.90	4.61	2.53	7.96	2.57
SUMMED FEATURE 4[d]	-	-	-	-	-	-
SUMMED FEATURE 7[e]	52.69	6.91	41.85	15.48	68.09	4.98
SUMMED FEATURE 9[f]	0.01	0.04*	0.01	0.06*	-	-

Feature	Known bacterial strains					
	Rhizobium "hedysari"					
	Mean	SD	Mean	SD	Mean	SD
10:0	-	-	-	-	-	-
10:0 3OH	-	-	-	-	-	-
11:0 2OH	-	-	-	-	-	-
unknown 11.541	-	-	-		-	-
unknown 14.966	-	-	-	-	-	-
12:0 3OH	-	-	-	-	-	-
13:0 ISO 3OH	-	-	-	-	-	-
13:1 AT 12-13	0.15	0.20*	-	-	-	-
14:0	0.01	0.04*	-	-	-	-
15:0	0.01	0.07*	-	-	-	-
15:0 ANTEISO	-	-	-	-	-	-
15:0 ISO	-	-	-	-	-	-
15:0 2OH	0.08	0.15*	-	-	-	-
15:0 3OH	0.19	0.22*	-	-	-	-
15:0 ISO 3OH	-	-	-	-	-	-
15:1 $_{w}$8C	-	-	-	-	-	-
16:0	4.90	1.45	-	-	-	-
16:0 3OH	2.68	0.25	-	-	-	-
16:0 ISO	-	-	-	-	-	-
16:1 $_{w}$7C	1.05	0.21	-	-	-	-
17:0	0.96	0.52	-	-	-	-
17:0 ISO	-	-	-	-	-	-
17:0 ISO 3OH	-	-	-	-	-	-
17:0 CYCLO	0.53	0.33	-	-	-	-
17:0 3OH	0.07	0.12*	-	-	-	-
17:1 $_{w}$6C	-	-	-	-	-	-
17:1 $_{w}$7C	-	-	-	-	-	-
17:1 $_{w}$8C	0.24	0.22*	-	-	-	-
18:0	5.50	1.53	-	-	-	-
18:1 2OH	-	-	-	-	-	-
18:1 $_{w}$9C	0.05	0.08*	-	-	-	-
19:0	-	-	-	-	-	-
19:0 CYCLO $_{w}$8C	19.96	7.94	-	-	-	-
19:0 10 METHYL	0.22	0.17*	-	-	-	-
20:0	-	-	-	-	-	-
20:1 $_{w}$9T	-	-	-	-	-	-
20:2 $_{w}$6, 9C	0.04	0.12*	-	-	-	-
20:3 $_{w}$6, 9, 12C	0.86	0.08	-	-	-	-
SUMMED FEATURE 1[a]	-	-	-	-	-	-
SUMMED FEATURE 2[b]	-	-	-	-	-	-
SUMMED FEATURE 3[c]	6.48	0.65	-	-	-	-
SUMMED FEATURE 4[d]	-	-	-	-	-	-
SUMMED FEATURE 7[e]	56.00	7.95	-	-	-	-
SUMMED FEATURE 9[f]	-	-	-	-	-	-

gr., group; a, not known; b, 15:1 ISO H/13:0 3OH, 15:1 ISO I/13:0 3OH, 13:0 3OH/15:1 i I/H; c, 12:0 ALDE ?, unknown 10.928, 16:1 ISO I/14:0 32OH, 14:0 3OH/16:1 ISO I; d, not known; e, 18:1 $_{w}$7C/$_{w}$9T/$_{w}$12T, 18:1 $_{w}$12T/$_{w}$9T/$_{w}$7C, 18:1 $_{w}$9C/$_{w}$12T/$_{w}$7C; f, not known; *, not used in the identification.

Table 9. Total fatty acid composition of unknown bacterial strains

Name	Unknown soil isolates		
	KJ44	KJ30	KJ27
	expressed as percentage (%)		
9:0	-	-	1.88
10:0	-	-	-
10:0 3OH	-	-	-
11:0 2OH	-	-	-
unknown 11.541	-	-	-
unknown 14.966	-	-	-
12:0 3OH	-	-	-
13:0 ISO 3OH	-	-	-
13:1 AT 12-13	-	-	-
14:0	-	-	-
15:0	-	-	-
15:0 ANTEISO	-	-	-
15:0 ISO	-	-	-
15:0 2OH	0.63	0.56	-
15:0 3OH	-	-	-
15:0 ISO 3OH	-	-	-
15:1 $_{w}$8C	-	-	-
16:0	3.62	3.56	9.56
16:0 3OH	1.10	0.93	3.87
16:0 ISO	-	-	-
16:1 $_{w}$7C	0.63	0.69	-
17:0	0.41	0.50	-
17:0 ISO	-	-	-
17:0 ISO 3OH	-	-	-
17:0 CYCLO	0.37	-	-
17:0 3OH	-	-	-
17:1 $_{w}$6C	-	-	-
17:1 $_{w}$7C	-	-	-
17:1 $_{w}$8C	-	-	-
18:0	5.93	5.72	5.03
18:1 2OH	-	-	2.28
18:1 $_{w}$9C	0.52	0.58	-
19:0	-	-	-
19:0 CYCLO $_{w}$8C	16.57	15.30	7.82
19:0 10 METHYL	0.51	-	-
20:0	-	-	-
20:1 $_{w}$9T	-	-	-
20:2 $_{w}$6, 9C	-	-	-
20:3 $_{w}$6, 9, 12C	2.53	2.03	2.27
SUMMED FEATURE 1[a]	-	-	-
SUMMED FEATURE 2[b]	-	-	-
SUMMED FEATURE 3[c]	8.03	7.62	6.72
SUMMED FEATURE 4[d]	-	-	-
SUMMED FEATURE 7[e]	59.16	62.50	60.58
SUMMED FEATURE 9[f]	-	-	-
Identified as	*Rhizobium leguminosarum*	*Rhizobium leguminosarum*	*Agrobacterium* biovar 2

Name	Unknown soil isolates		
	KJ17	KJ26	KJ203
	expressed as percentage (%)		
9:0	-	-	-
10:0	-	-	-
10:0 3OH	-	-	-
11:0 2OH	-	-	-
unknown 11.541	-	-	-
unknown 14.966	-	-	-
12:0 3OH	-	-	-
13:0 ISO 3OH	-	-	-
13:1 AT 12-13	0.34	0.29	-
14:0	-	-	-
15:0	-	-	-
15:0 ANTEISO	-	-	-
15:0 ISO	-	-	-
15:0 2OH	-	-	0.67
15:0 3OH	-	-	-
15:0 ISO 3OH	-	-	-
15:1 $_{w}$8C	-	-	-
16:0	4.95	4.93	3.78
16:0 3OH	2.33	2.44	0.88
16:0 ISO	-	-	-
16:1 $_{w}$7C	1.58	1.59	0.62
17:0	0.32	0.35	0.53
17:0 ISO	-	-	-
17:0 ISO 3OH	-	-	-
17:0 CYCLO	0.70	0.70	-
17:0 3OH	-	-	-
17:1 $_{w}$6C	-	-	-
17:1 $_{w}$7C	-	-	-
17:1 $_{w}$8C	0.21	0.20	-
18:0	3.74	3.40	6.97
18:1 2OH	-	-	-
18:1 $_{w}$9C	-	-	0.62
19:0	-	-	-
19:0 CYCLO $_{w}$8C	16.72	18.73	17.61
19:0 10 METHYL	0.38	0.41	-
20:0	-	-	-
20:1 $_{w}$9T	-	-	-
20:2 $_{w}$6, 9C	-	-	-
20:3 $_{w}$6, 9, 12C	0.97	1.05	2.03
SUMMED FEATURE 1[a]	-	-	-
SUMMED FEATURE 2[b]	-	-	-
SUMMED FEATURE 3[c]	6.65	6.70	7.64
SUMMED FEATURE 4[d]	-	-	-
SUMMED FEATURE 7[e]	61.10	59.19	58.66
SUMMED FEATURE 9[f]	-	-	-
Identified as	*Rhizobium "hedysari"*	*Rhizobium "hedysari"*	*Rhizobium leguminosarum*

Name	Unknown soil isolates		
	KJ19	KJ23	OR168
	expressed as percentage (%)		
9:0	-	-	-
10:0	-	-	-
10:0 3OH	-	-	-
11:0 2OH	-	-	-
unknown 11.541	-	-	-
unknown 14.966	-	-	-
12:0 3OH	-	-	-
13:0 ISO 3OH	-	-	-
13:1 AT 12-13	-	-	-
14:0	-	-	-
15:0	-	-	-
15:0 ANTEISO	-	-	-
15:0 ISO	-	-	-
15:0 2OH	-	0.59	0.60
15:0 3OH	-	-	-
15:0 ISO 3OH	-	-	-
15:1 $_{w}$8C	-	-	-
16:0	7.41	3.45	3.76
16:0 3OH	4.13	0.95	1.00
16:0 ISO	-	-	-
16:1 $_{w}$7C	-	0.62	0.57
17:0	-	-	0.39
17:0 ISO	-	-	-
17:0 ISO 3OH	-	-	-
17:0 CYCLO	-	-	0.38
17:0 3OH	-	-	-
17:1 $_{w}$6C	-	-	-
17:1 $_{w}$7C	-	-	-
17:1 $_{w}$8C	-	-	-
18:0	2.84	5.45	7.00
18:1 2OH	1.70	-	-
18:1 $_{w}$9C	-	0.47	0.58
19:0	-	-	-
19:0 CYCLO $_{w}$8C	13.04	14.84	18.09
19:0 10 METHYL	-	-	0.48
20:0	-	-	-
20:1 $_{w}$9T	-	-	-
20:2 $_{w}$6, 9C	-	-	0.23
20:3 $_{w}$6, 9, 12C	2.05	2.03	2.38
SUMMED FEATURE 1[a]	-	-	-
SUMMED FEATURE 2[b]	-	-	-
SUMMED FEATURE 3[c]	6.72	7.48	7.39
SUMMED FEATURE 4[d]	-	-	-
SUMMED FEATURE 7[e]	62.10	64.12	57.15
SUMMED FEATURE 9[f]	-	-	-
Identified as	*Agrobacterium* biovar 2	*Rhizobium leguminosarum*	*Rhizobium leguminosarum*

Name	Unknown soil isolates		
	KJ5	KJ13	NR40
	expressed as percentage (%)		
9:0	-	-	-
10:0	-	-	-
10:0 3OH	-	-	-
11:0 2OH	-	-	-
unknown 11.541	-	-	-
unknown 14.966	-	-	-
12:0 3OH	-	-	1.24
13:0 ISO 3OH	-	-	1.12
13:1 AT 12-13	0.50	0.58	-
14:0	0.19	0.19	-
15:0	0.51	0.47	-
15:0 ANTEISO	-	-	-
15:0 ISO	-	-	-
15:0 2OH	-	-	-
15:0 3OH	-	-	-
15:0 ISO 3OH	-	-	-
15:1 $_{w}$8C	-	-	-
16:0	8.57	8.53	18.91
16:0 3OH	3.42	3.38	-
16:0 ISO	-	-	-
16:1 $_{w}$7C	1.92	2.07	1.72
17:0	0.40	0.38	-
17:0 ISO	-	-	4.81
17:0 ISO 3OH	-	-	-
17:0 CYCLO	2.43	2.36	0.91
17:0 3OH	0.26	0.26	-
17:1 $_{w}$6C	-	-	-
17:1 $_{w}$7C	-	-	-
17:1 $_{w}$8C	0.42	0.41	-
18:0	0.28	0.27	5.91
18:1 2OH	-	-	0.94
18:1 $_{w}$9C	-	-	0.78
19:0	-	-	-
19:0 CYCLO $_{w}$8C	15.41	14.37	15.47
19:0 10 METHYL	0.85	0.88	1.62
20:0	-	-	-
20:1 $_{w}$9T	-	-	-
20:2 $_{w}$6, 9C	-	-	-
20:3 $_{w}$6, 9, 12C	0.25	0.24	-
SUMMED FEATURE 1[a]	-	-	-
SUMMED FEATURE 2[b]	-	-	-
SUMMED FEATURE 3[c]	6.29	6.44	-
SUMMED FEATURE 4[d]	-	-	-
SUMMED FEATURE 7[e]	58.28	59.18	46.56
SUMMED FEATURE 9[f]	-	-	-
Identified as	*Agrobacterium* biovar 1	*Agrobacterium* biovar 1	*Rhizobium loti*

Name	Unknown soil isolates		
	KJ57	NR42	NR64
	expressed as percentage (%)		
9:0	-	-	-
10:0	-	-	-
10:0 3OH	-	-	-
11:0 2OH	-	-	-
unknown 11.541	0.18	-	-
unknown 14.966(503)	-	-	0.42
12:0 3OH	-	-	-
13:0 ISO 3OH	-	-	-
13:1 AT 12-13	0.38	-	1.82
14:0	-	-	-
15:0	-	-	-
15:0 ANTEISO	-	-	-
15:0 ISO	-	-	-
15:0 2OH	-	-	-
15:0 3OH	-	-	-
15:0 ISO 3OH	3.63	-	-
15:1 $_{w}$8C	-	-	-
16:0	5.12	5.71	3.00
16:0 3OH	4.10	3.09	1.25
16:0 ISO	-	-	-
16:1 $_{w}$7C	0.27	1.03	0.49
17:0	-	0.47	0.53
17:0 ISO	0.70	-	-
17:0 ISO 3OH	0.43	-	-
17:0 CYCLO	0.34	1.23	-
17:0 3OH	-	-	-
17:1 $_{w}$6C	-	-	-
17:1 $_{w}$7C	-	-	-
17:1 $_{w}$8C	-	-	-
18:0	1.71	3.59	10.11
18:1 2OH	0.61	-	-
18:1 $_{w}$9C	-	-	-
19:0	-	-	-
19:0 CYCLO $_{w}$8C	42.57	36.69	13.04
19:0 10 METHYL	-	0.42	-
20:0	-	-	-
20:1 $_{w}$9T	-	-	0.53
20:2 $_{w}$6, 9C	0.38	0.36	-
20:3 $_{w}$6, 9, 12C	1.32	1.46	-
SUMMED FEATURE 1[a]	-	-	-
SUMMED FEATURE 2[b]	-	-	-
SUMMED FEATURE 3[c]	1.87	7.22	6.99
SUMMED FEATURE 4[d]	-	-	-
SUMMED FEATURE 7[e]	36.39	38.74	61.21
SUMMED FEATURE 9[f]	-	-	-
Identified as	*Rhizobium tropici*	*Rhizobium meliloti*	*Rhizobium leguminosarum*

a, not known; b, 15:1 ISO H/13:0 3OH, 15:1 ISO I/13:0 3OH, 13:0 3OH/15:1 i I/H; c, 12:0 ALDE ?, unknown 10.928, 16:1 ISO I/14:0 3OH, 14:0 3OH/16:1 ISO I; d, not known; e, 18:1 $_{w}$7C/$_{w}$9T/$_{w}$12T, 18:1 $_{w}$12T/$_{w}$9T/$_{w}$7C, 18:1 $_{w}$9C/$_{w}$12T/$_{w}$7C; f, not known.

Soil isolate KJ57 was identified as *Rhizobium tropici* based on its content of the following fatty acids: 15:0 ISO 3OH, 16:0, 16:0 3OH, 17:0 ISO, 17:0 CYCLO, 18:0, 18:1 2OH, 19:0 CYCLO $_{w}$8C, 20:2 $_{w}$6, 9C, 20:3 $_{w}$6, 9, 12C, summed feature 3 and summed feature 7, with a similarity index of 0.773.

NR42 was identified as *Rhizobium meliloti* based on their content of the following fatty acids: 16:0, 16:0 3OH, 16:1 $_{w}$7C, 17:0, 17:0 CYCLO, 18:0, 19:0 CYCLO $_{w}$8C, 19:0 10 METHYL, 20:3 $_{w}$6, 9, 12C, summed feature 3 and summed feature 7, with a similarity index of 0.447.

Soil isolates KJ23, KJ30, KJ44, NR64, OR168 and KJ203 were identified as *Rhizobium leguminosarum* based on their content of following fatty acids: 15:0 2OH, 16:0, 16:0 3OH, 16:1 $_{w}$7C, 18:0, 19:0 CYCLO $_{w}$8C, summed feature 3 and summed feature 7, with a similarity indices of 0.864, 0.879, 0.845, 0.582, 0.901 and 0.906 respectively.

Soil isolates KJ5 and KJ13 were identified as *Agrobacterium* biovar 1 (*Agrobacterium radiobacter*) based on the following fatty acids: 13:1 AT 12-13, 15:0 ISO 3OH, 16:0, 16:0 3OH, 16:1 $_{w}$7C, 17:0 CYCLO, 19:0 CYCLO $_{w}$8C, 19:0 10 METHYL, 20:3 $_{w}$6, 9, 12C, summed feature 3 and summed feature 7, with a similarity indices of 0.852 and 0.843 respectively.

Soil isolate KJ19 was identified as *Agrobacterium* biovar 2 (*Agrobacterium rhizogenes*) based on its content of the following fatty acids: 13:1 AT 12-13, 16:0, 16:0 3OH, 16:1 $_{w}$7C, 17:0 ISO, 17:0 CYCLO, 18:0, 18:1 2OH, 19:0 CYCLO $_{w}$8C, 20:3 $_{w}$6, 9, 12C, summed feature 3 and summed feature 7, with a similarity index of 0.488. It also showed a match to *Rhizobium etli*, with a similarity index of 0.469.

Soil isolate KJ27 was identified as *Agrobacterium rhizogenes* based on its contents of the following fatty acids: 16:0 3OH, 16:0 ISO, 18:1 2OH, 18:1$_{w}$9C, 19:0 CYCLO$_{w}$8C, summed feature 3 and summed feature 7, but a poor match to the data base, with a similarity index of 0.234.

Soil isolates KJ17 and KJ26 were identified as *Rhizobium "hedysari"* based on their content of the following fatty acids: 16:1 $_{w}$7c, 16:0, 17:0 CYCLO, 17:0, 16:0 3OH, 18:0, 19:0 CYCLO $_{w}$8C, 20:3 $_{w}$6, 9, 12C, summed feature 3 and summed feature 7 with similarity indices of 0.835 and 0.818 respectively.

NR40 was identified as *Rhizobium loti* based on its content of the following fatty acids: 13:0 ISO 3OH, 16:0, 17:0, 17:0 ISO, 17:0 CYCLO, 18:0, 19:0 CYCLO $_{w}$8C, 19:0 10 METHYL and summed feature 7, with a similarity index of 0.450.

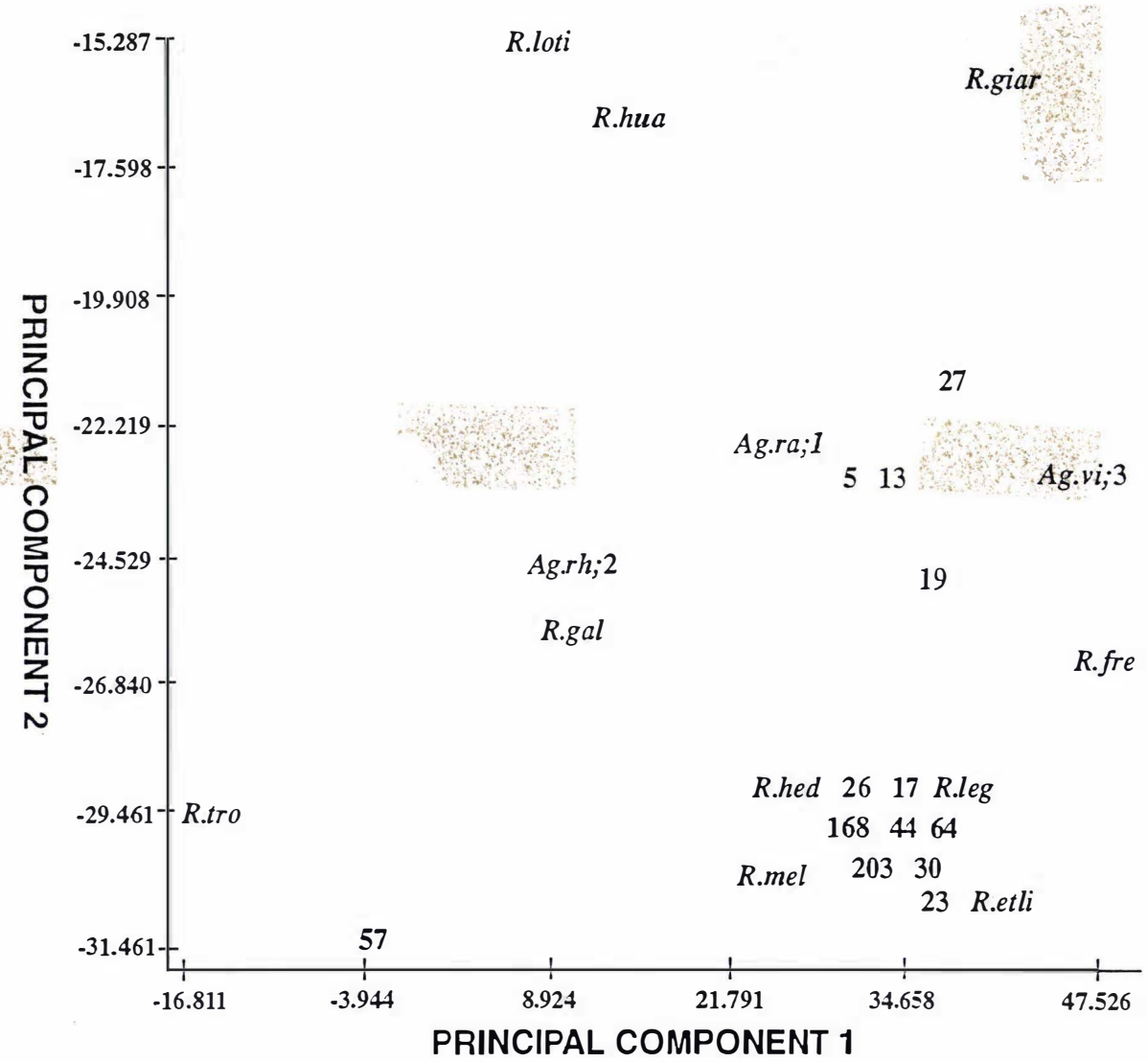
3. 11. 3 The 2-D plots of principal component analysis

The 2-D plot shown in Figure 20 uses principal component analysis of fatty acid methyl esters (FAME) to group entries in a two dimensional space. The x-axis represents principal component 1, and the y-axis represents principal component 2. (These may be changed to plot 1 vs, 3, or 2 vs. 3 to gain additional perspective). Such a plot is useful for examining the relationships between taxa, and in particular for distantly related organisms. Large numbers of taxa can be analyzed, and the resulting plot (s) used to define relatedness. The approach can be particularly helpful even when isolates match species currently unnamed by MIS.

Figure 20 shows a 2-D plot of soil isolates and previously characterized species. The plot shows two main clusters. Cluster one consists of strains KJ26, KJ17, OR168, KJ44, N64, KJ203, KJ30 and KJ23. Cluster one are all rhizobia. Cluster two consists of strains KJ27, KJ3, KJ13 and KJ19. Cluster two also indicates affinities to rhizobia but does not resolve between species of agrobacteria and rhizobia.

Soil isolate KJ57 shows a 2D plot value very distinct from the other soil isolates. However it also corresponds well to rhizobial species *R. tropici*.

Figure 20. 2-D plot of Principal Components 1 and 2 derived from fatty acid analysis, showing the location of known species profiles and their relationship to the unknown soil isolates. *R. loti*: *Rhizobium loti*; *R. hua.*: *Rhizobium huakuii*; *R. giar.*: *Rhizobium giardini*; *R. tro.*: *Rhizobium tropici*; *Ag. rh.*;2: *Agrobacterium rhizogenes* (group 2); *R. gal.*: *Rhizobium galegae*; *Ag. ra.*;1: *Agrobacterium radiobacter* (group 1); *Ag. vi.*;3: *Agrobacterium vitis* (group 3); *R. fre.*: *Rhizobium fredii*; *R. hed.*: *Rhizobium "hedysari"*; *R. leg.*: *Rhizobium leguminosarum;* *R. mel.*: *Rhizobium meliloti*, *R. etli*: *Rhizobium etli.*


-15.287
-17.598
-19.908
-22.219
-24.529
-26.840
-29.461
-31.461
PRINCIPAL COMPONENT 2
R.loti
R.giar
R.hua
27
Ag.ra;1
5 13
Ag.vi;3
Ag.rh;2
19
R.gal
R.fre
R.hed 26 17 R.leg
R.tro
168 44 64
R.mel
203 30
23 R.etli
57
-16.811
-3.944
8.924
21.791
34.658
47.526
PRINCIPAL COMPONENT 1

ERRATUM

Page	Line	Incorrect text	Correct text
109	7	numbered about 200	numbering 100
111	24	to investigate	to identify
116	9	for	from
118	32	ad then	and then
120	1	were identified	were respectively identified

4. DISCUSSION

4. 1 Isolation of Gram negative soil bacteria

In this study a collection of soil bacteria were isolated and purified from four different soil types namely Ramiha silt loam, Tokomaru silt loam, Kairanga silt loam (under white clover-ryegrass pastures) and Manawatu sandy loam (under fallow land with shrubs of *Lupinus* sp.). The soil bacteria which showed varying colony morphology, numbered about 200 were checked for their ability to nodulate white clover (*Trifolium repens*) cultivar Grasslands Huia. Only four strains nodulated. The remaining 196 strains did not nodulate. The medium used in our study was not selective for rhizobia. The experimental design of the project was intended to allow detection of any soil bacteria (not only rhizobia) which could accept and express pSym following conjugation with *Escherichia coli* strain PN200 containing plasmid pPN1 (pRtr514a::R68.45).

The present work differed from earlier studies where the object was to isolate non-symbiotic *Rhizobium* strains from soil. In these, Soberón-Chávez and Nájera, (1989) and Jarvis *et al.*, (1989) isolated strains from soil that were chromosomally similar to *R. leguminosarum*. Laguerre *et al.*, (1993a) isolated from soil both symbiotic and non-symbiotic *Rhizobium leguminosarum*. In an earlier study non-symbiotic strains were also isolated from the rhizosphere of bean plants (Segovia *et al.*, 1991). These authors specifically went looking for non-nodulating rhizobia whereas in the present study we screened for any Gram negative soil bacteria unable to nodulate white clover.

The existence of self-transmissible plasmids coding for symbiotic functions in strains of *Rhizobium leguminosarum* is also well known and established (Djordjevic *et al.*, 1983; Hooykaas *et al.*, 1981; Johnston *et al.*, 1978; Lamb *et al.*, 1982) and there is indirect evidence for plasmid transfer between strains of *R. leguminosarum* in soil (Schofield *et al.*, 1987). Conjugative plasmids like RP4 and its derivative R68.45 are readily transmitted between bacterial strains in natural environments such as soil and water (Sayre and Miller, 1991). Plasmids conferring different plant specificities can occur in rhizobia of the same electrophoretic type (Young, 1985). When *Rhizobium leguminosarum* biovar trifolii isolates from a field population were characterized with DNA probes, the same symbiotic plasmids were found in related strains (Schofield *et al.*, 1987). Young and Wexler, (1988) examined two field populations of *R. leguminosarum* biovar viceae and found that although the distribution of symbiotic

plasmids across chromosomal backgrounds was far from random, indistinguishable plasmids could be found in genetically unrelated bacteria. Each of these authors concluded that genetic exchange occurs between rhizobia in soil. Transfer of the symbiotic plasmid pJB5JI between strains of *Rhizobium* at frequencies of upto 10^{-4} per recipient in sterile and non-sterile soil has also been observed (Kinkle and Schmit, 1991).

Interspecies transfer of symbiotic plasmids has been reported from *R. leguminosarum* biovar trifolii (pRtr5a) (Hooykaas *et al.*, 1981) and from *Rhizobium meliloti* (pRMe416) (Kondorosi *et al.*, 1982) to *Agrobacterium tumefaciens*. These species are as closely related as are some rhizobia (Jarvis *et al.*, 1986), and there is evidence for a close ancestral relationship between the plasmids of *Rhizobium* and *Agrobacterium* species based on plasmid incompatibility studies (O'Connell *et al.*, 1987) as well as on phylogenetic analyses of the family *Rhizobiaceae* and related bacteria by sequencing of 16S rRNA genes (Willems and Collins, 1993; Yanagi and Yamasato, 1993). Nevertheless despite this close relatedness, nodules formed by *Agrobacterium* transconjugants have been found to be ineffective (Kondorosi *et al.*, 1982).

Conjugative transfer of symbiotic plasmids to bacteria not normally associated with root nodules has been reported for *Agrobacterium tumefaciens* (Hooykaas *et al.*, 1981; Kondorosi *et al.*, 1982), *Enterobacter agglomerans* (Dohler and Klingmuller, 1988), *Pseudomonas aeruginosa* and a *Lignobacter* sp. (Plasinski and Rolfe, 1985) and soil bacteria (Jarvis *et al.*, 1989). These and similar observations have stimulated the present study to assess the diversity of Gram negative soil bacteria capable of expressing a pSym plasmid pRtr514a.

4. 2 Transfer of pPN1

In this study, transfer of pPN1 (pSym::R68.45) to soil bacteria was demonstrated by (i) growth on antibiotic media, (ii) plant test (Vincent, 1970), (iii) Eckhardt gels (Eckhardt, 1978), (iv) the presence of bacteroids in nodules (Pankhurst *et al.*, 1979) and (v) by probing transconjugant soil bacteria isolated from the nodules with a radioactive *nod*A gene sequence.

4. 2. 1 Bacteria from several soil types can express pRtr514a

In earlier work, Segovia *et al.*, (1991) investigated the soil population of symbiotic and non-symbiotic rhizobia isolated from the root region of six week old nodulated plants harvested from a field in Tepoztlán, Morelos, Mexico. They reported that the

symbiotic isolates were found at a relative frequency of 1 symbiotic isolate to 40 non-symbiotic *R. leguminosarum* strains. In a different study of bacteria in other soil types, Jarvis *et al.*, (1989) found that rhizobial transconjugants which formed nodules occurred in 6 of 18 (33%) strains whose DNA hybridized with that of strain PN165 and in 1 of 9 (11%) strains containing DNA which did not hybridize with that of PN165. These soil isolates had been collected from ryegrass-white clover permanent pastures on river flats consisting of recent alluvial soil and two neighboring terrace soils which consisted of yellow-grey earths derived from loess and alluvial material.

In this study a total of 12 soil isolates from 100 conjugations (12%) formed nodules on white clover. Approximately 50% of the soil isolates which expressed pRtr514a (KJ5, KJ23, KJ26, KJ44, KJ57 and KJ203) were isolated from Tokomaru silt loam (a weakly leached, moderate to strong gleyed yellow-grey earth derived from loess). 25% (KJ3, KJ17 and KJ30) of the soil isolates were isolated from Ramiha silt loam (a soil derived from loess, solifluction material, and slope deposits), 17% (KJ13 and KJ27) from Kairanga silt loam (a soil derived from alluvium and weakly leached slowly accumulating gley recent soil) and 8% (KJ19) from Manawatu sandy loam (derived from alluvium and weakly leached slowly accumulating recent soils).

Although Tokomaru silt loam shows the largest percentage of soil isolates, 40 isolates were tested from this soil type. From the remaining soil types only 20 isolates were tested for each type. These sample sizes are small and hence a more accurate estimate for the extent of the microbial diversity needs further study. However the results do show that different soil types harbour non-nodulating soil bacteria, including leached, low P and low pH soil such as Ramiha silt loam. The purpose of this study, however, was to investigate the types of bacteria involved.

4. 2. 2 The frequency of symbiotic plasmid transfer

Rao *et al.*, (1994) showed five *R. leguminosarum* biovar trifolii strains including the type strain ATCC10004 and three New Zealand inoculant strains (ICMP2163, ICMP2668 and ICMP2666), could transfer their symbiotic plasmids to a pSym-cured *R. leguminosarum* biovar trifolii strain PN165 at frequencies of 1 in 10^{-3} to 1 in 10^{-5} and nodules were formed on white clover by transconjugant bacteria and not the donor bacteria resistant to the antibiotics used.

Further the existence in soil of rhizobia which lack a symbiotic plasmid has been inferred by Young (1985) in relation to *R. leguminosarum* and demonstrated for biovar phaseoli by Soberón-Chávez and Nájera (1989) and by Segovia *et al.*, (1991).

Ronson and Scott, (1983) transferred plasmid pPN1 to a variety of different bacterial strains at frequencies as high as 10^{-3} to 10^{-4}. Jarvis *et al.*, (1989) conjugated *Escherichia coli* strain PN200 with a Rifampicin-resistant derivative of NR41 (BJ1) with an observed frequency of 10^{-6}. Because of these relatively high rates of transfer, in the present study we also used *Escherichia coli* strain PN200 containing plasmid pPN1 (pRtr514a::R68.45) as our donor for conjugation experiments.

We showed that nine strains of soil bacteria formed transconjugants with a frequency of transfer of 2.91×10^{-5} (mean value) on conjugation with *Escherichia coli* strain PN200 containing plasmid pPN1. Further, 7 out of 9 such strains nodulated white clover. We could not calculate the frequency of transfer for the remaining five isolates, as antibiotic resistance could not be obtained for them but the transconjugant mixture was inoculated on white clover seedlings and the five strains nodulated white clover. The frequencies of transfer we obtained were quite similar to the ones mentioned by the above authors.

When pPN1 is used the pSym is mobilized whereas in soils Sym plasmids are probably self-transmissible Sym plasmid. It was concluded from these results that transconjugation between rhizobia probably occurs in different soil types.

4. 2. 3 The nature of the nodules formed

Attempts to transfer symbiotic plasmids conferring nodulation and nitrogen fixation to recipient *Rhizobium* strains closely related to the donor usually result in the formation of effective nodules which may help extend the host range of the recipient strain (Djordjevic *et al.*, 1983; Johnston *et al.*, 1978; Lamb *et al*, 1982). Jarvis *et al.*, (1989) transferred by conjugation the co-integrate plasmid pPN1 (pSym::R68.45) to strains of non-nodulating, Gram negative rod shaped soil bacteria (NR42, NR41, NR64 and OR168) and the transconjugants formed were $Nod^{+}Fix^{-}$. In a different study of type strains, the co-integrate plasmid pPN1 was used to transfer a Sym plasmid from *Rhizobium leguminosarum* biovar trifolii (pRtr514a) to *Lignobacter* strain K17 and *Pseudomonas aeruginosa* strain PAO5 by conjugation, and they formed nodule-like structures (Plazinski and Rolfe, 1985).

In our conjugation experiments using plasmid pPN1, nodulation by transconjugant soil bacteria was verified by plant tests in nitrogen-deficient medium. Both positive control (inoculated with *Rhizobium leguminosarum* biovar trifolii strain ICMP2163 and negative control (inoculated with sterile water, *Escherichia coli* strain PN200

containing pPN1 or *E. coli* strain ATCC9637 and the recipient soil bacteria) plants were examined. Only in the case of the positive control and transconjugant soil isolates were true nodules formed on white clover. Within 4-6 weeks of incubation 50 to 60% of the clover plants nodulated and 3 to 6 nodules were formed by each transconjugant soil bacterium except for KJ1. Soil bacteria KJ3, KJ5, KJ13, KJ17, KJ19, KJ23, KJ26, KJ27, KJ30, KJ44, KJ57 and KJ203 were Nod^+ but nitrogen fixation was not studied. The host range of these transconjugants is not known because the strains were tested only on white clover.

4. 2. 4 Stability of pRtr514a in soil bacteria

The Eckhardt gels showed that transconjugants contained different amounts of symbiotic genetic material. Strains KJ1 and KJ3 contained R68.45 only, transconjugants of strains KJ13, KJ19, KJ23, KJ26, KJ30 and KJ44 contained pPN1 and R68.45 and transconjugants of strains KJ5, KJ17, KJ27, KJ57, KJ203 and PN165 contained pPN1. Similar results were obtained by Jarvis *et al.*, 1989. They point out that the maintenance of the co-integrate depends on the host bacterium, and an intact co-integrate is not required for the expression of nodulation genes.

4. 2. 5 Nodule cytology

In previous studies nodule cytology has proven useful to examine the extent of bacteroid formation. For example Konodorosi *et al.*, (1982); Plasinski and Rolfe, (1985) showed that transconjugant *Agrobacterium* and *Lignobacter* strains were recovered from root nodules. They invaded plant cells and became enclosed in plant cell membrane but were unable to fix atmospheric nitrogen. *Nif* genes have been expressed in these bacterial hosts. It is possible that Tn5 insertion in the plasmid (Hooykaas *et al.*, 1981) or the use of broad host range plasmids containing RP4 genes to mobilize symbiotic plasmids (Konodorosi *et al.*, 1982; Plasinski and Rolfe, 1985) may have affected *nif* genes expression in the host plant. Hynes and O'Connell, (1988) found that RP4 severely inhibited nodulation and resulted in the formation of ineffective nodules on peas by several strains of *R. leguminosarum* biovar viceae.

In our experiments, microtome sections of nodule tissue were examined by light and electron microscopy to determine the distribution of infected plant cells and verify that these cells contained bacteroids enclosed in plant cell membranes. The only difference we observed was that the nodule cells formed by the inoculant *R. leguminosarum* biovar trifolii strain ICMP2163 and transconjugants were filled with bacteroids but a few nodule cells formed by the transconjugants were devoid of bacteroids.

4. 2. 6 Probing for *nod* genes

Total genomic DNA from each of the transconjugants formed by the soil bacteria, was isolated from the nodules and digested with restriction endonucleases. The fragments separated by gel electrophoresis, were then transferred to a hybridization membrane and probed with a radioactive amplified 590 bp *nod*A sequence. All eleven strains of transconjugant soil bacteria produced a band at 11.7 Kb indicating the presence of the transferred symbiotic plasmid. This shows at the molecular level that soil harbours non-nodulating soil bacteria which can maintain symbiotic genes and probably symbiotic plasmids. KJ1 and KJ3 failed to hybridize with the 590 bp *nod*A sequence and KJ1 also did not nodulate white clover. The reason for this discrepancy may be the loss of pPN1 due to subsequent sub-culturing.

4. 3 The identity of the bacteria involved

The application of modern taxonomic methods to the classification of root nodule bacteria has led to the recognition of an increasing number of new species and genera (Jarvis and Tighe, 1994). The methods used include numerical taxonomy, multi-locus enzyme electrophoresis, DNA-DNA relatedness, rRNA:DNA hybridization and 16S rRNA sequence analysis (Graham *et al.*, 1991). Symbiotic performance, which has been the most practical trait, although not absolute, for differention between species has become used less with the recognition of non-nodulating bacteria in the soil. There is at present a pressing need for uniformly accepted criteria for identification which would enable rhizobiologists, and especially ecologists, to identify the rhizobia they are working with (Laguerre *et al.*, 1994).

In this study to assess the diversity of soil isolates expressing pSym, soil isolates were identified by several methods including: rRNA fingerprinting, partial 16S rRNA sequencing, DNA-DNA hybridization and fatty acid analysis.

4. 3. 1 rRNA fingerprinting as a method

The usefulness of rRNA fingerprinting as an identification method for bacteria was first shown by Grimont *et al.*, (1987) and Saunders *et al.*, (1988). They found that *Legionella* species could be identified by their rRNA patterns. However, in another study Verger *et al.*, (1987) found that *Brucella* sp. could not be distinguished from one another on their rRNA patterns. Similarly Segovia *et al.*, 1991 who examined non-

nodulating rhizobial strains from the rhizosphere regions of six week old bean plants, found no variability among the isolates or between the isolates and a type strain.

In the present study the fingerprint patterns of soil isolates and reference rhizobial strains were also investigated. However unlike the studies of Segovia *et al.*, (1991) and Verger *et al.*, (1987) heterogeneity was observed between isolates and different rhizobial type strains. Six soil isolates out of 15 (40%) were similar to known rhizobial type strains by this method. Other isolates could not be unambiguously identified on the basis of their observed fingerprinting patterns. A general problem with this approach at the present is that diagnostic features of many type strains are still to be determined. Hence, a more detailed study of the pattern of variability in rhizobia and agrobacteria is required before such an approach could have general applicability in rhizobial studies.

Since the 16S rRNA sequences database for rhizobial and other bacteria has recently been greatly extended (Jarvis *et al.*, 1992; Sawada *et al.*, 1993; Willems and Collins, 1993; Yanagi and Yamasato, 1993; Young *et al.*, 1991) further efforts to characterize the soil isolates were made based on partial sequence determinations of 16S rDNA genes.

4. 3. 2 rRNA sequencing

Sawada *et al.*, (1993) determined the 16S rRNA sequences of seven representative *Agrobacterium* strains, eight representative *Rhizobium* strains, and the type strains of *Azorhizobium caulinodans* and *Bradyrhizobium japonicum*. These strains included the type strains of *Agrobacterium tumefaciens*, *Agrobacterium rhizogenes*, *Agrobacterium radiobacter*, *Agrobacterium vitis*, *Agrobacterium rubi*, *Rhizobium fredii*, *Rhizobium galegae*, *Rhizobium huakuii*, *Rhizobium leguminosarum*, *Rhizobium loti*, *Rhizobium meliloti* and *Rhizobium tropici*. A phylogenetic analysis (Figure 8d), with the neighbour joining method which used genetic distances estimated as K_{nuc} values (Kimura, 1980), showed that the 15 strains of *Agrobacterium* and *Rhizobium* species formed a phylogenetic cluster clearly separated from the other members of the alpha subclass of the *Proteobacteria*.

In a further study Willems and Collins, (1993) also showed that the genera *Rhizobium* and *Agrobacterium* were phylogenetically intermixed. Several subgroupings were evident in which *Rhizobium* and *Agrobacterium* species were most closely related. The comparative analysis also confirmed that the genera *Bradyrhizobium* and *Azorhizobium* belonged to phylogenetic lineages distinct from rhizobia and

agrobacteria. Willems and Collins, (1993) present trees obtained using the Fitch and Margoliash algorithm and parsimony analysis (Figure 8a, b). In both these trees *R. meliloti* and *R. fredii* were closely related as were *R. leguminosarum* and *R. tropici*. *R. galegae* and *R. loti* showed significant divergence from these species and from each other.

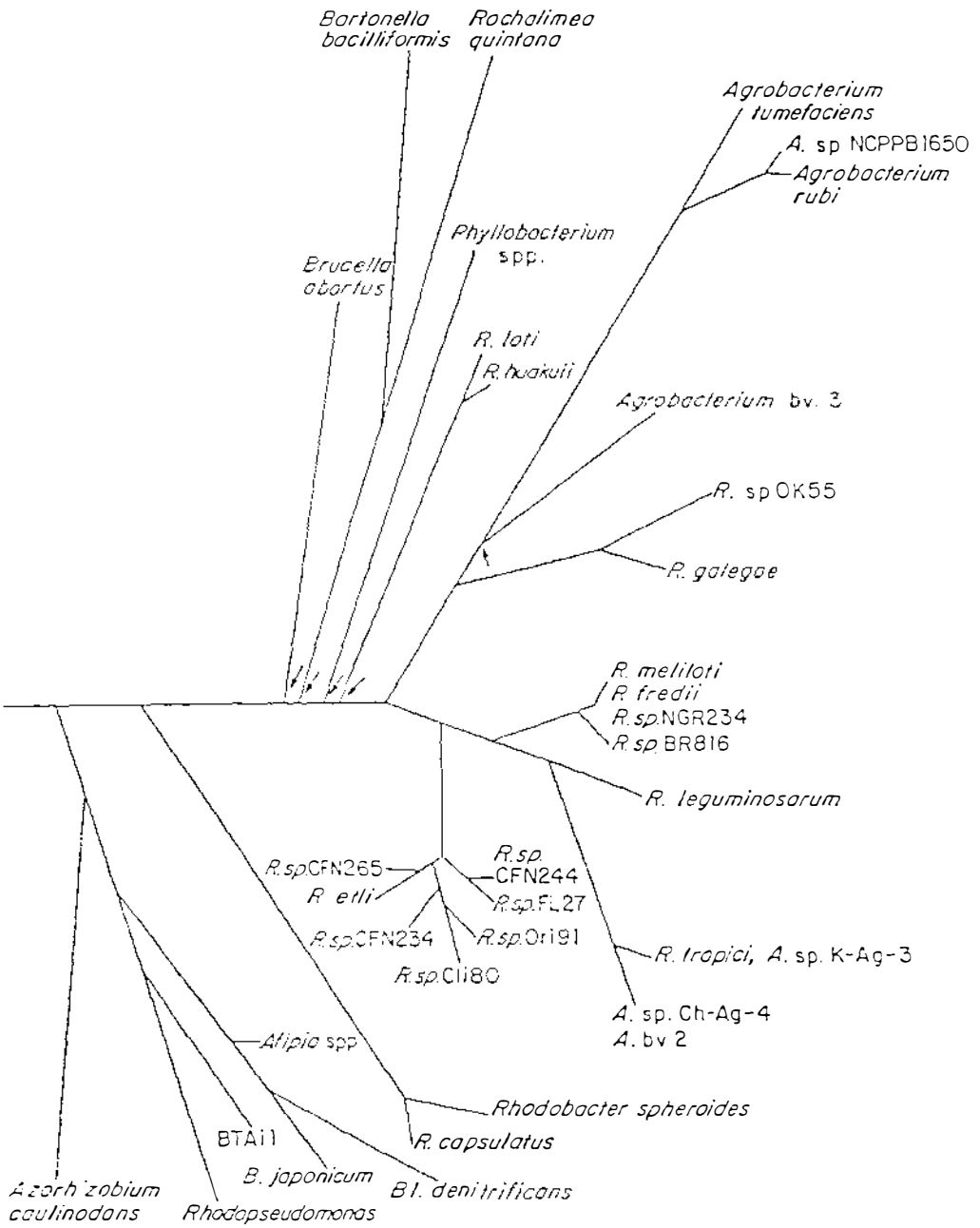
This lack of phylogenetic distinctiveness between agrobacteria and rhizobia was also found by Yanagi and Yamasato, (1993). They studied the phylogenetic relationships between 16S rDNA genes from *Rhizobiaceae* and related bacteria. Their data included 19 strains for the genera *Rhizobium, Sinorhizobium, Agrobacterium, Phyllobacterium, Mycoplana (M. dimorpha), Ochrobacterium, Brucella and Rochalimaea* (a rickettsia) (Figure 8c). They found the *Rhizobium* species except *Rhizobium loti* and *Rhizobium huakuii* were intermingled with *Agrobacterium*. These latter two *Rhizobium* species constituted a separate divergent line of descent from the other *Rhizobium* species.

The results of the above three studies and Figure 21 (Martínez-Romero, 1994) are relevant to present study. However, as discussed below the issue over the phylogenetic relatedness of some rhizobial and agrobacterial species does not affect identification of most soil isolates as particular described species.

The very high bootstrap values obtained for soil isolates and particular rhizobial type sequences in our tree (Figure 19) show that for the purpose of species identification to known type strains partial sequencing appears adequate (e.g. soil isolates KJ203, NR42, KJ44). In contrast, as shown from the low bootstrap values in the same tree, partial sequences were not sufficient to resolve the phylogenetic relationship between other species (e.g. soil isolates KJ17, 26, OR168, NR64). Consequently if the sample contained isolates for which type strains had not been determined the placement of such soil isolates in the tree could be ambiguous.

Our phylogenetic tree showed that all strains isolated are *Rhizobium* sp. and some (those whose edges are placed in the tree with low bootstrap support) may be undescribed species of *Rhizobium* (e.g. soil isolates KJ17, KJ26, NR64 and OR168). Our sequencing study identified soil isolates KJ5 and KJ13 as *Rhizobium loti*. Soil isolate KJ203 was *Rhizobium etli*. Soil isolate NR42 was *Rhizobium fredii* or *Rhizobium meliloti*. Soil isolate KJ57 was *Rhizobium tropici* IIA. Soil isolates KJ44, KJ30, KJ19, NR41, KJ27, and KJ23 were *Rhizobium leguminosarum* biovar trifolii. These results were also tested by DNA-DNA hybridization.

Figure 21. Phylogenetic tree derived from results obtained by Hernandez Lucas *et al.*, unpublished; Sawada *et al.*, 1993; Willems and Collins, 1993; Yanagi and Yamasato, 1993 and Young *et al.*, 1991. Genetic distances were used to construct the tree by Neighbor-Joining method (Saitou and Nei, 1987). Position of nodes indicated with arrows is not definitive (Martínez-Romero, 1994).


Bartonella bacilliformis
Rochalimea quintana
Agrobacterium tumefaciens
A. sp NCPPB1650
Agrobacterium rubi
Phyllobacterium spp.
Brucella abortus
R. loti
R. huakuii
Agrobacterium bv. 3
R. sp OK55
R. galegae
R. meliloti
R. fredii
R. sp. NGR234
R. sp. BR816
R. leguminosarum
R. sp. CFN265
R. etli
R. sp. CFN244
R. sp. FL27
R. sp. CFN234
R. sp. Ori91
R. sp. Cli80
R. tropici, A. sp. K-Ag-3
A. sp. Ch-Ag-4
A. bv 2
Alipia spp
Rhodobacter spheroides
R. capsulatus
BTAi1
B. japonicum
Bl. denitrificans
Azorhizobium caulinodans
Rhodopseudomonas

4. 2. 3 DNA-DNA hybridization

DNA-DNA hybridization is a standard method for the designation of species (Wayne *et al.*, 1987). Using this approach we found that the relationship between known reference strains was in good agreement with previous taxonomic classifications based on DNA-DNA homology (Crow *et al.*, 1981, Jarvis *et al.*, 1982, Laguerre *et al.*, 1993b, Martinez *et al.*, 1991 and Segovia *et al.*, 1991) and sequence analysis of 16S rRNA (Sawada *et al.*, 1993, Willems and Collins, 1993 and Yanagi and Yamasato, 1993).

DNA-DNA hybridization showed that soil isolates KJ5 and KJ13 were *Rhizobium loti* consistent with sequence analysis. The largest group of soil isolates expressing pRtr514a were KJ19, KJ23, KJ27, KJ30, KJ44 and NR41. DNA hybridization showed these were likely to be *Rhizobium leguminosarum* biovar trifolii a result which was also consistent with the 16S rRNA sequence analysis. Soil isolate KJ57 was found to be *Rhizobium tropici* IIA and a result which was also consistent with the 16S rDNA phylogenetic tree. Soil isolate NR42 was identified as *Rhizobium meliloti* based on the DNA relatedness data this was also confirmed by sequence analysis. In the phylogenetic tree KJ203 was identified as *Rhizobium etli* but this could not be confirmed on DNA-DNA relatedness. Since we did not have the *R. etli* type strains. Soil isolates KJ17, KJ26, NR64 and OR168 were also not identified unambiguously. They showed less than 80% relatedness to *Rhizobium leguminosarum* biovar trifolii. It is suggested that they may represent unknown species of *Rhizobium*

Another step was taken to identify the soil isolates expressing pSym based on total fatty acid analysis.

4. 2. 4 Fatty acid analysis

As an independent method, the fatty acid content of the strains was analyzed by gas - liquid chromatography, which is also considered a powerful method to differentiate between bacterial species (Lechevalier, 1977). A comparison of the species names assigned to unnamed soil bacteria by fatty acids together with DNA analyses is shown in Table 10. The table is based on a recent study by Jarvis and Tighe, (1994). They examined 123 strains of *Rhizobium* : *Rhizobium fredii* (19), *Rhizobium galegae* (20), *Rhizobium leguminosarum* (22), *Rhizobium loti* (17), *Rhizobium meliloti* (21), and *Rhizobium tropici* (18) and six unknowns. The authors developed fatty acid profiles for each species ad then used them to identify unknown species.

Table 10. A comparison of the species names assigned to unnamed soil bacteria by fatty acids and DNA analyses

Soil isolate	Ribosomal hybridization	16S rRNA sequence data	Total DNA-DNA relatedness	Total fatty acid analysis
KJ5	not identified	*Rhizobium loti*	*Rhizobium loti*	*Agrobacterium radiobacter*
KJ13	not identified	*Rhizobium loti*	*Rhizobium loti*	*Agrobacterium radiobacter*
KJ17	not identified	not identified	not identified	*Rhizobium "hedysari"*
KJ19	*Rhizobium leguminosarum*	*Rhizobium leguminosarum*	*Rhizobium leguminosarum*	*A. rhizogenes/ Rhizobium etli*
KJ23	*Rhizobium leguminosarum*	*Rhizobium leguminosarum*	*Rhizobium leguminosarum*	*Rhizobium leguminosarum*
KJ26	not identified	not identified	not identified	*Rhizobium "hedysari"*
KJ27	*Rhizobium leguminosarum*	*Rhizobium leguminosarum*	*Rhizobium leguminosarum*	*Agrobacterium rhizogenes**
KJ30	*Rhizobium leguminosarum*	*Rhizobium leguminosarum*	*Rhizobium leguminosarum*	*Rhizobium leguminosarum*
NR41	not identified	*Rhizobium leguminosarum*	*Rhizobium leguminosarum*	not done
NR42	not identified	*Rhizobium meliloti*	*Rhizobium meliloti*	*Rhizobium meliloti*
KJ44	*Rhizobium leguminosarum*	*Rhizobium leguminosarum*	*Rhizobium leguminosarum*	*Rhizobium leguminosarum*
KJ57	*Rhizobium tropici*	*Rhizobium tropici*	*Rhizobium tropici*	*Rhizobium tropici*
NR64	not identified	not identified	not identified	*Rhizobium leguminosarum*
OR168	not identified	not identified	not identified	*Rhizobium leguminosarum*
KJ203	not identified	*Rhizobium etli*	not identified	*Rhizobium leguminosarum*

Rhizobium etli, *Rhizobium "hedysari"*, *Agrobacterium radiobacter* and *Agrobacterium rhizogenes* were not used in the ribosomal hybridization and DNA-DNA relatedness study. *: poor match to data base 0.234. Each fatty acid profile has an identification and Similarity Index (SI) listed. A SI of 0.400 or higher indicates a very good match to the Microbial Identification System (MIS) data base; 0.250 or lower indicates a poor profile match.

Using their criteria, in our study soil isolates NR42 and KJ57 were identified as *Rhizobium meliloti* and *Rhizobium tropici* by fatty acid analysis. This agreed with identifications based on rRNA fingerprinting, 16S rRNA sequences and DNA-DNA hybridization respectively.

Soil isolates KJ23, KJ30 and KJ44 were identified as *Rhizobium leguminosarum* by fatty acid analysis and this agreed with identifications based on rRNA fingerprinting, 16S rRNA sequences and DNA-DNA hybridization.

Soil isolates KJ17 and KJ26 were identified as *Rhizobium "hedysari"* by fatty acid analysis. To confirm the fatty acid based identification we need 16S rDNA sequence from a *R. "hedysari"* reference strain.

Soil isolates NR64 and OR168 were also unclassified *Rhizobium* sp. by primary sequence data. They are identified as *R. leguminosarum* by fatty acids analysis.

Soil isolate KJ203 was shown by primary sequence analysis to be *Rhizobium etli.* However fatty acid analysis (Figure 20) did not resolve its identity between possible type strains. The 2D-plot shows *R. leguminosarum, R. etli* and *R. meliloti* are all close together and have overlapping ranges (as discussed in section 4. 3. 2) and that this is reflected in identification problems.

Soil isolate KJ19 was identified as *Rhizobium leguminosarum* by ribosomal hybridization, 16S rRNA sequence and total DNA-DNA relatedness but not by cellular fatty acids (CFA). KJ19 was identified by CFA as *Agrobacterium* biovar 2, revised as *Agrobacterium rhizogenes* (Sawada *et al.*, 1993). The reason for this could be that *Agrobacterium* and *Rhizobium* are infact not phylogeneticaly distinct (Jarvis *et al.*, 1986; Sawada *et al.*, 1993; Willems and Collins, 1993; Yanagi and Yamasato, 1993). Further, the following fatty acids of strain KJ19: 16:0, 16:0 3OH, 18:0, 18:0, 18:1 2OH, 19: CYCLO$_w$8C, 20:3$_w$6, 9, 12C and summed features 3 and 7 were similar to both *Agrobacterium rhiozgenes* and *Rhizobium leguminosarum.* In addition fatty acids such as 13:1 AT 12-13 and 17:0 CYCLO were found in *Agrobacterium rhizogenes* and 15:0 2OH and 18:1 2OH were found in *Rhizobium leguminosarum.* Fatty acid 16:1$_w$7C was found in both *Agrobacterium rhizogenes* and *Rhizobium leguminosarum* but not found in soil isolate KJ19. Thus the misidentification is probably due to the similarity of the fatty acid content of *Agrobacterium rhizogenes* and *Rhizobium leguminosarum.*

KJ19 also has a match to *Rhizobium etli*. CFA profiles for *Rhizobium leguminosarum* and *Rhizobium etli* are available but the microbial identification system (MIS) confuses them on occasions. It can be also be seen from the 2D-plot that *R. leguminosarum, R. etli* and *R. meliloti* are all close together and have overlapping ranges. Hence this may also explain the difficulty in identifying strain KJ19.

Soil isolate KJ27 was identified as *Rhizobium leguminosarum* based on ribosomal hybridization, 16S rRNA sequence data and total DNA-DNA relatedness but its total fatty acid profile as *Agrobacterium rhizogenes* was a poor match to the data base. This was quite a surprising result as the 16S rDNA sequence of this isolate has identical sequence to *R. legumionsarum*. This observation suggests there may be need for revision of some of the criteria used by Jarvis and Tighe, (1994).

Soil isolate NR40 was identified as *Rhizobium loti* by fatty acid analysis (Rao *et al.*, 1994). However sequence characterization and DNA-DNA hybridization was not under taken.

Soil isolates KJ5 and KJ13 were identified as *Rhizobium loti* based on 16S rRNA sequence analysis and DNA-DNA hybridization but this disagrees with the identification based on cellular fatty acid analysis. CFA identified strains KJ5 and KJ13 as *Agrobacterium radiobacter*. Again this suggests a problem with the fatty acid profiles suggested by Jarvis and Tighe, (1994). The fatty acids of strains KJ5 and KJ13: 16:0, 17:0 CYCLO, 19:0 CYCLO$_{w}$8C, 19:0 10 Methyl and summed feature 7 were similar to *Agrobacterium radiobacter* and *Rhizobium loti* but fatty acids of strains KJ3 and KJ5: 13:1 AT 12-13, 16:0 3OH, 16:1$_{w}$7C, 20:3$_{w}$6, 9, 12C and summed feature 3 were similar to *Agrobacterium radiobacter* and not *Rhizobium loti*. In addition fatty acids 13:0 ISO 3OH and 17:0 were found in *Rhizobium loti* and not in strains KJ5 and KJ13. The misidentification could be due to the similarity of the fatty acid content of *Agrobacterium radiobacter* and *Rhizobium loti*. Another reason could be that *Agrobacterium* and *Rhizobium* are closely related (Jarvis *et al.*, 1986, Sawada *et al.*, 1993, Willems and Collins, 1993; Yanagi and Yamasato, 1993).

(During the preparation of this thesis a judicial opinion appeared (Bouzar, 1994) with which Sawada *et al.*, (1993) concurred, indicating that biovar 1 was incorrectly called *Agrobacterium radiobacter* and should be designated *Agrobacterium tumefaciens*.)

4. 3 Summary and conclusions

The present study demonstrates that several soil types (including leached, low P and low pH soil such as Ramiha silt loam) harbour non-nodulating soil bacteria which can maintain symbiotic genes and probably symbiotic plasmids. We demonstrated such bacteria could accept and express the plasmid pPN1. All bacteria isolated were non-nodulating rhizobia. These included: *R. leguminosarum, R. loti, R. meliloti, R. etli and R. tropici.*

Four methods of identification were employed to characterize soil isolates. (a) Close agreement was obtained between DNA-rRNA fingerprint, 16S rRNA sequence analysis and DNA-DNA hybridization results. (b) The identity of 6 (40%) strains out of 15 were identified by rRNA fingerprinting. (c) Partial sequencing provided the strongest evidence for identification. Although in a few cases the identity of the bacteria, although of a rhizobial/agrobacterial type remained unclear. (d) DNA-DNA hybridization data agreed with the identification method of partial sequencing. (e) A comparison of the species names assigned to unnamed soil bacteria by fatty acids and DNA analyses showed an agreement of 50%.

4. 4 Theoretical and practical implications

It has long been recognized that spreading soil, taken from a field cropped with legumes, over a virgin field can increase the yield of a legume crop. Once it became understood that the soil bacteria constituted the fertility factor in such top soil, the inoculation of legumes with rhizobial cultures became a routine practice in both developed and developing countries.

The two media and the selection of colonies from the media used in this study may have introduced some bias in the collection of soil bacteria used in the conjugation experiments. Identifying some of the strains which did not express pPN1 as a means of assessing whether the media used were able to support a wide range of soil bacteria. Such bias might be overcome by (i) selecting colonies at random using random number table, (ii) use of media selective for specific soil bacteria, (iii) use of named strains from the culture collections as recipients in conjugation experiments. However the two media used in this study were not selective for rhizobia but were used to select a diverse group of soil bacteria which could express the symbiotic genes.

Thies *et al.*, (1991) have noted the improved yields of N-fertilized crops when compared to inoculated or uninoculated crops. Clearly, greater crop yields could be

expected from improvements in inoculation technology and the development of inoculant strains with increased nodulation and nitrogen-fixing capacity. Further research is necessary to identify more non-nodulating strains from a variety of soils which can accept and express symbiotic genes. These strains could be identified with modern molecular methods such as PCR and direct sequencing of partial 16S rDNA. This could be expected to result in the discovery of new species of rhizobia. A great deal about the non-nodulating bacteria which can express pSym is still not known. Segovia *et al.,* (1991) determined nitrogen fixation by acetylene reduction assay. They reported that the nonsymbiotic isolates, when complemented with an *R. legumionsarum* bv. phaseoli symbiotic plasmid were able to fix nitrogen in symbiosis with bean roots at levels similar to those of the parental strain. In this study, it would be particularly interesting to know how well these tranconjugants fix atmospheric nitrogen and how stable the symbiotic plasmid is in them.

It is not clear how important symbiotic plasmid transfer is in relation to the maintenance of the plasmid in the soil environment and efficient nitrogenase expression. It may be possible to identify the genes involved in the conjugation and transfer of symbiotic plasmids. These questions could then be approached by comparing the expression and survival of pSym fixed in a background which efficiently expresses the *nod* genes on a clover plant by the insertion of Tn5 in a *tra* gene sequence with the expression and survival of the same pSym free to transfer into another soil bacterium in glasshouse and field experiments.

When land is brought into cultivation and sown to pasture indigenous rhizobia usually appear after seed mixtures containing legumes have been inoculated with a suitable strain of *Rhizobium*. Indigenous rhizobia may arise by transfer of the symbiotic plasmid from the inoculum strain to soil bacteria. These bacteria could be rhizobia currently lacking a symbiotic plasmid or soil bacteria not usually associated with root nodules. If these soil bacteria receive the symbiotic plasmid by conjugation they may also possess a variable ability to express symbiotic genes but they are likely to be well adapted to the local environment and better able to survive than the introduced inoculant *Rhizobium* strains. Inoculation of successive crops could thus generate large populations of indigenous rhizobia which would compete with the inoculant strains to form nodules and fix atmospheric nitrogen in the roots of pasture legumes. This may decrease the overall efficiency of nitrogen fixation and the probability of enhancing yield with existing inoculation technology may decrease dramatically. This study has shown that pSym expression occurs in a diverse group of *Rhizobium* sp. and there is evidence to show that this occurs in different soil types. Although a variety of soil bacteria may be recipients [including *Sphingobacterium multivorum* (Fenton and

Jarvis, 1994)] we have not found any non-rhizobial recipient from soil in five years of searching which express a symbiotic plasmid.

Three DNA based methods and one based on fatty acid analysis, were used to identify soil bacteria which could express a symbiotic plasmid from *R. leguminosarum* biovar trifolii. Partial 16S rRNA sequencing was a particularly useful identification method because it was accurate and quick. Total DNA could be readily isolated, and partial 16S rRNA sequences be amplified and sequenced and a database search completed to get an answer with a high probability of being correct. Some of the primary 16S rDNA sequences from the unknown soil bacteria were identical with sequences in the database and this allowed these strains to be clearly identified. Some groupings in the reconstructed phylogenetic tree were also observed to occur with high probabilities also indicating unambiguous identification. For others, alternate node positions having equal and low probabilities are possible, making the phylogenetic positions of those isolates uncertain. Some strains did not cluster with any named strains. They may be new, previously unrecognized species. Therefore it seems unlikely that they are not rhizobia although they may belong to undiscribed *Rhizobium* sp.

The following authors have used rRNA fingerprinting successfully: Grimont and Grimont, 1986; Grimont *et al.*, 1987; Saunders *et al.*, 1988; Segovia *et al.*, 1991. In the present work rRNA fingerprinting showed heterogeneity between and within species. Only 40% of the soil isolates used here were recognizably similar to known rhizobial species. Further experimental work is required to characterize known type strains on the pattern of variability before this approach is generally useful. However, there was good agreement between rRNA fingerprinting and other DNA based methods.

DNA-DNA hybridization is considered a reliable means of establishing the relationship between bacterial species. The advantage of this approach is that it utilizes the whole genome. The problems are: (i) the need to store reference DNAs, (ii) the inability to store reference data as can be done for sequence DNA data, (iii) it is only useful for species limits and (iv) in some strains of *Agrobacterium* and *Rhizobium* upto 25% of the DNA has been reported as extrachromosomal (Martínez-Romero, 1994; Prakash and Atherly, 1986). Hence estimates of overall DNA similarity may not accurately reflect phylogenetic relationships. However in the present study if *R. etli* reference DNA had been used, it would have been possible to distinguish *R. etli* form *R. leguminosarum.*

The identification of some soil isolates based on CFA did not agree completely with the molecular methods and the 2-D plot indicates overlapping ranges for some rhizobia

and agrobacteria strains. This is another expression of the "interwining" of *Rhizobium* and *Agrobacterium*. The system discriminates successfully between named *Rhizobium* and *Agrobacterium* (Jarvis and Tighe, 1994). Inability to identify 50% of soil strains is surprising, but this may be due to lack of their pSym or Ti plasmid.

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APPENDIX

Aligned sequences of part of the 16S rRNA gene from fifteen Gram negative soil bacteria which could express pSym from *Rhizobium leguminosarum* biovar trifolii. Sequences are aligned to known sequence from the Genbank + EMBL Data Library. *Bradyrhiz.*, *Bradyrhizobium japonicum* strain USDA31 (GenBank accession number M55487); *Azo. cauli.*, *Azorhizobium caulinodans* strain ORS571 (GenBank accession number M55491); *Rho. vanni.*, *Rhodomicrobium vannielii* (GenBank accession number M34127); *R. loti*, *Rhizobium loti* strain NZP2213 (EMBL accession number X67229);, *R. galega*, *Rhizobium galegae* strain HAMBI540 (EMBL accession number X67226); *A. vitis*, *Agrobacterium vitis* strain NCPPB3554 (EMBL accession number X67225); *Agro. tum.*, *Agrobacterium tumefaciens* strain DMS30150 (GenBank accession number M11223); *R. etli*, *Rhizobium etli* strain Or191 (GenBank accession number M55236); *R. melilot.*, *Rhizobium meliloti* strain NZP4017 (GenBank accession number M55495); *R. fredii*, *Rhizobium fredii* strain USDA205 (GenBank accession number M74163); *A. rhizoge.*, *Agrobacterium rhizogenes* strain ATCC15834 (EMBL accession number X67224); *R. tropici*, *Rhizobium tropici* IIA strain CFN299 (EMBL accession number X67233); *R. legumin.*, *Rhizobium leguminosarum* biovar trifolii strain ATCC14480 (EMBL accession number X67227).

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Bradyrhiz AGGCTTAACA CATGCAAGTC GAGCGGGCAT AGCAATATGT CAGCGGCAGA CGGGTGAGTA ACGCGTGGGA ACGTACCTTT TGGTTCGGAA CAACTGAGGG AAACTTCAGC TAATACCGGA TAAGCCCTTA
Azo cauli AGGCTTAACA CATGCAAGTC GAACGGGC.. .CCTTCGGGT CAGTGGCAGA CGGGTGAGTA ACGCGTGGGA ACGTGCCCTT CAGTTCGGAA TAACCCAGGG AAACTTGGGC TAATACCGGA TACGTCCGAA
Rho vanne AGGCTTAACA CATGCAAGTC GAACGCAC.. ..CGCAAGGT GAGTGGCAGA CGGGTGAGTA ACGCGTGGGA ACCTTCCCTT TGGTACGGAA TAACTTCGGG AAACCGAAGC TAATACCGTA TATCTCCTCC
KJ5 AGGCTTAACA CATGCAAGTC GAGCGCCT.. ..CGCAAGAG GAGCGGCAGA CGGGTGAGTA ACGCGTGGGA ATCTACCCAT CTCTACGGAA CAACTCCGGG AAACTGGAGC TAATACCGTA TACGTCCTTC
KJ13 AGGCTTAACA CATGCAAGTC GAGCGCCT.. ..CGCAAGAG GAGCGGCAGA CGGGTGAGTA ACGCGTGGGA ATCTACCCAT CTCTACGGAA CAACTCCGGG AAACTGGAGC TAATACCGTA TACGTCCTTC
R. loti AGGCTTAACA CATGCAAGTC GAGCGCCT.. ..CGCAAGAG GAGCGGCAGA CGGGTGAGTA ACGCGTGGGA ATCTACCCAT CTCTACGGAA CAACTCCGGG AAACTGGAGC TAATACCGTA TACGTCCTTC
R. galega AGGCTTAACA CATGCAAGTC GAGCGCCT.. ..CGCAAGAG GAGCGGCAGA CGGGTGAGTA ACGCGTGGGA ATCTACCCAT CCCTACGGAA CAACTCCGGG AAACTGGAGC TAATACCGTA TACGCCCTTC
A. vitis AGGCTTAACA CATGCAAGTC GAGCGCCT.. ..CGCAAGAG GAGCGGCAGA CGGGTGAGTA ACGCGTGGGA ATCTACCGTA CCCTACGGAA TAGCTCCGGG AAACTGGAAT TAATACCGTA TACGCCCTTC
Agro tum AGGCTTAACA CATGCAAGTC GAACGCCC.. ..CGCAAGGG GAGTGGCAGA CGGGTGAGTA ACGCGTGGGA ATCTACCGTG CCCTGCGGAA TAGCTCCGGG AAACTGGAAT TAATACCGCA TACGCCCTAC
R. etli AGGCTTAACA CATGCAAGTC GAGCGCCC.. ..CGCAAGGG GAGCGGCAGA CGGGTGAGTA ACGCGTGGGA ACGTACCCTT TACTACGGAA TAACGCATGG AAACGTGTGC TAATACCGTA TGTGCCCTTT
KJ203 AGGCTTAACA CATGCAAGTC GAGCGCCC.. ..CGCAAGGG GAGCGGCAGA CGGGTGAGTA ACGCGTGGGA ACGTACCCTT TACTACGGAA TAACGCATGG AAACGTGTGC TAATACCGTA TGTGCCCTTT
KJ17 AGGCTTAACA CATGCAAGTG GAACGCCC.. ..CGCAAGGG GAGTGGCACA CGGGTGAGTA ACACGTGGGA ACGTACCCTT TTCTACGGAA TAACCCAGGG AAACTTGGAC TAATACCGTA TGTGCCCTTC
KJ26 AGGCTTAACA CATGCAAGTG GAACGCCC.. ..CGCAAGGG GAGTGGCACA CGGGTGAGTA ACACGTGGGA ACGTACCCTT TTCTACGGAA TAACCCAGGG AAACTTGGAC TAATACCGTA TGTGCCCTTC
NR64 AGGCTTAACA CATGCAAGTC GAGCGCCC.. ..CGCAAGGG GAGCGGCAGA CGGGTGAGTA ACACGTGGGA ACGTACCCTT TACTACGGAA TAACGCAGGG AAACTTGTGC TAATACCGTA TGTGCCCTTC
R.melilot AGGCTTAACA CATGCAAGTC GAGCGCCC.. ..CGCAAGGG GAGCGGCAGA CGGGTGAGTA ACGCGTGGGA ATCTACCCTT TTCTACGGAA TAACGCAGGG AAACTTGTGC TAATACCGTA TGAGCCCTTC
NR42 AGGCTTAACA CATGCAAGTC GAGCGCCC.. ..CGCAAGGG GAGCGGCAGA CGGGTGAGTA ACGCGTGGGA ATCTACCCTT TTCTACGGAA TAACGCAGGG AAACTTGTGC TAATACCGTA TGAGCCCTTC
R. fredii AGGCTTAACA CATGCAAGTC GAGCGCCC.. ..CGCAAGGG GAGCGGCAGA CGGGTGAGTA ACGCGTGGGA ATCTACCCTT TTCTACGGAA TAACGCAGGG AAACTTGTGC TAATACCGTA TGAGCCCTTC
A.rhizoge AGGCTTAACA CATGCAAGTC GAGCGCCC.. ..CGCAAGGG GAGCGGCAGA CGGGTGAGTA ACGCGTGGGA ATCTACCCTT TTCTACGGAA TAACGCAGGG AAACTTGTGC TAATACCGTA TGTGTCCTTC
R.tropici AGGCTTAACA CATGCAAGTC GAGCGCCC.. ..CGCAAGGG GAGCGGCAGA CGGGTGAGTA ACGCGTGGGA ATCTACCTTT TGCTACGGAA TAACGCAGGG AAACTTGTGC TAATACCGTA TGTGTCCTTC
KJ57 AGGCTTAACA CATGCAAGTC GAGCGCCC.. ..CGCAAGGG GAGCGGCAGA CGGGTGAGTA ACGCGTGGGA ATCTACCTTT TGCTACGGAA TAACGCAGGG AAACTTGTGC TAATACCGTA TGTGTCCTTC
OR168 AGGCTTAACA CATGCAAGTC GAACGCCC.. ..CGCAAGGG GAGTGGCAGA CGGGTGAGTA ACGCGTGGGA ATCTACCCTT TACTACGGAA TAACGCATGG AAACGTGTGC TAATACCGTA TGTGTCCTTC
R.legumin AGGCTTAACA CATGCAAGTC GAGCGCCC.. ..CGCAAGGG GAGCGGCAGA CGGGTGAGTA ACGCGTGGGA ATCTACCCTT GACTACGGAA TAACGCAGGG AAACTTGTGC TAATACCGTA TGTGTCCTTC
NR41 AGGCTTAACA CATGCAAGTC GAGCGCCC.. ..CGCAAGGG GAGCGGCAGA CGGGTGAGTA ACGCGTGGGA ATCTACCCTT GACTACGGAA TAACGCAGGG AAACTTGTGC TAATACCGTA TGTGTCCTTC
KJ19 AGGCTTAACA CATGCAAGTC GAGCGCCC.. ..CGCAAGGG GAGCGGCAGA CGGGTGAGTA ACGCGTGGGA ATCTACCCTT GACTACGGAA TAACGCAGGG AAACTTGTGC TAATACCGTA TGTGTCCTTC
KJ27 AGGCTTAACA CATGCAAGTC GAGCGCCC.. ..CGCAAGGG GAGCGGCAGA CGGGTGAGTA ACGCGTGGGA ATCTACCCTT GACTACGGAA TAACGCAGGG AAACTTGTGC TAATACCGTA TGTGTCCTTC
KJ23 AGGCTTAACA CATGCAAGTC GAGCGCCC.. ..CGCAAGGG GAGCGGCAGA CGGGTGAGTA ACGCGTGGGA ATCTACCCTT GACTACGGAA TAACGCAGGG AAACTTGTGC TAATACCGTA TGTGTCCTTC
KJ44 AGGCTTAACA CATGCAAGTC GAGCGCCC.. ..CGCAAGGG GAGCGGCAGA CGGGTGAGTA ACGCGTGGGA ATCTACCCTT GACTACGGAA TAACGCAGGG AAACTTGTGC TAATACCGTA TGTGTCCTTC
KJ30 AGGCTTAACA CATGCAAGTC GAGCGCCC.. ..CGCAAGGG GAGCGGCAGA CGGGTGAGTA ACGCGTGGGA ATCTACCCTT GACTACGGAA TAACGCAGGG AAACTTGTGC TAATACCGTA TGTGTCCTTC

Bradyrhiz CGGGGAAAGA TTTATCGCCG AAAGATCGGC CCGCGTCTGA TTAGCTAGTT GGTGAGGTAA TGGCTCACCA AGGCGACGAT CAGTAGCTGG TCTGAGAGGA TGATCAGCCA CATTGGGACT GAGACACGGC CCAA
Azo cauli AGGAGAAAGA TTGATCGCTG AAGGATCGGC CCGCGTCTGA TTAGCTAGTT GGTGAGGTAA TGGCTCACCA AGGCGACGAT CAGTAGCTGG TCTGAGAGGA TGATCAGCCA CATTGGGACT GAGACACGGC CCAA
Rho vanne GGGAGAAAGA TTTATCGCCA AAGGATGGGC CCGCGTTGGA TTAGCTAGTT GGTGTGGTAA CGGCGCACCA AGGCGACGAT CCATAGCTGG TCTGAGAGGA TGATCAGCCA CACTGGGACT GAGACACGGC CCAG
KJ5 GGGAGAAAGA TTTATCGGAG ATGGATGAGC CCGCGTTGGA TTAGCTAGTT GGTGGGGTAA TGGCCTACCA AGGCGACGAT CCATAGCTGG TCTGAGAGGA TGATCAGCCA CATTGGGACT GAGACACGGC CCAA
KJ13 GGGAGAAAGA TTTATCGGAG ATGGATGAGC CCGCGTTGGA TTAGCTAGTT GGTGGGGTAA TGGCCTACCA AGGCGACGAT CCATAGCTGG TCTGAGAGGA TGATCAGCCA CATTGGGACT GAGACACGGC CCAA
R. loti GGGAGAAAGA TTTATCGGAG ATGGATGAGC CCGCGTTGGA TTAGCTAGTT GGTGGGGTAA TGGCCTACCA AGGCGACGAT CCATAGCTGG TCTGAGAGGA TGATCAGCCA CATTGGGACT GAGACACGGC CCAA
R. galega GGGGGAAAGA TTTATCGGGG ATGGATGAGC CCGCGTTGGA TTAGCTAGTT GGTGGGGTAA AGGCCTACCA AGGCGACGAT CCATAGCTGG TCTGAGAGGA TGATCAGCCA CATTGGGACT GAGACACGGC CCAA
A. vitis GGGGGAAAGA TTTATCGGGG TATGATGAGC CCGCGTTGGA TTAGCTAGTT GGTGGGGTAA AGGCCTACCA AGGCGACGAT CCATAGCTGG TCTGAGAGGA TGATCAGCCA CATTGGGACT GAGACACGGC CCAA
Agro tum GGGGGAAAGA TTTATCGGGG TATGATGAGC CCGCGTTGGA TTAGCTAGTT GGTGGGGTAA AGGCCTACCA AGGCGACGAT CCATAGCTGG TCTGAGAGGA TGATCAGCCA CATTGGGACT GAGACACGGC CCAA
R. etli GGGGGAAAGA TTTATCGGTA AAGGATCGGC CCGCGTTGGA TTAGCTAGTT GGTGGGGTAA AGGCCTACCA AGGCGACGAT CCATAGCTGG TCTGAGAGGA TGATCAGCCA CATTGGGACT GAGACACGGC CCAA
KJ203 GGGGGAAAGA TTTATCGGTA AAGGATCGGC CCGCGTTGGA TTAGCTAGTT GGTGGGGTAA AGGCCTACCA AGGCGACGAT CCATAGCTGG TCTGAGAGGA TGATCAGCCA CATTGGGACT GAGACACGGC CCAA
KJ17 GGGGGAAAGA TTTATCGGAA AAGGATCGGC CCGCGTTGGA TTAGCTAGTT GGTGGGGTAA AGGCCTACCA AGGCGACGAT CCATAGCTGG TCTGAGAGGA TGATCAGCCA CATTGGGACT GAGACACGGC CCAA
KJ26 GGGGGAAAGA TTTATCGGAA AAGGATCGGC CCGCGTTGGA TTAGCTAGTT GGTGGGGTAA AGGCCTACCA AGGCGACGAT CCATAGCTGG TCTGAGAGGA TGATCAGCCA CATTGGGACT GAGACACGGC CCAA
NR64 GGGGGAAAGA TTTATCGGTA AGGGATCGGC CCGCGTTGGA TTAGCTAGTT GGTGGGGTAA AGGCCTACCA AGGCGACGAT CCATAGCTGG TCTGAGAGGA TGATCAGCCA CATTGGGACT GAGACACGGC CCAA
R.melilot GGGGGAAAGA TTTATCGGGA AAGGATGAGC CCGCGTTGGA TTAGCTAGTT GGTGGGGTAA AGGCCTACCA AGGCGACGAT CCATAGCTGG TCTGAGAGGA TGATCAGCCA CATTGGGACT GAGACACGGC CCAA
NR42 GGGGGAAAGA TTTATCGGGA AAGGATGAGC CCGCGTTGGA TTAGCTAGTT GGTGGGGTAA AGGCCTACCA AGGCGACGAT CCATAGCTGG TCTGAGAGGA TGATCAGCCA CATTGGGACT GAGACACGGC CCAA
R. fredii GGGGGAAAGA TTTATCGGGA AAGGATGAGC CCGCGTTGGA TTAGCTAGTT GGTGGGGTAA AGGCCTACCA AGGCGACGAT CCATAGCTGG TCTGAGAGGA TGATCAGCCA CATTGGGACT GAGACACGGC CCAA
A.rhizoge GGGAGAAAGA TTTATCGGGA AAGGATGAGC CCGCGTTGGA TTAGCTAGTT GGTGGGGTAA AGGCCTACCA AGGCGACGAT CCATAGCTGG TCTGAGAGGA TGATCAGCCA CATTGGGACT GAGACACGGC CCAA
R.tropici GGGAGAAAGA TTTATCGGCA AGAGATGAGC CCGCGTTGGA TTAGCTAGTT GGTGGGGTAA AGGCCTACCA AGGCGACGAT CCATAGCTGG TCTGAGAGGA TGATCAGCCA CATTGGGACT GAGACACGGC CCAA
KJ57 GGGAGAAAGA TTTATCGGCA AGAGATGAGC CCGCGTTGGA TTAGCTAGTT GGTGGGGTAA AGGCCTACCA AGGCGACGAT CCATAGCTGG TCTGAGAGGA TGATCAGCCA CATTGGGACT GAGACACGGC CCAA
OR168 GGGAGAAAGA TTTATCGGTA AAGGATGAGC CCGCGTTGGA TTAGCTAGTT GGTGGGGTAA AGGCCTACCA AGGCGACGAT CCATAGCTGG TCTGAGAGGA TGATCAGCCA CATTGGGACT GAGACACGGC CCAA
R.legumin GGGAGAAAGA TTTATCGGTC AAGGATGAGC CCGCGTTGGA TTAGCTAGTT GGTGGGGTAA AGGCCTACCA AGGCGACGAT CCATAGCTGG TCTGAGAGGA TGATCAGCCA CATTGGGACT GAGACACGGC CCAA
NR41 GGGAGAAAGA TTTATCGGTC AAGGATGAGC CCGCGTTGGA TTAGCTAGTT GGTGGGGTAA AGGCCTACCA AGGCGACGAT CCATAGCTGG TCTGAGAGGA TGATCAGCCA CATTGGGACT GAGACACGGC CCAA
KJ19 GGGAGAAAGA TTTATCGGTC AAGGATGAGC CCGCGTTGGA TTAGCTAGTT GGTGGGGTAA AGGCCTACCA AGGCGACGAT CCATAGCTGG TCTGAGAGGA TGATCAGCCA CATTGGGACT GAGACACGGC CCAA
KJ27 GGGAGAAAGA TTTATCGGTC AAGGATGAGC CCGCGTTGGA TTAGCTAGTT GGTGGGGTAA AGGCCTACCA AGGCGACGAT CCATAGCTGG TCTGAGAGGA TGATCAGCCA CATTGGGACT GAGACACGGC CCAA
KJ23 GGGAGAAAGA TTTATCGGTC AAGGATGAGC CCGCGTTGGA TTAGCTAGTT GGTGGGGTAA AGGCCTACCA AGGCGACGAT CCATAGCTGG TCTGAGAGGA TGATCAGCCA CATTGGGACT GAGACACGGC CCAA
KJ44 GGGAGAAAGA TTTATCGGTC AAGGATGAGC CCGCGTTGGA TTAGCTAGTT GGTGGGGTAA AGGCCTACCA AGGCGACGAT CCATAGCTGG TCTGAGAGGA TGATCAGCCA CATTGGGACT GAGACACGGC CCAA
KJ30 GGGAGAAAGA TTTATCGGTC AAGGATGAGC CCGCGTTGGA TTAGCTAGTT GGTGGGGTAA AGGCCTACCA AGGCGACGAT CCATAGCTGG TCTGAGAGGA TGATCAGCCA CATTGGGACT GAGACACGGC CCAA
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